Heliocobacter pylori lipopolysaccharide enhances the expression of NADPH oxidase components in cultured guinea pig gastric mucosal cells

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Abstract Recently, we showed that cultured guinea pig gastric pit cells possess a phagocyte NADPH oxidase-like activity, which was up-regulated by \textit{Heliocobacter pylori} lipopolysaccharide. We demonstrate here that these cells express all of the phagocyte NADPH oxidase components (gp91-, p22-, p67-, and p40-phoxes). Treatment with lipopolysaccharide increased the expression of gp91-, p22-, and p67-phoxes, but not that of the p47- and p40-phoxes. Intriguingly, the p67-phox expression consistently correlated with up-regulation of superoxide anion-producing ability. Thus, the gastric pit cell NADPH oxidase may play an important role in regulation of the inflammatory response associated with \textit{H. pylori} infection.

Key words: NADPH oxidase; Superoxide anion; Lipopolysaccharide; Gastric mucosal cell; \textit{Heliocobacter pylori}

1. Introduction

\textit{Helicobacter pylori} is now recognized as a crucial pathogen for chronic gastritis type B and peptic ulcer, and as a possible contributor to the development of adenocarcinoma of the stomach [1,2]. \textit{H. pylori} first comes into contact with gastric pit cells. Our efforts to find the signal molecules which regulate the interactions between this Gram-negative bacterium and gastric pit cells revealed that primary cultures of guinea pig gastric pit cells secrete an abundant amount of superoxide anion through the NADPH oxidase-like system [3]. In addition, \textit{H. pylori} lipopolysaccharide (LPS) potentiated the cells for enhanced capacity of $O_2^-$ generation, resulting in the activation of nuclear factor \textit{kB} in an autoregulatory manner [3].

With regard to modulation of inflammation and immune responses, including pyrogenicity, B-cell mitogenicity [4], and activation of endothelial cells [5], \textit{H. pylori} LPS has been considered to be less active than LPS derived from Enterobacteriaceae, such as \textit{Escherichia coli}. The concentration of \textit{H. pylori} LPS required for the priming of phagocytic cells is 1000–10000-fold higher than that of \textit{E. coli} LPS [6]. However, recently, \textit{H. pylori} LPS was shown to induce atrophic gastritis [7] and modulate the functions of several types of gastric mucosal cells, such as stimulation of pepsinogen release by guinea pig gastric chief cells [8], histamine secretion from rat enterochromaffin-like cells [9], and apoptosis of rat gastric surface mucous cells [10].

Phagocyte NADPH oxidase is a complex electron transport chain, which is switched to transfer a single electron from NADPH to molecular oxygen to form $O_2^-$ upon cell stimulation. The NADPH oxidase consists of membrane-bound cytochrome $b_558$ heterodimer (gp91-phox and p22-phox) and four cytosolic components (p67-phox, p47-phox, and p40-phox), and is modulated by p21 Rac1/2 (see [11] for a review).

In a previous report [3], we found the interesting phenomenon that unstimulated gastric pit cells possess a membrane-associated NADPH oxidase activity, which is negligible in resting phagocytes. They also contained p47-phox and p67-phox proteins in the cytoplasm, suggesting that the $O_2^-$-generating system in gastric pit cells may share functional and structural features with that of phagocytes. However, the precise molecular characterization of the pit cell oxidase has not been done.

In this study, we further characterized the gastric pit cell NADPH oxidase system by examining the expression of all the phox components by immunoblot analysis and the subcellular localization of p47- and p67-phoxes by confocal laser microscopy. Furthermore, we demonstrated that \textit{H. pylori} LPS significantly increased the amounts of p67-, p22-, and gp91-phoxes, but not p47- and p40-phoxes, in the cells in association with the up-regulation of $O_2^-$ release.

2. Materials and methods

2.1. Reagents and media

\textit{H. pylori} (NCTC 11637) was kindly provided by Dr. Nakazawa (University of Yamaguchi, Japan). An enhanced chemiluminescence Western blotting detection system and Fluorolink Cy3-labelled goat anti-rabbit IgG were purchased from Amersham Japan (Tokyo). LPS from \textit{E. coli} K-335, superoxide dismutase (SOD, from horse heart), ferricytochrome $c$, vancomycin, and amphotericin B were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of \textit{H. pylori} LPS

\textit{H. pylori} (NCTC 11637) was cultured in 10 ml of brucella broth (Gibco, Grand Island, NY, USA), supplemented with 5\% (w/v) FCS, 10 $\mu$g/ml vancomycin, and 2 $\mu$g/ml amphotericin B, for 48 h at 37°C under microaerobic conditions (12\% CO$_2$–5\% O$_2$–83\% N$_2$). The viability and shape of the organisms were monitored by phase-contrast microscopy. \textit{H. pylori} LPS was prepared by the hot-phenol water method as previously reported [3]. All procedures were performed with the approval of the respective institutional biosafety review committees and in compliance with their guidelines for biohazards.

2.3. Preparation and culture of gastric mucosal cells

Male guinea pigs weighing approximately 250 g were purchased from Shizuoka Laboratory Animal Center Inc. (Shizuoka, Japan). Gastric mucosal cells were isolated aseptically from guinea pig fundic glands and cultured, as described previously [12,13]. The cultured cells were characterized by cytochemical and immunocytochemical analyses as well as transmission electron microscopic examination [3,13]. The cultured cells consisted of pit cells (about 90\%), parietal cells (5\%), mucous neck cells (less than 1\%), fibroblasts (less than 1\%), and granule-free progenitor cells (5\%) [3]. Among these cells populations, only matured pit cells expressed detectable levels of p47- and p67-phoxes, which is switched to transfer a single electron from NADPH to molecular oxygen to form $O_2^-$ upon cell stimulation. The NADPH oxidase consists of membrane-bound cytochrome $b_{558}$ heterodimer (gp91-phox and p22-phox) and four cytosolic components (p67-phox, p47-phox, and p40-phox), and is modulated by p21 Rac1/2 (see [11] for a review). In a previous report [3], we found the interesting phenomenon that unstimulated gastric pit cells possess a membrane-associated NADPH oxidase activity, which is negligible in resting phagocytes. They also contained p47-phox and p67-phox proteins in the cytoplasm, suggesting that the $O_2^-$-generating system in gastric pit cells may share functional and structural features with that of phagocytes. However, the precise molecular characterization of the pit cell oxidase has not been done.

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phoxes [3]. These cells were identified as the cells responsible for O$_2^-$ production by nitroblue tetrazolium staining [3].

2.4. Preparation of guinea pig peritoneal neutrophils

Guinea pig peritoneal neutrophils were obtained by flushing peritoneal cavities with saline 12 h after an intraperitoneal injection of 25 ml of 3% thioglycolate broth as described previously [3]. Microscopic observation after Giemsa staining revealed that more than 90% of peritoneal exudated cells were neutrophils.

2.5. Measurement of O$_2^-$ release from gastric mucosal cells

O$_2^-$ release from gastric mucosal cells was measured as the SOD-inhibitable reduction of ferricytochrome c as described previously [3]. The reduction of ferricytochrome c was spectrophotometrically determined at 550 nm, and the amount of O$_2^-$ release was expressed as nmol/mg protein/h. Cell protein was determined by the method of Lowry et al. using bovine serum albumin as a standard.

2.6. Immunoblot analysis

Antisera against the human NADPH oxidase components were raised in rabbits by injection of keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to gp91-phox (residues 536–555), p22-phox (residues 177–195), p47-phox (residues 376–390), and p40-phox (residues 1–15), as described previously [14,15]. Polyclonal antibodies against recombinant human p47-phox and human p67-phox were gifts from Dr. Babior (The Scripps Research Institute, California, USA). Whole-cell extracts were prepared from gastric mucosal cells or neutrophils, as described previously [3]. The whole-cell proteins (50 µg of protein per lane) were then separated by SDS-PAGE in a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride filter. The filter was blocked with 4% purified milk casein for 30 min at room temperature and then incubated for 1 h with each of the above antibody at a dilution of 1:1000. After washing with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20, reacted antibodies were revealed by an enhanced chemiluminescence Western blotting detection system.

2.7. Confocal microscopic analysis

Untreated cells were fixed in 3% paraformaldehyde in PBS for 20 min. After washing with PBS, the cells were permeabilized with 0.01% (v/v) Triton X-100 for 30 s, and then incubated in 4% (v/v) milk casein for 30 min. After washing, the cells were incubated with 50 times diluted antibody against either p67- or p47-phox for 1 h at room temperature. Immuno-reacted antibodies were detected with fluorescein (rhodamine)-linked anti-rabbit IgG. Subcellular localization of either p67- or p47-phox was imaged by confocal laser scanning microscopy ($\lambda_{ex} = 550$ nm and $\lambda_{em} = 570$ nm) (Leica TCS NT, Heidelberg, Germany).

3. Results

3.1. Expression of the phagocyte NADPH oxidase components in gastric mucosal cells

Before analyzing the expression of gp91-, p22-, p67-, p47-, and p40-phoxes by immunoblotting with antibodies against human phox proteins in guinea pig gastric mucosal cells, we tested whether each antibody could specifically recognize the respective phox protein of guinea pig neutrophils. As shown in Fig. 1, all phox proteins in guinea pig neutrophils were detected by the anti-human phox antibodies. The molecular mass of gp91-phox, p67-phox, p47-phox, p40-phox, or p22-phox in neutrophils was determined to be 54–60 kDa [16], 63 kDa [17], 47 kDa [17], 39 kDa [17], or 22 kDa [16], respectively, being consistent with those in macrophages. The difference between human (91 kDa) and guinea pig (54–60 kDa) in the molecular mass of gp91-phox is attributed to the degree of glycosylation [16].

Gastric mucosal cells contained immunoreactive proteins corresponding to all of the phox proteins; however, they expressed gp91- and p40-phoxes at much lower levels than neutrophils (Fig. 1). The molecular mass of guinea pig neutrophil p67-phox is known to be 63 kDa, but gastric mucosal cells contained an immunoreactive protein with a molecular mass of 67 kDa. This intriguing 67 kDa protein was further confirmed to be p67-phox by immunoblotting with two distinct antibodies against human p67-phox [18] and porcine p63-phox [19] (data not shown).

Subcellular localization of p67- or p47-phox in resting gastric pit cells was analyzed by confocal laser scanning microscopy after immunofluorescence staining (Fig. 2). Significant amounts of p67-phox were localized along the plasma membrane, as well as in the cytoplasm (Fig. 2B). A small amount of p47-phox was also seen in the peripheral membrane region, while it was mainly distributed in the cytosol (Fig. 2D). It is known that translocated p47-phox to the plasma membrane anchors p67-phox for the activation of the phagocyte NADPH oxidase [15]. Thus, this unique translocation profile of p67- and p47-phoxes in a resting state may explain spontaneous O$_2^-$ release from gastric pit cells.

3.2. Effect of LPS from H. pylori or E. coli on expression of NADPH oxidase components

Untreated gastric mucosal cells released 53 ± 4 nmol O$_2^-$/mg protein/h (mean ± S.D., n = 12). Their treatment with H. pylori LPS (0.5 ng/ml) or E. coli LPS (10 ng/ml) for 12 h significantly increased O$_2^-$ release to 153 ± 9 (n = 12) or 161 ± 12 nmol O$_2^-$/mg protein/h (n = 12), respectively. The effect of a protein synthesis inhibitor, cycloheximide, on each LPS-primed O$_2^-$ release was examined. Cycloheximide at 100 ng/ml did not change the basal amount of O$_2^-$ production (52 ± 4 nmol O$_2^-$/mg protein/h, n = 12). However, it completely inhibited

![Fig. 1. Expression of neutrophil NADPH oxidase components in gastric mucosal cells.](image-url)
the primed increases in $\mathrm{O}_2^-$ production by $H. \text{pylori}$ or $E. \text{coli}$ LPS, leaving them at the basal levels: $56 \pm 9 (n=12)$ for $H. \text{pylori}$ LPS and $59 \pm 8 \text{nmol } \mathrm{O}_2^/\text{mg protein/h} (n=12)$ for $E. \text{coli}$ LPS. These results suggest that the LPSs induced certain phox proteins for the enhanced capacity of $\mathrm{O}_2^-$ release. Cells stayed viable throughout the experiments, based on continued trypan blue exclusion, adherence to the culture plates, and unchanged morphological features (data not shown).

To elucidate the cellular events leading to the priming of $\mathrm{O}_2^-$ release with the LPSs, we compared the expression of the NADPH oxidase components before and after treatment (Fig. 3). In response to $H. \text{pylori}$ LPS (Fig. 3A,B) or $E. \text{coli}$ LPS (Fig. 3D,E), gp91- and p22-phoxes were somewhat induced (Fig. 3C,F), while the expression of p47- and p40-phoxes was not changed (data not shown). Of phox components, the expression of p67-phox consistently correlated with up-regulation of $\mathrm{O}_2^-$-producing ability (Fig. 3C,F) with both LPSs. Thus, p67-phox is likely to be a key molecule to regulate the $\mathrm{O}_2^-$-producing capacity of gastric pit cells, as shown in the HL-60 cell line [33].

4. Discussion

In our previous study [3], cell-free reconstitution experiments and immunoblot analysis with antibodies against p47-and p67-phoxes suggested that gastric pit cells in culture have a phagocyte NADPH oxidase-like enzyme. However, the presence of other critical components of the phagocyte oxidase, particularly a redox center of cytochrome b$_{558}$, had not been determined. Cultured gastric pit cells can spontaneously secrete a larger amount of $\mathrm{O}_2^-$ ($1.08 \text{nmol/10}^6 \text{cells/min}$) than murine resident peritoneal macrophages stimulated by phorbol diester ($0.07 \text{nmol/10}^6 \text{cells/min}$), and their oxidase activity was markedly sensitive to $H. \text{pylori}$ LPS. These features have not been documented in any cell types including phagocytes, implying that the pit cells have a unique NADPH oxidase system.

To address these issues, we examined the expression of gp91-, p22-, p67-, p47-, and p40-phoxes, using specific antibodies against the human neutrophil phox components. To our knowledge, this is the first report that gastric mucosal cells express all of these essential components of the neutrophil NADPH oxidase. Several non-phagocytic cells, including fibroblasts [20], endothelial cells [21], and glomerular mesangial cells [22], are known to produce low levels of $\mathrm{O}_2^-$ (less than 0.2 nmol/10$^6$ cells/min). Among the phox components, p22-, p47-, and p67-phoxes are ubiquitously expressed in non-phagocytic cells [20-22]. However, the expression of gp91-phox has not been documented. Lower $\mathrm{O}_2^-$-generating activities in those cells are suggested to be due to the lack of this component [20-22]. The present finding that gastric mucosal cells expressed a detectable level of gp91-phox may explain their unexpectedly higher capacity for $\mathrm{O}_2^-$ production, compared to the above non-phagocytic cells.

The p40-phox expression in gastric mucosal cells was at an extremely lower level in contrast to that in neutrophils (Fig. 1). Neutrophil NADPH oxidase activity can be reconstituted in a cell-free system in the absence of p40-phox [23], p40-phox that interacts with p67-phox is proposed to inhibit translocation of p67-phox to plasma membrane in resting neutrophils [15], being a negative regulator of the NADPH oxidase, rather than an essential component for $\mathrm{O}_2^-$ production. Thus, the gastric pit cell oxidase may be constitutively active because...
of the insufficiency of the negative regulation by p40-phox, resulting in spontaneous secretion of O$_2^-$. At present, there is no evidence that directly verifies this concept, but this might be supported by the finding that significant amounts of p67-phox were detected on the plasma membrane of resting gastric pit cells (Fig. 2).

The kinetics of _E. coli_ LPS-induced up-regulation of O$_2^-$ production by gastric mucosal cells resembles that of the macrophage NADPH oxidase [24,25]. In the latter case, the priming appeared 8 h after exposure to _E. coli_ LPS and required de novo synthesis of proteins, including the NADPH oxidase components [26-28]. The priming of gastric mucosal cells was more sensitive to _H. pylori_ LPS than _E. coli_ LPS and also associated with de novo protein synthesis as demonstrated by cycloheximide experiments. Cycloheximide completely blocked the LPS-induced increase in O$_2^-$ release, but did not affect the basic one. Among the _phox_ components examined, the magnitude and time course of the increase in p67-phox coincided with those of up-regulation of O$_2^-$ production with _H. pylori_ LPS or _E. coli_ LPS. p67-phox has been considered to be one of the critical components to regulate the activity of phagocyte NADPH oxidase [29]. In addition, the expression of gp91- and p22-phoxes was also up-regulated by each LPS to a small degree. These observations suggest that the regulation of _phox_ proteins, particularly p67-phox, with each LPS may be a critical step to control the O$_2^-$-producing ability of gastric pit cells. Therefore, it would be important to reveal LPS-mediated intracellular signals that up-regulate these components.

Gastric pit cells were reported to phagocytose neighboring dead cells [30], express MHC class II antigen with these components. The priming of gastric mucosal cells was the first line of defense against _H. pylori_ infection. They may actively bear inflammatory and immune responses of gastric mucosa through the unique NADPH oxidase.

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**References**