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REVIEW

Nox enzymes and oxidative stress in the immunopathology of the gastrointestinal tract

Kazuhito Rokutan · Tsukasa Kawahara · Yuki Kuwano · Kumiko Tominaga · Keisei Nishida · Shigetada Teshima-Kondo

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Abstract Chronic inflammation caused by *Helicobacter pylori* infection or inflammatory bowel disease (IBD) is closely linked to cancer development. Innate immune abnormalities and enhanced production of reactive oxygen species through a phagocyte NADPH oxidase (Nox2) are key issues in understanding the pathogenesis of inflammation-dependent carcinogenesis. Besides Nox2, functionally distinct homologues (Nox1, Nox3, Nox4, Nox5, Duox1, and Duox2) have been identified. Nox1 and Duox2 are highly expressed in the gastrointestinal tract. Although the functional roles of Nox/Duox in the gastrointestinal tract are still unclear, we will review their potential roles in the gastrointestinal immunopathology, particularly in *H. pylori*-induced inflammation, IBD, and malignancy.

Keywords Nox1 · Nox2 · Duox2 · Innate immunity · Inflammation · Carcinogenesis

Introduction

The gastrointestinal epithelium functions as an innate physical and immune barrier against commensal or pathogenic microbes. The epithelial response to both specific and nonspecific microbes is one of the key issues in understanding the mechanism for maintenance of mucosal homeostasis and the pathogenesis of chronic inflammation and possibly malignancy of the gastrointestinal tract [42, 63, 94, 96, 97]. Although, neutrophils, macrophages, and dendritic cells are main players in the innate immune system, gastrointestinal epithelial cells recognize pathogen-associated molecular patterns (PAMPs) by specific host pattern-recognition receptors (PRRs) such as plasma membrane Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (NOD) proteins [2]. Upon recognition of PAMPs, the epithelial cells can trigger inflammation and injury by releasing inflammatory mediators such as cytokines, reactive oxygen species (ROS), and nitric oxide (NO).

Six homologues of the catalytic core ($gp91^{phox}$/Nox2) of phagocyte NADPH oxidase have recently been identified as sources of ROS and named systematically as the *N*ADPH *ox*idase (Nox)/*du*al *ox*idase (Duox) family [18, 47, 72]. These novel enzymes have been proposed to have a variety of functions (for reviews see [18, 47, 51, 72, 73, 112]). In addition to phagocyte Nox2, two Nox/Duox members (Nox1 and Duox2) are highly expressed in the gastrointestinal tract. Nox1 is often called "colon NADPH oxidase", and its mRNA levels are low in the ileum, intermediate in the right colon, and high in the left colon [71]. Duox1 and Duox2 were cloned from human and porcine thyroid glands [33, 35]. The Duox-based hydrogen peroxide (H_2O_2)-generation system is essential for thyroid hormone synthesis [79]. Duox2 expression is not restricted to the thyroid. Geiszt et al. reported high expression in the salivary glands and rectum, and low expression in the cecum and ascending colon [48]. Recently, El Hassani et al. have shown that Duox2 protein is expressed in all segments of the porcine digestive tract and in the human colon, small intestine, and duodenum [37]. Duox2 has an intrinsic Ca^{2+}-, NADPH-dependent H_2O_2-generating activity [7] that may play a potential role in inflammation and host defense, particularly in the cecum and colon.

K. Rokutan (✉) · T. Kawahara · Y. Kuwano · K. Tominaga · K. Nishida · S. Teshima-Kondo
Department of Stress Science, Institute of Health Biosciences, University of Tokushima Graduate School,
3-18-15 Kuramoto-cho,
Tokushima 770-8503, Japan
e-mail: rokutan16@basic.med.tokushima-u.ac.jp

Although the understanding of the functional role of the Nox/Duox family in the gastrointestinal tract is still at the preliminary stage, we will review the potential role of these superoxide (O_2^-)-producing enzymes in immunopathology of the gastrointestinal tract, with particular focus on possible roles of Nox enzymes in innate immunity, inflammation, and carcinogenesis in the stomach and colon.

Nox1 in guinea pig gastric epithelium

Before the discovery of Nox1 [101], a potent NADPH oxidase-like activity in epithelial cells was first documented in primary cultures of guinea pig gastric epithelial cells (surface mucous epithelial cells) [106]. Due to the cross reactivity of the anti-Nox2 antibody used, the enzyme was initially reported as a Nox2-based oxidase-like system [106]. The catalytic core of the oxidase was then molecularly identified as guinea pig Nox1 [105]. The first report [106], however, already showed several features characteristic of the Nox1-based oxidase system. First, without any stimulus, the gastric pit cells spontaneously secrete a large amount of O_2^- (100 nmol/mg protein/h) nearly equivalent to that of mouse peritoneal macrophages. Activation of nuclear factor κB (NF-κB) by ROS, particularly H_2O_2, plays an essential role in cell growth, survival of aged cells, and inflammatory responses [105]. Second, guinea pig gastric Nox1 is extremely sensitive to lipopolysaccharide (LPS) from *Helicobacter pylori* (*H. pylori*) as well as *Escherichia coli* [106]. Under LPS-free conditions, these cells respond to LPS from type I *H. pylori* (2.1 endotoxin units/ml or higher), but not to less virulent type II strains, and up-regulate O_2^- generation tenfold. Lipid A is a bioactive component involved in the priming [67]. Although the molecular mechanisms linking TLR4 and Nox1 activation remain to be elucidated, the evidence suggests for the first time a possible role of Nox1 in the innate immune response of gastric epithelial cells. Third, the membrane-bound enzyme of the cells required undefined cytosolic factors for activation, which were partially replaceable by the cytoplasm from neutrophils [106]. After discovery of the novel homologues of $p47^{phox}$ and $p67^{phox}$, designated NOXO1 (Nox organizer 1) and NOXA1 (Nox activator 1), respectively, as essential cofactors for Nox1 activity [15, 26, 46, 104], the cytosolic factors were then identified as guinea pig NOXO1 and NOXA1 [64]. Finally, guinea pig gastric epithelial cells provided a possible mechanism for *H. pylori* LPS-induced activation of Nox1 [64]. The guinea pig cells constitutively express Nox1, $p22^{phox}$, $p67^{phox}$, NOXA1, and Rac1. *H. pylori* LPS treatment not only increases the Nox1 mRNA, but also newly induces expression of the transcript encoding NOXO1 [64]. In addition, *H. pylori* LPS independently activates Rac1, providing the first evidence that Rac1 may be involved in activation of the Nox1-based oxidase system [64], similar to the effect of Rac2 in neutrophils. The essential role of Rac1 in Nox1-based oxidase activity has now been established in subsequent studies [25, 78, 111]. Nox1-based oxidase is now recognized as a multicomponent enzyme system, consisting of a membrane-bound, functional heterodimer (Nox1 and $p22^{phox}$) [6, 55, 66], associated with NOXO1, NOXA1, and Rac1. Thus, a series of studies on Nox1 in primary cultures of guinea pig gastric mucosal cells [64, 67, 105, 106] has provided substantial information on putative functions of the Nox1-based oxidase system in immunopathology of the stomach. In particular, the series of experiments suggested that *H. pylori* infection might stimulate the expression of Nox1 and NOXO1, thus facilitating ROS-dependent injury of gastric mucosa. On the other hand, the expression of Nox1 mRNA has been systemically examined in various human tissues [24, 69, 101] and the stomach [16, 46, 95], but it has not yet been documented in the human stomach (Fig. 1). It is likely that expression of Nox1 in the stomach is species specific [18].

H. pylori infection and gastric disorders

Before describing potential roles of Nox enzymes in *H. pylori* infection, we will provide a brief review of this important infection for readers unfamiliar with this area of research. *H. pylori* is a noninvasive, non-spore-forming, spiral-shaped Gram-negative rod bacterium [75]. *H. pylori* infects the stomach of half of the human population worldwide and causes chronic active gastritis, which can lead to peptic ulcer disease, gastric adenocarcinoma, and

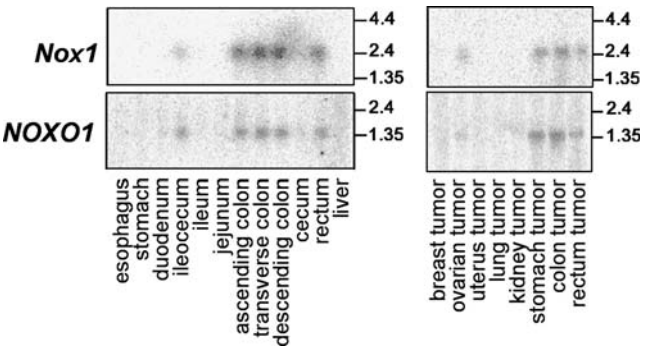

Fig. 1 Expression of Nox1 and NOXO1 mRNAs in the gastrointestinal tract. Using a multiple tissue Northern blot membrane for the human digestive system or human tumors (MTN™) from Clontech (Palo Alto, CA,USA), expression levels of Nox1 and NOXO1 transcripts were measured by Northern hybridization with use of a ^{32}P-labeled cDNA probe for human Nox1 or NOXO1 according to the manufacturers' protocols. It should be noted that Nox1 and NOXO1 mRNAs show a similar expression pattern

mucosa-associated lymphoid tissue lymphoma [84, 87]. However, only 10–15% of individuals infected with *H. pylori* develop peptic ulcer disease, and the risk of gastric cancer is estimated to be 1–3%. *H. pylori* infection activates epithelial cells, neutrophils, dendritic cells, monocytes, and macrophages, and leads to a Th1 type of adaptive immune response [115]. However, the host immune response is ineffective, as the bacterium persists, and the inflammation continues for decades [115]. After the International Agency for Research on Cancer (IARC) classified *H pylori* infection as a class I carcinogen in 1994, a considerable amount of confirmatory evidence has accumulated. Bacterial factors, environmental insults, and the host immune response drive the initiation and progression of mucosal atrophy, metaplasia, and dysplasia toward gastric cancer. Major virulence factors include the cytotoxin-associated gene pathogenicity island (*cag* PAI), which encodes a type IV bacterial secretion system that injects bacterial products into gastric epithelial cells (Fig. 2). The presence of these bacterial products results in signaling events that lead to increased inflammation and neoplastic risk [8, 19, 81, 83, 86, 93]. Vacuolating toxin A (VacA) is also strongly associated with cellular damage and inflammation [8, 81, 86, 93]. Most of *H. pylori* exist in a mucus layer. About 10% of *H. pylori* adhere to gastric epithelial cells, while organisms are rarely found intracellularly. Adherence of *H. pylori* to the gastric epithelium is a complicated process that involves a number of bacterial cell-surface receptors [11]. Recently, it has been shown that gastric epithelial cells sense *H. pylori* peptidoglycan through NOD1 and trigger innate immune responses of gastric epithelial cells [114]. The NOD1 detection of *H. pylori* depends on the delivery of peptidoglycan into host cells by the type IV secretion system encoded by the *cag* PAI [114] (Fig. 2). As for the host factors, single nucleotide polymorphisms in a combination of IL-1β, IL-1β receptor antagonist, TNF-α, and IL-10, which eventually result in enhanced production of IL-1β and TNF-α, and low levels of IL-10 confer a 27-fold increased risk of gastric cancer when infected with *H. pylori* [36]. In addition, the combination of high-risk host genotypes and high-risk bacterial phenotypes further elevate the risk up to 87-fold over base line levels [40].

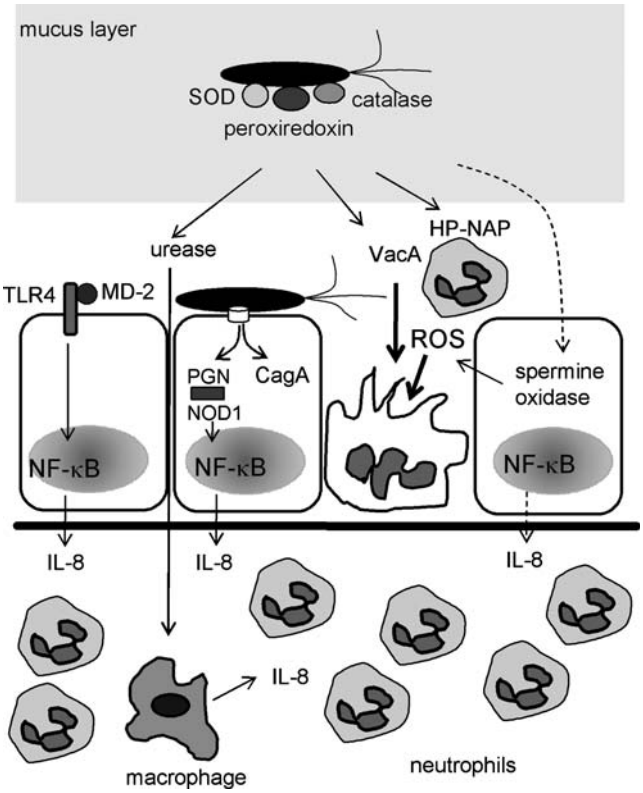

Fig. 2 Interactions between *H. pylori* and gastric epithelial cells for inflammation. Most of *H. pylori* exist in a mucus layer. *H. pylori* accidentally adheres to gastric epithelial cells and injects CagA and peptidoglycan (PGN) into the cells through the type IV secretion system, which triggers production of inflammatory mediators by gastric epithelial cells through activation of NF-κB. In addition to VacA, *H. pylori*-derived urease recruits macrophage, and *H. pylori* neutrophil-activating protein (HP-NAP) directly activates Nox2-based oxidase. *H. pylori* expresses SOD, catalase, and a unique peroxiredoxin on the outer membrane to protect against damage caused by ROS. Ingested bacteria can escape from phagocytosis-dependent killing, while they can dysregulate and activate the Nox2-based oxidase system. Bacteria are not eliminated, and tissue damage is enhanced by ROS. LPS-TLR4/MD-2 is another important route for initiation of inflammatory response of gastric epithelial cells. Spermine oxidase is also considered to be a possible source of ROS in gastric epithelial cells

Nox2 and *H. pylori*-induced inflammation

Usually, during the course of an infection, neutrophils are recruited early, macrophages are more abundant at the later stages, and the numbers of neutrophils decline upon elimination of the invading microbe. In the case of *H. pylori* infection, inflammation is characterized by a massive recruitment of neutrophils that can persist for decades. A large number of neutrophils traverses gastric epithelial cells and encounters *H. pylori* in the mucus layer and at ulcer margins [4], but they are unable to eliminate the noninvasive *H. pylori*. Neutrophil numbers do not decline until bacterial eradication by antibiotic treatment. According to the histological criteria, the gastritis is called "active" when neutrophils are found, representing acute inflammation. Thus, *H. pylori* infection results in a state of "chronic active inflammation". The fact that *H. pylori* remains in the stomach at a high density despite the host response indicates that innate immunity and/or adaptive immunity are ineffective against this bacterium [115].

A massive influx of neutrophils into the gastric mucosa [50, 75] is one of the key factors in *H. pylori*-induced

inflammation and carcinogenesis. The mechanism of the initial insult to the host DNA is unknown; however, it has been suggested that the neoclassical outcome is related to oxidative stress. *H. pylori* induces DNA damage [13, 39, 85] and mutations [89, 109] in the gastric mucosa, which may be important in the pathogenesis of gastric cancer. From this standpoint, ROS derived from Nox2-based oxidase are crucial. Enhanced ROS production has been well documented in the stomach of patients with *H. pylori* infection [32, 102]. Davies et al. [32] found a positive association between ROS production and the infective load of *H. pylori*, and neutrophils are a major source of ROS production. Suzuki et al. [102] have shown that ROS production in gastric mucosa is enhanced by the infection of *cagA*-positive *H. pylori* species together with an extensive accumulation of neutrophils in patients with gastric ulcer.

The interactions between *H. pylori* and gastric epithelial cells are summarized in Fig. 2. *H. pylori* and its products can activate neutrophils [3, 91]. Multiple bacterial virulence factors are known to exacerbate *H. pylori*-induced inflammation, including LPS, PicB, urease, and the vacuolating cytotoxin VacA [30]. Among bacterial products, a virulence factor called *H. pylori* neutrophil-activating protein (HP-NAP) is particularly interesting. HP-NAP is a 150-kDa dodecameric iron-binding protein that promotes adhesion of neutrophils to endothelial cells [38, 108]. Recently, Satin et al. have demonstrated that purified recombinant HP-NAP is a highly antigenic protein that stimulates phagocyte chemotaxis, assembly of Nox2 oxidase, and production of ROS [98]. Another interesting piece of evidence has been provided by Allen et al. [5]. They suggest that *H. pylori* dysregulates Nox2-based phagocyte oxidase. In general, soluble stimuli such as fMLP or phorbol 12-myristate 13-acetate (PMA) promote oxidase assembly at the plasma membrane, and rapid release of O_2^- primarily into the extracellular milieu. In contrast, microbes cause the NADPH oxidase to form phagosomes and release O_2^- into the phagosome lumen, where it has maximal toxicity to the ingested microbe and cause minimal damage to host tissues. In the case of *H. pylori*, the bacterium is readily ingested by neutrophils and induces a rapid and strong respiratory burst similarly to that stimulated by PMA. *H. pylori* is a more potent activator of neutrophils than are opsonized zymosan, *Staphylococcus aureus*, or *Salmonella*. Furthermore, *H. pylori* disrupts NADPH oxidase targeting so that O_2^- is released into the extracellular milieu and do not accumulate inside phagosomes [5]. In addition, Allen et al. suggest that nascent *H. pylori* phagosomes acquire flavocytochrome b_{558}, while $p47^{phox}$ and $p67^{phox}$ are not efficiently recruited or retained on the phagosome membrane [5]. These results suggest that *H. pylori* may cause aberrant activation of Nox2-based oxidase, resulting in accelerated oxidative damage to the gastric mucosa. The dysregulation of Nox2-based oxidase by *H. pylori* may constitute an important part of the strategy for this bacterium to escape from the oxidant-dependent killing system. In addition, *H. pylori* possesses a unique bacterial alkyl hydroperoxide reductase (peroxiredoxin) that helps to escape from ROS-mediated injury [14, 23] (Fig. 2).

ROS derived from neutrophils are believed to damage the gastric mucosa and promote carcinogenesis in patients with *H. pylori* infection. However, Keenan et al. disagreed with this hypothesis [68]. They examined the gastric pathology of wild-type mice and mice with chronic granulomatous disease (CGD) after infection with a mouse-adapted strain of *H. pylori*. They found that glandular atrophy and epithelial cell proliferation (precancerous signs) were rather accelerated in CGD mice in association with increased numbers of neutrophils in the gastric mucosa [68]. They suggest that a functional Nox2-based oxidase is likely to have a protective role by dampening inflammation, and that the release of granule constituents from neutrophils contributes to tissue damage. In fact, there is evidence that inflammatory responses are rather prolonged and enhanced in CGD [44, 54, 80, 118]. Furthermore, CDG animals exhibit altered cytokine production at the inflammatory site [21, 80], and activated T cells from the animals produce more Th1-type cytokines [62]. Thus, there can be no doubt that neutrophils play a central role in *H. pylori*-induced gastric damage; however, the role of the Nox2-based oxidase system should be more carefully assessed. In addition, ROS from other sources should also be considered with regard to the tissue damage. The potential roles of other Nox enzymes have not been fully examined. However, a recent report suggests that the generation of ROS in the gastric epithelial cells may also contribute to dysfunction of these cells [117]. The generation of H_2O_2 by the induction of spermine oxidase in *H. pylori*-stimulated gastric epithelial cells has been suggested as one of the specific causes of the oxidative stress that leads to both apoptosis and DNA damage [117] (Fig. 2).

Nox1 in *H. pylori*-related gastric disorders

Guinea pig gastric epithelial cells possess a Nox1-based oxidase system that can be up-regulated by *H. pylori* LPS. However, there is still disagreement about whether Nox1 is expressed in the human stomach. Salles et al. examined the expression of Nox1, Nox2, and Nox5 in gastric biopsies of hospitalized geriatric patients [95]. They found expression of Nox2 and Nox5 mRNA, but not Nox1 mRNA. Because Nox5 is generally found in lymphoid tissues [17], this observation suggests the possibility that Nox5 may be located in the mucosa-associated lymphoid tissue (MALT).

But at present, there is no direct evidence to support this hypothesis. As for Nox1, Salles et al. [95] examined 30 patients (7 men and 23 women, mean age: 84 years ±6.1, range: 67–95 years), some of whom had peptic ulcer (n=6) or gastric cancer (n=2). Chronic inflammation was present in 24 (80%) patients; however, all samples were negative for Nox1 mRNA expression. In a subsequent study, Szanto et al. [103] examined Nox1 mRNA expression in human normal stomachs and gastric tumors using a tumor cDNA array [103] but did not detect any significant Nox1 transcript signals. In contrast, August et al. [12] analyzed a total of 50 patients with gastritis (n=20), gastric ulcer (n=20), and gastric carcinoma (n=10) and found that Nox1 transcript was significantly expressed in gastritis, gastric ulcer caused by *H. pylori* virulent strains, and gastric cancer. Thus, there is still disagreement on Nox1 expression and its association with gastric diseases.

H. pylori infection and gastric cancer

H. pylori infection increases the risk of developing both intestinal- and diffuse-type gastric adenocarcinomas. The diffuse-type gastric cancer is less common and occasionally associated with germline mutations in the E-cadherin gene [53]. This type of cancer also develops during the progression of atrophic gastritis with *H. pylori* infection, particularly with active gastritis [110]. The primary stages in the development of intestinal-type gastric adenocarcinomas are: chronic active non-atrophic gastritis → multifocal atrophy → intestinal metaplasia (first complete, then incomplete) → dysplasia → invasive carcinoma [28] (Fig. 3). Intestinal metaplasia is a crucial precancerous stage. At this stage, the original glands and the foveolar epithelium are replaced by cells with intestinal phenotype. The metaplastic intestinal cells in the initial phases resemble the small intestinal mucosa: they are lined by eosinophilic absorptive enterocytes with a well-developed "brush border" composed of myriads of microvilli. Goblet cells are found at regular intervals. This type of metaplasia is called "complete", reflecting the fact that it secretes the normal set of digestive enzymes such as sucrase, trehalase, and alkaline phosphatase [76]. At later stages, the metaplastic cells lose their small intestinal phenotypes, acquire morphologic features of the large intestine, and are lined only by goblet cells of different sizes and shapes. This type is called "incomplete". Thus, the dynamics of the precancerous process show a gradual phenotypic transformation from normal epithelium to metaplastic cells with small intestinal morphology and then to cells resembling colonic mucosa that express both gastric and colonic mucins. Inappropriately activated intestine-specific factors, including *caudal*-related homeodomain proteins (CDX1 and CDX2), MUC6, and trefoil factor family peptide 2, have been suggested to participate in the development of intestinal-type cancer [100, 119]. Among them, gastric expression of CDX2 alone was shown to be sufficient to induce intestinal metaplasia [100] and gastric polyps consisting of intestinal-type adenocarcinoma in mice [82]. Nox1 is not expressed in the small intestine, whereas the human colon constitutively expresses abundant Nox1. It might be possible that Nox1 is expressed in "incomplete" metaplasia. The proximal 5′-flanking region of the human Nox1 gene contains potential binding sites for GATA factors and the *caudal*-related homeodomain proteins (CDX1 and CDX2), and these transcription factors facilitate basal promoter activity of this gene in the colon cancer cell line, $CaCo_2$ [20, 113]. Furthermore, the Th1 cytokine,

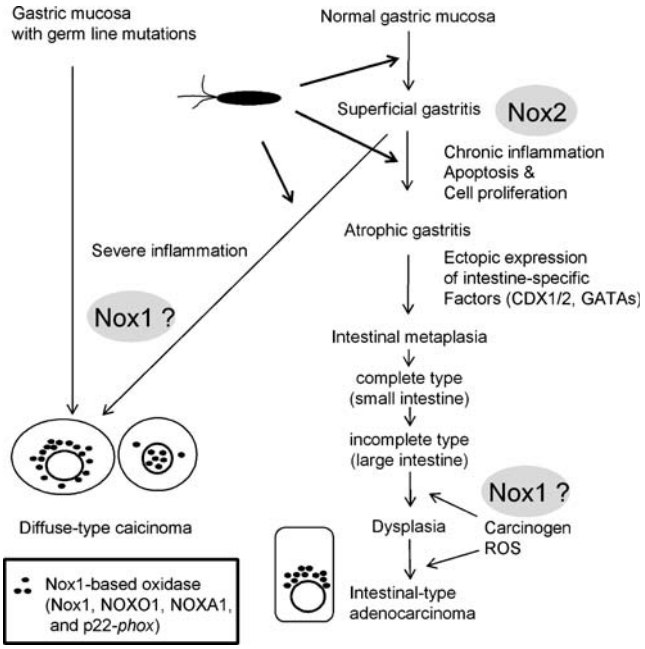

Fig. 3 Possible pathway of pathological events in development of gastric adenocarcinomas. *H. pylori* infection increases the risk of developing both intestinal- and diffuse-type gastric adenocarcinomas. The diffuse-type gastric cancer is less common and occasionally associated with germline mutations in the E-cadherin gene [53]. This type of cancer also develops during the progression of atrophic gastritis with *H. pylori* infection, particularly with active gastritis [110]. In the case of intestinal-type adenocarcinomas, *H. pylori* colonization leads to superficial gastritis, chronic atrophic gastritis, intestinal metaplasia (complete and then incomplete type), and finally to dysplasia and adenocarcinomas. During chronic inflammation and tissue regeneration, intestine-specific factors (CDX1/2, GATAs, etc.) are inappropriately expressed. ROS and carcinogens are believed to trigger malignant transformation to dysplasia and cancer. Nox2-based oxidase plays a crucial role during chronic inflammation. Nox1-based oxidase components are specifically expressed in both diffuse- and intestinal-type adenocarcinomas. The components are preferentially associated with Golgi apparatus in these cancer cells, while diffuse-type adenocarcinomas also contained cancer cells having Nox1 and its partner proteins in their nuclei [107]

interferon (IFN)-γ, stimulates transcription of the human Nox1 gene in a colon cancer cell line, T84 [71]. These data implicate that an enhanced Th1 response may cause dysregulated expression of Nox1 in the presence of GATA factors and CDX2. However, Nox1 transcript [95] and protein [107] have not been detected in atrophic gastritis with intestinal metaplasia.

Nox1 in gastric cancer

As shown in Fig. 1, the expression of Nox1 mRNA coincides with that of NOXO1 mRNA in the gastrointestinal tract. Neither of these mRNAs is present in the normal stomach, but a gastric tumor expresses significant amounts of both transcripts, as also observed in colon and rectal tumors (Fig. 1). Recently, Tominaga et al. [107] carefully investigated the expression of Nox1-based oxidase components in the human stomach. Using an immunohistochemical technique, they showed that Nox1 and NOXO1 proteins were absent from chronic atrophic gastritis, adenomas, or surrounding tissues of adenocarcinomas. In contrast, Nox1 and its partner proteins (NOXO1, NOXA1, and $p22^{phox}$) were expressed in intestinal-type adenocarcinomas (19 out of 21 cases), diffuse-type adenocarcinomas (15 out of 15 cases), and signet-ring cell carcinomas (9 out of 9 cases). In addition, the study showed that NOXO1, NOXA1, and $p22^{phox}$ as well as Nox1 were predominantly associated with the Golgi apparatus in these cancer cells. At the same time, they found that diffuse-type adenocarcinomas also contained cancer cells having Nox1 and its partner proteins in their nuclei. Nox1-expressing cancer cells exhibited both gastric and intestinal phenotypes. At present, it is unknown whether the increased Nox1-based oxidase system is actually functional in human gastric cancer tissues. The mechanism(s) responsible for cancer-specific expression of Nox1 and its essential partner proteins are also unknown. As shown in Fig. 3, phagocyte Nox2 is crucial in the stages of chronic atrophic gastritis. The concept of the multistep progression of gastric cancer implicates an important role of ROS particularly in the course of transformation into dysplasia and cancer [27, 41, 58] (Fig. 3). Although the study by Tominaga et al. [107] did not precisely examine the expression of Nox1 in the stages of incomplete metaplasia or dyspresia, the results suggest an unproven hypothesis that Nox1 might be involved in the ROS-mediated transformation. The study suggests that the Nox1-base oxidase may be a potential marker of neoplastic transformation and play an important role in oxygen radical- and inflammation-dependent carcinogenesis in the human stomach. Further studies are needed to address these issues.

Nox1 expression in the colon

Nox1 is highly expressed in the colon [16, 69, 101]. Nox1 mRNA was detected in human colon epithelial cells along the crypt–villus axis [103, 113], while another study suggested preferential expression of the transcript in the lower part of the crypts [45]. On the other hand, immunohistochemistry showed that Nox1 protein exhibits maturation-dependent expression along with upward migration and that surface mucous cells possess the most abundant Nox1 protein in the human [43] and guinea pig [65] colons. Regarding the mechanism for preferential expression of this Nox homologue in colonic epithelial cells, a recent study has identified a complex element between −422 and −291 of the Nox1 promoter in the $CaCo_2$ cell line, which is critical for basal promoter activity [113]. This study shows that the intestinal specific transcription factors GATA-6 and CDX1/2, and hepatocyte nuclear factor-1α (HNF-1α) cooperatively stimulate transactivation of the Nox1 promoter [113]. Nox1 mRNA expression follows a gradient with low levels in the proximal colon and high levels in the distal colon [103, 113]. The expression gradients of Nox1 mRNA in the colon paralleled those of GATA-6, HNF-1α, and CDX1 [113], suggesting that developmental, tissue-restricted transcription factors play a key role in Nox1 expression in vivo.

Differentiation-dependent expression of Nox1 in colonic epithelial cells

Nox1-derived ROS were initially thought to have tumorigenic and angiogenic functions [9, 10, 101]. However, subsequent studies have revealed that the Nox1-transfected NIH 3T3 cells carry a mutation of Ras that may account for the abnormal cell growth and transformation [72]. In cultured large intestinal epithelial cells, one study found the highest Nox1 levels in the subconfluent state [88], while another study showed that NOX1 levels were increased in colonocytes upon differentiation and growth arrest [45]. In addition to the maturation-dependent expression of Nox1 protein in the human [43] and guinea pig [65] colons, induction of differentiation of colon cancer cell lines ($CaCo_2$ and HT29 cells) with 1α,25-dihydroxyvitamin D_3 or IFN-γ enhances Nox1 expression and decreases cell proliferation [45], suggesting that colonic Nox1 has distinct functions other than mitogenic properties. The enhanced expression of Nox1 in the subconfluent state may be explained by a recent report showing that Nox1-dependent O_2^- production stimulates the migration of HT29-D4 colonic adenocarcinoma cells on collagen-I during cell spreading [31].

Role of Nox1 in innate immunity of the colon

The human gastrointestinal tract contains between 10 and approximately 100 trillion microorganisms, with concentrations increasing along the gastrointestinal tract from stomach (~10^3), jejunum (~10^4), ileum (10^5~10^8), to the colon (10^{10}~10^{14}). The intestinal mucosa play a crucial role in maintaining barrier function and protecting against bacterial invasion while permitting commensal bacteria to aid in nutrient metabolism. The interaction between commensal bacteria and the innate immune system plays a central role in the maintenance of mucosal homeostasis, and innate immune abnormalities in response to commensal bacteria have been suggested to be responsible for chronic inflammatory disorders and malignancies [42, 63]. There is increasing evidence that TLR and NOD signaling plays a central role in chronic gastrointestinal inflammation. Although, the expression of TLRs in the gastrointestinal tract has been examined, expression, localization, and function of individual TLRs remain unclear. In the human stomach, TLR2, TLR4, TLR5, and TLR9 are known to be expressed by epithelial cells [99]. The small intestine (including duodenum, jejunum, and ileum) and the large intestine express most of the TLRs, but usually TLR signaling in intestinal cell lines appear to be down-regulated [1]. For example, intestinal epithelial cell lines express detectable amounts of TLR 4 transcript, while TLR4 protein is undetectable, and these cells lack CD14 and MD2 required for recognition of LPS. However, TLR signaling especially by TLR4 has been suggested to be up-regulated in the setting of chronic intestinal inflammation [42, 63]. At the same time, Nox/Duox family members may act as a downstream effectors of PRRs such as TLRs and NOD proteins (for details see [51] and a review of this series).

It has been suggested that Nox1 may play a pivotal role in local innate immune and inflammatory responses. This idea was originally proposed based on a series of experiments using guinea pig gastric epithelial cells in culture [64, 67, 105, 106]. A possible link between Nox1 and TLRs in large intestinal epithelial cells was also first reported by Kawahara et al. [65]. They studied TLR-mediated induction of Nox1 using a colon cancer cell line (T84). T84 cells express massages for TLR2, TLR4, and TLR9. They respond to a recombinant structural protein of flagella filament (rFliC) from *Salmonella enteritidis* and up-regulate O_2^- generation fourfold. Neither LPS from *H. pylori* or *E. coli*, peptidoglycan from *Staphylococcus aureus*, nor CpG DNA augments the O_2^- production [65]. T84 cells show highly polarized expression of TLR5 on the basolateral surface [49, 57]. Certain flagellated bacteria are capable of translocating flagellin and stimulating TLR5, leading to the activation of pro-inflammatory signals, particularly those associated with the NF-κB pathway [49, 56, 57]. Guinea pig gastric mucosal cells are sensitive to LPS [67, 106], while large intestinal epithelial cells may be preferentially stimulated by flagellin for augmentation of Nox1-mediated O_2^- production [65]. Although the molecular mechanism linking Nox1 and TLR4/TLR5 remains to be explored, the difference in the sensitivity may reflect the luminal environments: colonic epithelial cells are always exposed to Gram-negative bacteria. While TLR4 may be useful in the stomach because no bacteria except for *H. pylori* can colonize in the severe acidic conditions. In fact, TLR4 has been suggested to play a potential role in regulation of inflammation caused by *H. pylori* infection [8].

Human colon cancer cell lines such as T84 or $CaCo_2$ cells produce only small amounts of O_2^- (<2 nmol/mg protein/h), as NOXO1 and NOXA1 are absent and poorly expressed, respectively. However, it is possible that colonic epithelial cells may actually produce significant amounts of O_2^- in vivo. Freshly isolated and cultured colonic epithelial cells from guinea pigs constitutively express Nox1, NOXO1, $p67^{phox}$, NOXA1, $p22^{phox}$, and Rac1 [65]. This Nox1 is fully activated and spontaneously secretes O_2^- at a higher rate (about 160 nmol/mg protein/h) than does guinea pig gastric Nox1 primed with *H. pylori* LPS (about 100 nmol/mg protein/h). However, the amount of ROS was not enough to inhibit the growth of *Salmonella enteritidis* or to kill the bacterium in vitro [65], thus indicating that ROS derived from a Nox1-based oxidase system may be only a warning signal that recruits neutrophils to kill pathogenic microbes. Surface mucous epithelial cells of the stomach and colon might use different TLR members in recognizing respective pathogenic microbes, activate Nox1, and finally produce defensive mediators. These results suggest that the Nox1 activation constitutes an early response in epithelial cell host defense against pathogens. However, functional and mechanistic studies would be essential to demonstrate the role of Nox1 in host defense.

Role of Nox enzymes in inflammation of the colon

Another important feature of colonic Nox1 is the regulation of its expression. IFN-γ activates transcription of the human Nox1 gene in T84 cells through signal transducers and activators of transcription 1 (STAT1) and a γ-activated sequence (GAS) element located between −3818 and −3810 bp of the Nox1 5′-flank [71]. In addition to IFN-γ [45, 71], IL-1β, IL-18, and TNF-α similarly induce Nox1 in T84 cells, suggesting that Nox1 may be inducible in response to a variety of proinflammatory cytokines (Fig. 4). On the other hand, TNF-α acts as the most potent activator

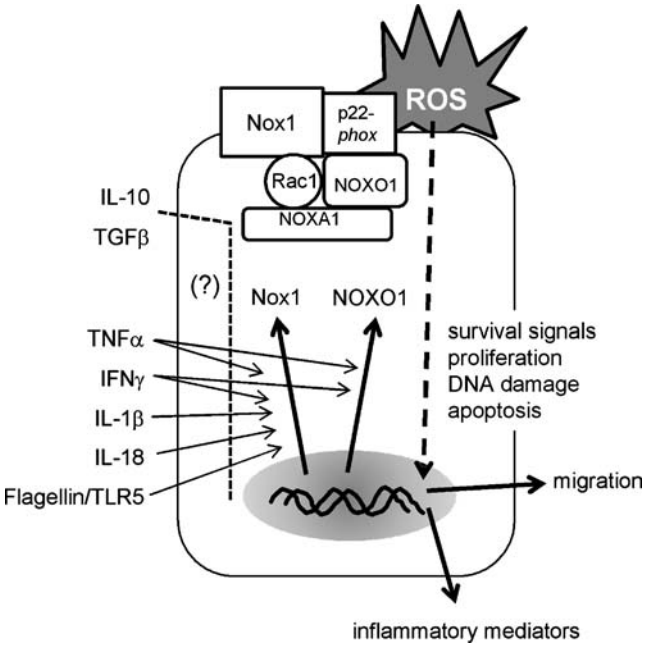

Fig. 4 Expression and possible functions of Nox1-based oxidase in large intestinal epithelial cells. A variety of pro-inflammatory mediators such as IL-1β, IL-18, IFNγ, TNFα, and TLR5 ligand (flagellin) stimulates Nox1 expression, while only distinct cytokines such as IFNγ and TNFα are able to induce NOXO1 to enhance O_2^- generation in a colon cancer cell line (T84). Anti-inflammatory cytokines (IL-10 and TGFβ) inhibit the stimulatory actions of pro-inflammatory cytokines (unpublished observations). ROS produced by Nox1 cause DNA damage and enhance proliferation, survival, and apoptosis depending on the amounts of ROS produced. Nox1-derived ROS initiate and enhance production of inflammatory mediators by large intestinal epithelial cells. Nox1-derived ROS are considered to stimulate cell migration

of Nox1-based oxidase activity in T84 cells in association with marked induction of NOXO1 (unpublished observations). Furthermore, immunosuppressive cytokines such as TGF-β or IL-10 effectively block this TNF-α-stimulated activation of Nox1 (unpublished observations). These findings indirectly suggest a potential role of Nox1 in inflammatory disorders of the colon. Crohn's disease and ulcerative colitis, known as inflammatory bowel disease (IBD), are common chronic inflammatory conditions of the gastrointestinal tract. Virtually all inflammatory mediators investigated to date seem to be dysregulated in the inflamed intestinal mucosa of patients with IBD. TNF-α is now considered to be one of the crucial inflammatory mediators [22]. ROS produced in large amounts by the massively infiltrating phagocytes are also believed to constitute a major tissue-destructive force and may contribute significantly to the pathogenesis of inflammatory bowel disease [70, 92]. On the other hand, CGD patients develop an inflammatory bowel disease, suggesting that NOX2-derived ROS might play a role in the defense against colon inflammation [59, 74]. The discovery of Nox1, highly expressed in the colon, raised the question whether Nox1-based oxidase system could be involved in the pathogenesis of inflammatory bowel disease. However, to date, little information is available in this regard. An in situ hybridization study by Szanto et al. demonstrated the presence of Nox1-positive lymphocytes in the appendix, and in lesions of Crohn's disease and ulcerative colitis [103]. However, at present, the pathological significance of Nox1-positive lymphocytes is unknown. Further studies are required to confirm this surprising finding.

Nox1 and colon cancer

ROS generation has been implicated in the progression from inflammatory bowel disease to cancer [29, 61]. Nox1 might participate in development of colon cancer through ROS-dependent DNA damage and stimulation of cell proliferation. However, at present, no convincing data support this possibility. Nox1 shows a differentiation-dependent expression pattern. In situ hybridization and transcriptome analyses [45, 103] failed to detect consistent differences in Nox1 transcript expression levels between normal and tumor samples of the colon. Down-regulation of Nox1 using antisense oligonucleotides did not inhibit proliferation of $CaCo_2$ cells [45]. Thus, it is unlikely that Nox1 directly stimulates cell proliferation. Fukuyama et al. [43] carefully examined the Nox1 protein expression in human colon tissues from patients with adenomas or adenocarcinomas. Nox1 showed a differentiation-dependent expression pattern in adenocarcinomas: well differentiated adenocarcinomas expressed abundant Nox1 protein, whereas poorly differentiated adenocarcinomas scarcely expressed Nox1. The relationship between Nox1 and mitogenic activity was analyzed by a double-immunostaining method using anti-Nox1 antibody and a proliferation marker (Ki-67). The analysis showed that Ki-67-negative, well-differentiated tumor cells contained abundant Nox1, while Ki-67-positive, proliferating cells did not express Nox1 [43], thus supporting the concept that Nox1 may not be directly linked to mitogenic stimulation of large intestinal epithelial cells. However, NF-κB is predominantly activated in adenoma and adenocarcinoma cells expressing abundant Nox1 [43], suggesting a putative role of Nox1-derived ROS in the activation of NF-κB. Although Nox1 protein is expressed in both normal and malignant colon tissues, Nox1 is overproduced in the precancerous stage (benign polyps), and ROS produced by overexpressed Nox1 may increase a risk of colon cancer due to their genotoxic and proinflammatory properties. Recent evidence has now demonstrated an association between NF-κB activity and cancer-promoting action [52, 60, 90]. Abnormally active NF-κB inhibits apoptosis, which eliminates defective cells,

thus contributing to cancer cell survival and resistance to drug and radiation therapies [52, 60, 90]. ROS over-produced in inflamed tissues may cause carcinogenic mutations and activate NF-κB and other crucial components involved in mitogen signaling [34, 77]. Thus, ROS-mediated alterations in DNA, imbalance between epithelial cell proliferation and apoptosis as well as tumor angiogenesis [116] may play pivotal roles in the development of inflammation-associated cancer.

DUOX2 in the gastrointestinal tract

DUOX2 is also expressed in the distal gastrointestinal tract, in particular, cecum, sigmoidal colon, and rectal glands [35, 37, 48]. The more precise anatomical location is suggested in one study to be highly differentiated enterocytes within the apical membrane of the brush border [37], while another study suggested the lower half of the rectal glands [48]. The possible function of DUOX2 in the distal gastrointestinal tract includes a role in the host defense. Duox2 may provide H_2O_2 that supports lactoperoxidase-mediated antimicrobial defense mechanisms on the mucosal surface possibly in collaboration with lactoperoxidase [37, 48]. Definitely, further studies are needed to understand the pathophysiology of Duox2 in the gastrointestinal tract.

Conclusion

Recently available data strongly suggest that Nox1 in gastrointestinal epithelial cells and Nox2 in phagocytes and dendritic cells may play important roles in regulation of innate immunity. Although clear functional and mechanistic data are still missing, Nox1, Nox2, and Nox4 are suggested to be major downstream effectors of TLRs. Innate immune abnormalities are now considered to be key issues in understanding the pathogenesis of chronic atrophic gastritis and IBD as well as inflammation-dependent carcinogenesis. Specific induction of the Nox1-based oxidase system in gastric cancer and up-regulation of this oxidase with proinflammatory mediators suggest a potential role of Nox1 in inflammation- and ROS-mediated carcinogenesis in the gastrointestinal tract. These preliminary observations should be functionally and mechanistically confirmed by upcoming experiments to fully understand the role of Nox/Duox in innate immunity and carcinogenesis in the gastrointestinal tract.

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