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Forum Review

NADPH Oxidases in the Gastrointestinal Tract: A Potential Role of Nox1 in Innate Immune Response and Carcinogenesis

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ABSTRACT

The gastrointestinal epithelium functions as physical and innate immune barriers against commensal or pathogenic microbes. NADPH oxidase 1 (Nox1) and dual oxidase 2 (Duox2), highly expressed in the colon, are suggested to play a potential role in host defense. Guinea-pig gastric pit cells and human colonic epithelial cells (T84 cells) express Nox1. With regard to activation of Nox1, the gastric epithelial cells are primed with *Helicobacter pylori* lipopolysaccharide, whereas T84 cells preferentially use the Toll-like receptor (TLR) 5, rather than TLR4, against *Salmonella enteritidis* infection. Thus, gastric and colonic epithelial cells may use different TLR members to discern pathogenicities among bacteria, depending on their environments and to activate Nox1 appropriately for host defense. Nox1-derived reactive oxygen species (ROS) have been implicated in the pathogenesis of inflammation-associated tumor development. The human stomach does not express Nox1. *Helicobacter pylori* infection alone does not induce it, whereas Nox1 is specifically expressed in gastric adenocarcinomas. In the human colon, Nox1 is differentiation-dependently expressed, and its expression is upregulated in adenomas and well-differentiated adenocarcinomas. Although Nox1 expression may not be directly linked to mitogenic activity, Nox1-derived ROS may exert a cancer-promoting effect by increasing resistance to programmed cell death of tumor cells. *Antioxid. Redox Signal.* 8, 1573–1582.

INTRODUCTION

In addition to functions in digestion, nutrient transport, water and mineral absorption, and endocrine and paracrine hormone production, the gastrointestinal epithelium serves a primary protective role against irritants derived from foods and commensal or pathogenic microbes. Physical barrier and innate immune barrier functions regulate the density of luminal microorganisms. At the same time, the intestinal epithelium takes an active part in the induction of adaptive immune surveillance at the mucosal surface. Thus, homeostasis of the epithelium is maintained by a finely tuned balance between response and tolerance to the luminal microbes. Disruption of innate and/or adaptive responses results in chronic, persistent inflammation. Epidemiologic data show a clear association between chronic inflammatory conditions and subsequent malignant transformation in the inflamed tissue (17).

The gastrointestinal tract is the site of a significant proportion of the inflammation-associated tumor development. A well-known example is gastric cancer that is associated with chronic inflammation caused by infection of a spiral-shaped, gram-negative bacterium, *Helicobacter pylori* (*H. pylori*) (39, 63, 75). *H. pylori* colonizes in mucous layer of the stomach. *H. pylori* attaches selectively to surface mucous epithelial cells (pit cells) and triggers inflammation of gastric mucosa, leading to the development of gastritis and ulcer diseases. Chronic, persistent infection with *H. pylori* causes a temporal sequence of pathologic changes: superficial gastritis, chronic atrophic gastritis, intestinal metaplasia, and dysplasia, and eventual development of gastric cancer (39). The strong association is also observed in colon carcinogenesis

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arising in individuals with inflammatory bowel diseases, particularly chronic ulcerative colitis (41).

Recent evidence has now tied nuclear factor (NF)-κB activity into cancer-promoting action (34, 40, 64). Abnormally active NF-κB inhibits apoptosis that eliminates defective cells, thus contributing to cancer development and resistance to drug and radiation therapies (34, 40, 64). Reactive oxygen species (ROS) overproduced in inflamed tissues may cause carcinogenic mutations and activate NF-κB and other crucial components for mitogen signaling (21, 35). Thus, ROS-mediated alterations in DNA and imbalance between epithelial cell proliferation and apoptosis may play a pivotal role in the development of inflammation-associated cancer (13, 39). Transmigrated phagocytes are a primary source of ROS in the inflamed tissue. However, several lines of evidence suggest that NADPH oxidase 1 (Nox1) and probably dual oxidase 2 (Duox2) may play a crucial role in the initiation of local innate immune response and inflammation of the large intestine. It is also suggested that dysregulated expression of Nox1 may be associated with inflammation- and oxygen radical–dependent cancer development. Although the study on the function of the Nox/Duox family has just gotten under way, in this review, we focus mainly on roles of Nox1 in innate immune response and in the development of cancer in the gastrointestinal tract.

NADPH OXIDASE (Nox)/DUAL OXIDASE (DUOX) FAMILY IN THE GASTROINTESTINAL TRACT

Over the last several years, six homologues of gp91^{phox} have been identified and named systematically as the NADPH oxidase (Nox)/dual oxidase (Duox) family (31, 53, 54). These novel enzymes are proposed to serve a variety of functions, including regulation of cell growth (5, 69, 73), blood pressure (20, 57), atherosclerosis (36, 55, 68), thyroid hormone synthesis (59), host defense (32, 46, 48), and otoconia morphogenesis (8, 61). In the gastrointestinal tract, two Nox/Duox members (Nox1 and Duox2) are highly expressed. Nox1 is often called as colon NADPH oxidase, and its message is low in the ileum, intermediate in the right colon, and high in the left colon (71). Duox1 and Duox2, previously known as Thox1 and Thox2, respectively, were cloned from human and porcine thyroid glands (18, 22). The Duox-based hydrogen peroxide (H_2O_2)-generation system is essential for thyroid hormone synthesis, which has been confirmed by the recent report on congenital hypothyroidism in a patient with a biallelic inactivating mutation in the Duox2 gene (59). Duox2 expression is not restricted to the thyroid. Geiszt *et al*. (32) reported high expression in the salivary glands and rectum, and low expression in the cecum and ascending colon. Recently, El Hassani *et al*. (25) showed that Duox2 protein is expressed in all segments of the porcine digestive tract and in the human colon, small intestine, and duodenum (25). Duox2 has an intrinsic Ca^{2+}-, NADPH-dependent H_2O_2-generating activity (3) that may play a potential role in inflammation and host defense, particularly in the cecum and colon. In addition to the phagocyte NADPH oxidase (gp91^{phox}/Nox2), lymphoid cell–specific Nox5 is also found in the small intestine (9).

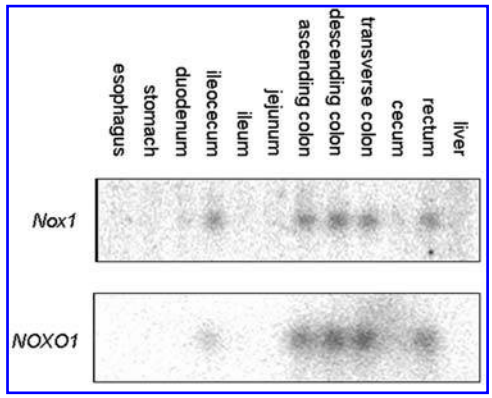

FIG. 1. Expression of Nox1 and NOXO1 mRNAs in human tissues. A multiple-tissue Northern blot membrane for the human digestive system (MTN; Clontech, Palo Alto, CA) was hybridized using the ^{32}P-labeled cDNA probe for human Nox1 or NOXO1.

Several Nox family NADPH oxidases are now recognized as multicomponent enzyme systems. Novel homologues of p47^{phox} and p67^{phox}, designated NOXO1 (Nox organizer 1) and NOXA1 (Nox activator 1), respectively, were identified as essential cofactors for Nox1 activity (7, 14, 30, 72).

As shown in Fig. 1, the Nox1 mRNA is constitutively expressed in the entire colon tissues and in the ileocecum at a lower level, whereas it is not detected in the other tissues examined (Fig. 1, upper panel). The expression of NOXO1 mRNA coincides with that of the Nox1 transcript in the human gastrointestinal tract (Fig. 1, lower panel), similarly as previously reported (30, 72). The NOXA1 mRNA is also highly expressed in the colon. However, compared with NOXO1, the NOXA1 transcript is more widely distributed in the gastrointestinal tissues, including the stomach and small intestine, and in other organs (30, 72).

Nox1 EXPRESSED IN GUINEA-PIG GASTRIC EPITHLIUM

Before the discovery of a novel homologues of gp91^{phox}, mitogen oxidase 1 (Mox1, now renamed as Nox1) (69), we had reported that primary cultures of guinea-pig gastric pit cells (surface mucous epithelial cells) possessed a potent NADPH oxidase–like activity (74). This oxidase was then molecularly identified as guinea-pig Nox1 (GenBank accession number AB099629) (45, 73). The membrane-bound Nox1 required undefined cytosolic factors that were partially replaced by the cytoplasm from neutrophils (74). Subsequently, the cytosolic factors were identified as guinea pig NOXO1 (GenBank, AB105906) and NOXA1 (GenBank, AB105907) (45). Some disadvantages occur with the use of the primary cultures. Guinea-pig gastric pit cells in primary culture cannot be maintained for longer periods (>4 days), reflecting the rapid cell-turnover rate *in vivo* (66). Furthermore,

this short-term culture is not applicable for specific knockdown experiments using short interfering RNAs.

Despite the limitations, the gastric pit cell Nox1 displays several important features characteristic of this enzyme. First, this enzyme has a potent superoxide anion (O_2^-)-producing capability easily detectable by the cytochrome *c* method. Second, the Nox1 can be primed by treatment with lipopolysaccharide (LPS) from *Helicobacter pylori* as well as *Escherichia coli* (*E. coli*), which makes it possible to study the underlying mechanism for Nox1 activation. Finally, this system is potentially useful to investigate functions of this enzyme.

ACTIVATION OF Nox1 IN GUINEA-PIG GASTRIC PIT CELLS

Primary cultures of guinea-pig gastric mucosal cells are extremely sensitive to LPS from *H. pylori* as well as *E. coli*. 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 upregulate O_2^- generation 10-fold (48). Lipid A is a bioactive component for the priming (48). The guinea-pig cells constitutively express Nox1, $p22^{phox}$, $p67^{phox}$, its homolog NOXA1, and Rac1. The LPS treatment not only increases the Nox1 mRNA to a greater extent but also newly induces expression of the transcript encoding NOXO1, required for the Nox1 activity (45). Because mRNAs for Nox2, Nox3, and Nox4 were not detected in guinea pig gastric mucosal cells, even after treatment with *H. pylori* LPS, as well as in the quiescent cells (45 and unpublished observations), it is likely that Nox1 is responsible for the enhanced ROS generation by gastric pit cells primed with LPS. In addition, *H. pylori* LPS activates Rac1 (*i.e.*, conversion of Rac1 to the GTP-bound state). A phosphoinositide 3-kinase (PI3K) inhibitor LY294002 blocked the *H. pylori* LPS-induced Rac1 activation and O_2^- generation without affecting the expression of Nox1 and NOXO1 mRNAs. This inhibition was completely restored by transfection of an adenoviral vector encoding a constitutively active Rac1 (G12V) (45) (Fig. 2).

As shown in Fig. 2A, for activation of the phagocyte NADPH oxidase containing $gp91^{phox}$ (Nox2) as the catalytic center, 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 $p22^{phox}$, which interaction is crucial for the oxidase activation; the SH3 domains are normally masked via intramolecular interaction with the autoinhibitory region.

A hypothetical model for activation of Nox1 in guinea-pig gastric pit cells is shown in Fig. 2B. In guinea-pig gastric pit cells, the *H. pylori* LPS-stimulated upregulation of O_2^- pro-

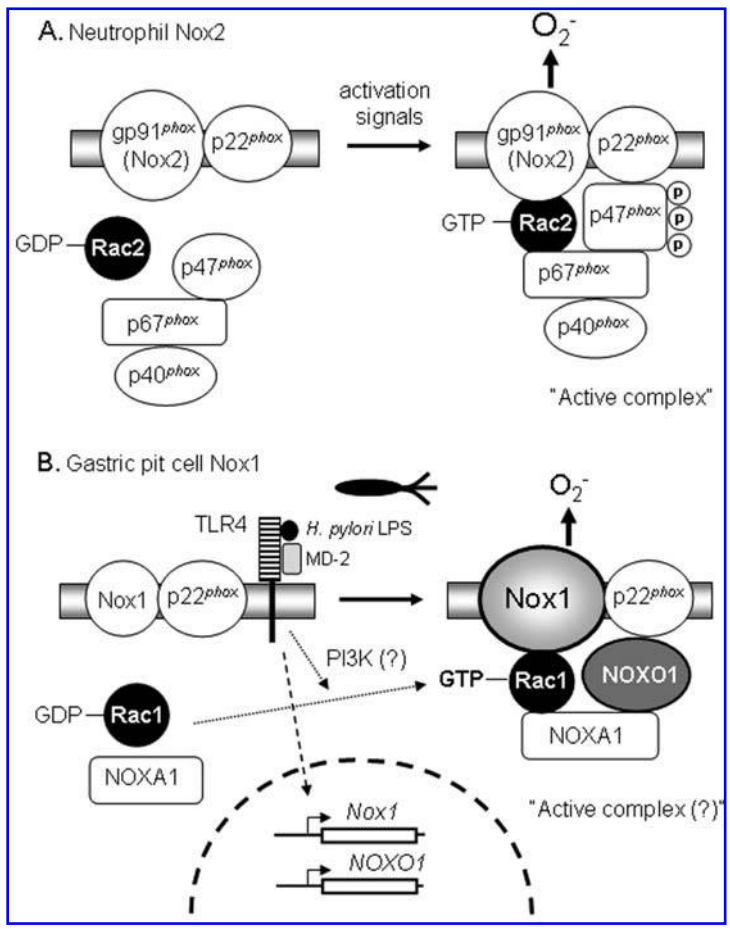

FIG. 2. A hypothetical model for Nox1 activation. (**A**) In response to appropriate stimuli, cytosolic components ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) assemble with $p22^{phox}$ and Nox2 in association with activation of Rac2, leading to stimulation of the respiratory-burst response. (**B**) Guinea-pig gastric pit cells respond to *H. pylori* LPS and markedly increase O_2^- generation. In this case, the LPS not only increases the Nox1 mRNA to a greater extent but also newly induces expression of the message encoding Nox organizer 1 (NOXO1), a novel $p47^{phox}$ homologue, required for the Nox1 activity. In addition, *H. pylori* LPS activates Rac1, probably in a PI3K-dependent manner. The activation of gastric Nox1 requires two distinct events: transcriptional upregulation of Nox1 and NOXO1, and activation of Rac1.

duction may require two distinct events: transcriptional activation of Nox1 and NOXO1, and activation of Rac1. Because NOXO1 lacks the autoinhibitory region (7, 30, 72), NOXO1 is capable of binding via its SH3 domains to $p22^{phox}$ without any conformational changes (72). Once Noxo1 is synthesized together with Nox1 as in the LPS-treated gastric mucosal cells, Nox1 complexed with $p22^{phox}$ is expected to interact constitutively with NOXO1, thereby being in a quasi-activated state. Because coexpression of Nox1 and $p22^{phox}$ results in enhanced O_2^- generation and increased stabilization of both components (2, 47, 72), Nox1 and $p22^{phox}$ are likely to function as a heterodimeric complex. Although each component is not yet proven to be part of the complex in the gastric pit cells, the mechanism for the regulation of Nox1 may be in some respects similar to that already well known for Nox2 (Fig. 2). In a previous study (48), we reported that the amount of a 67-kDa protein that cross-reacted with an antibody against human $p67^{phox}$ increased in parallel with elevation of O_2^- generation after treatment with *H. pylori* LPS. Using a new antibody against recombinant human $p67^{phox}$, it was subsequently confirmed that the guinea-pig $p67^{phox}$ with a molecular mass of 63 kDa was not affected by *H. pylori* LPS (45).

The activation of Rac may serve as a switch for activation of Nox1 as well as for that of $gp91^{phox}$. In agreement with this, the Nox activators $p67^{phox}$ (49) and NOXA1 (72) are capable of binding to GTP-bound Rac but not to GTP-bound Cdc42. At present, the mechanism for Toll-like receptor (TLR) 4–mediated activation of Rac1 is still unknown. The kinetics of activation of Rac2 in fMLP-stimulated neutrophils coincides with rapid and transient generation of O_2^- (1). Activation of the small GTPase is regulated by guanine-nucleotide exchange factor (GEF) (for a review, see ref. 77) and GTPase-activating protein (GAP) (for a review, see ref. 58). In neutrophils, Nox2 may be regulated by GAPs including breakpoint cluster region protein, p50RhoGAP, and p190RhoGAP (28). Recently, it has been shown that ROS production in growth factor–stimulated cells is mediated by the sequential activation of PI3K, a Rac-GEF (βPix), and Rac1 (62). More recently, using Rac1 mutants or Noxa1 mutants defective in Rac binding or short interfering RNA-mediated Rac1 silencing, Ueyama *et al.* (76) have also shown that the assembling and activation of multicomponent oxidase Nox1 are regulated by Rac1 as well as by NOXO1 and NOXA1 (76), also supporting an important role of Rac1 in the regulation of Nox1 activity in gastric pit cells. In contrast to the transient activation of Rac2 in neutrophils, the LPS-stimulated activation of Rac1 lasts for up to 16 h in parallel with persistent elevation of spontaneous O_2^- generation (45), suggesting that the gastric epithelium may have a unique regulatory system for Rac1. However, the system remains to be elucidated.

FUNCTIONS OF Nox1 IN THE STOMACH

The phagocyte NADPH oxidase is dormant in resting cells. Phagocytosis or stimulation by phorbol diesters initiates release of large amounts of O_2^-. This respiratory burst activity is essential for killing ingested microbes. Once phagocytosis has been completed, the respiratory burst declines and eventually ceases. In contrast, kinetics of O_2^- generation by Nox1 in guinea-pig gastric pit cells is strikingly different from that by phagocyte NADPH oxidase (Nox2). Gastric pit cells continue to produce O_2^- spontaneously for 48 h or longer, suggesting that Nox1 may regulate more-fundamental cell functions. After starting cultivation, the magnitude of O_2^--producing capability of guinea-pig gastric pit cells coincides with their mitogenic activity (73). Scavenging of ROS, particularly H_2O_2, suppresses their growth and accelerates the apoptosis of aged cells. Thus, ROS probably derived from Nox1 play an essential role in the cell growth and the maintenance of matured cells, at least *in vitro* (73). NF-κB activated by the ROS is an important survival signal in these cells (73).

Quiescent guinea-pig gastric pit cells in primary culture produce small amounts of O_2^- (<10 nmol/mg protein/h), but once primed with *H. pylori* LPS, they increased O_2^- generation 10-fold in association with the induction of Nox1 and NOXO1, and probably with activation of Rac1 (45). It takes about 8 h for this priming. The rate of O_2^- production by the LPS-primed cells (100 nmol/mg protein/h) is nearly equivalent to that of mouse peritoneal macrophages, although this amount was not enough to kill *H. pylori* directly *in vitro* (48, 74). Surface mucous epithelial cells serve a primary protective role against irritants by providing a mucus coat. They also play an important role in host defense as well, producing proinflammatory mediators after the interaction with the pathogenic microbe. Stimulation of TLR4 by *H. pylori* LPS activated NF-κB within 30 min, followed by upregulation of O_2^- production within 8 h (45, 48). Enhanced production of ROS further augmented NF-κB activation, leading to prolonged expression of the tumor necrosis factor (TNF)-α and cyclooxygenase II mRNAs (45, 73), suggesting that Nox1 may be one of the key molecules representing the initial trigger for host innate and immune response against *H. pylori*.

It should be noted that the Nox1-mediated cellular events may be peculiar to guinea-pig gastric epithelial cells, because the Nox1 has never been documented in the human stomach (Fig. 1 and ref. 30). According to the studies with guinea-pig gastric pit cells, we expected that *H. pylori* infection induced abundant Nox1 and NOXO1 proteins in the human gastric mucosa. However, Nox1 and NOXO1 could not be detected in superficial gastritis and chronic atrophic gastritis with intestinal metaplasia (unpublished observations). This is also supported by a recent report that the Nox1 transcript is absent in gastric biopsy specimens from elderly patients, even with chronic active gastritis (67). Thus, *H. pylori* infection alone does not induce Nox1 in the human stomach. Conversely, the mRNAs for Nox1 and NOXO1 were specifically coexpressed in both intestinal- and diffuse-type adenocarcinomas (Fig. 3).

We also confirmed that Nox1 and NOXO1 proteins were coexpressed in gastric adenocarcinomas, whereas they were absent in accompanying normal or chronic atrophic tissues (unpublished observations). These results raise the possibility that the dysregulated expression of Nox1 and NOXO1 may be associated with oxygen radical–related carcinogenesis in the stomach. Further studies are required to prove this hypothesis.

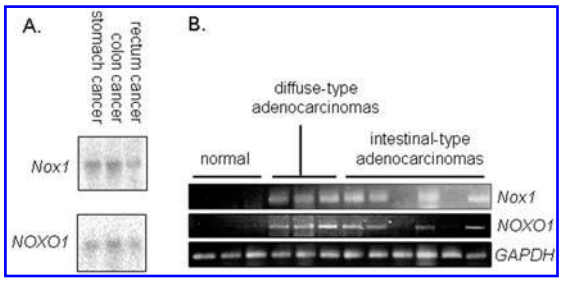

FIG. 3. Expression of Nox1 and NOXO1 mRNAs in human gastric cancer. (**A**) A multiple tissue Northern blot membrane (MTN; Clontech) was subjected to Northern blotting, and Nox1 and NOXO1 mRNAs are expressed in gastric adenocarcinoma. (**B**) Custom-designed human stomach tumor cDNA panels were purchased from BioChain Institute (Hayward, CA). Expression of mRNAs for Nox1, NOXO1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in normal and intestinal- or diffuse-type adenocarcinomas was examined by reversed transcription–polymerase chain reaction.

Nox1 IN LARGE INTESTINAL EPITHELIAL CELLS

In contrast to the guinea pig and human stomachs, Nox1 protein is constitutively expressed in surface mucous epithelial cells of the guinea pig and human colons. We have established primary cultures of guinea-pig large intestinal epithelial cells (LIECs), and found that freshly isolated and cultured guinea-pig LIECs constitutively express Nox1, NOXO1, p67^{phox}, NOXA1, p22^{phox}, and Rac1 (46). This Nox1 is fully activated and spontaneously secretes O_2^- at a higher rate (~160 nmol/mg protein/h) than guinea-pig gastric Nox1 primed with *H. pylori* LPS (~100 nmol/mg protein/h). The amount is again not enough to suppress the growth of *Salmonella enteritidis* or kill the bacterium *in vitro* (46).

In contrast, human colon cancer cell lines (T84 and Caco2 cells) produce only small amounts of O_2^- (<2 nmol/mg protein/h). In this case, NOXO1 and NOXA1 are absent or poorly expressed, respectively. T84 cells can be used for studying the mechanism for activation of Nox1 in LIECs. When the NOXO1 cDNA was transfected, T84 cells augmented O_2^- production in response to phorbol 12-myristate 13-acetate (PMA). Overproduction of NOXA1 additionally increased the O_2^--generating capability, whereas transfection of the p67^{phox} was not effective. NOXA1 lacks putative domains to interact with p40^{phox}, and colonic epithelial cells did not have p40^{phox}. Thus, NOXA1, rather than p67^{phox}, is likely to be a physiologic partner with Nox1 and NOXO1 in catalyzing an electron transfer from NADPH to molecular oxygen (46).

At present, physiologic roles of colonic Nox1 are not fully understood. Nox1 protein is expressed in the terminally differentiated cells in the colon, suggesting that Nox1 may have other roles besides mitogenic properties. Nox1 expression in Caco2 and T84 cells increases along with differentiation (29). Nox1 in LIECs appears to participate in the local innate immune response. When T84 cells overexpressing NOXO1 and NOXA1 were exposed to bacterial components, they responded to a recombinant structural protein of flagella filament (rFliC) from *S. enteritidis* and upregulated O_2^- generation fourfold. However, none of LPS from *H. pylori* or *E. coli,* peptidoglycan from *S. aureus*, and CpG DNA increased the O_2^- production (46). Intestinal epithelial cells, including T84 and Caco2 cells, express TLR5, and bacterial flagellin stimulates the TLR5 signaling, leading to the activation of proinflammatory signals, particularly NF-κB pathway (33, 37). T84 cells show highly polarized expression of TLR5 on the basolateral surface (33, 38). Certain flagellated bacteria are capable of translocating flagellin and stimulating TLR5 (38). Guinea-pig gastric pit cells are sensitive to LPS (48, 74), whereas large intestinal epithelial cells preferentially use TLR5 for augmentation of Nox1-mediated O_2^- production (46). This difference in the sensitivity may reflect luminal environments: LIECs are always exposed to gram-negative bacteria. Thus, surface mucous epithelial cells of the stomach and colon may use different TLR members in recognizing respective pathogenic microbes, activate Nox1, and finally produce defensive mediators. These results strongly suggest that the Nox1 activation constitutes early responses in epithelial cells against pathogens for the host defense. The evidence that interferon (IFN)-γ can stimulate induction of Nox1 in large intestinal epithelial cells further supports an important role in mucosal host defense (29, 46). At the same time, it is probable that Nox1-derived ROS are involved in the initiation and/or potentiation of mucosal inflammation and inflammatory bowel diseases. Studies of patients and mouse models have revealed that Crohn disease is driven by overproduction of interleukin (IL)-12, IFN-γ, and TNF-α (10). ROS derived from IFN-γ–activated Nox1 may be involved in the pathogenesis of T_H1-mediated inflammatory diseases such as Crohn disease. At present, we do not know whether the IFN-γ–dependent activation of the Nox1 expression actually participates in mucosal host defense or inflammation. Additional experiments are required to test the hypothesis.

REGULATION OF Nox1 EXPRESSION

The human Nox1 gene consists of 13 exons spanning approximately 31.49 kbp of genomic DNA. Nox1 shows tissue-specific distribution and has been recognized as an inducible O_2^--producing enzyme: IFN-γ (29, 46), 1α,25-dihydroxyvitamin D_3 (29), or TLR ligand (46) induces Nox1 in large intestinal epithelial cells, and angiotensin II (78), prostaglandin F2α (44), or platelet-derived growth factor (44) stimulates the induction in vascular smooth muscle cells. To the best of our knowledge, the upregulation of Nox1 transcript expression is accompanied by increase in its protein level. To understand the functions of Nox1, it is particularly important to elucidate the mechanism for transcription of this gene. However, the underlying mechanisms are not fully documented.

Recently, we have found that IFN-γ stimulates the transcription of the human Nox1 gene as well as the Nox2 gene (52). The regulatory mechanism for the transcription of the Nox1 gene expressed in a wide variety of nonphagocytic cells should be distinct from that of the Nox2 gene dominantly expressed in myeloid cells. IFN-γ has been proposed positively to regulate transcription of the Nox2 gene through at least

three different pathways. First, BID/YY 1 binds to the four binding sites in a region from −90 to −355 bp of the promoter after maturation-dependent dissociation of the transcriptional repressor CCAAT box binding protein (22, 42, 56). Second, IFN-γ stimulates the binding of PU.1 or the complex hematopoietic-associated factor-1 (HAF-1), consisting of PU.1, interferon regulatory factor 1, IFN consensus sequence-binding protein, and cAMP-responsive element-binding protein (CREB)-binding protein, to the PU.1/hematopoietic-associated factor-1–binding element centered at −53 bp of the promoter (16, 23, 70). Last, signal transducers and activators of transcription 1 (STAT1) associated with the γ-activated sequence (GAS) element at bp −100 of the promoter (16, 51).

Mutation of the PU.1/HAF-1–binding element (PU box) in the Nox2 promoter results in chronic granulomatous disease (50, 60, 70). The Nox1 promoter lacks this element and proximally (up to −2 kbp) does not contain any common binding sites for IFN-γ–responsive transcription factors. The proximal 2-kbp fragment does not respond to this cytokine. Within the 5′-flanking region of the Nox1 promoter, one GAS element located between −3818 and −3810 bp is crucial for the IFN-γ–induced transcription of the Nox1 gene (52). The mutation of GAS almost completely abrogates the IFN-γ–stimulated activation of the 4.8-kbp proximal promoter of the Nox1 gene (Fig. 4).

The 500 bp-proximal promoter of the Nox1 gene that contains binding sites for GATA or CDX factors appears to be essential for the basal promoter activity (unpublished observation).

With regard to intestinal-type gastric cancer, inappropriate activation of specific developmental pathways is likely to be involved in the development of intestinal metaplasia and intestinal-type gastric carcinomas—that is, gastric epithelial cells also deviate from their normal differentiation pathways toward an intestinal phenotype (39, 79). Inappropriate activation of intestine-specific factors, including GATA6 and CDX1/2, are likely candidates linked with the induction of intestinal metaplasia. These factors may participate in the inappropriate activation of the human Nox1 gene. However, intestinal metaplasia alone does not induce Nox1 in gastric mucosa. It is possible to speculate that enhanced T_H1 cytokine production might induce dysregulated expression of Nox1 in the presence of intestine-specific transcription factors. In any case, human gastric cancer specifically express Nox1, and Nox1 may be one of the key molecules for inflammation and ROS-associated carcinogenesis in the human stomach.

Recently it was shown that a fine-tuned crosstalk occurs between nitric oxide (NO) and ROS-generating systems. Glomerular mesangial cells can produce high amounts of NO and ROS (65). In this case, NO downregulates the expression of Nox1 in rat renal mesangial cells. Thus, the crosstalk may regulate the course and outcome of inflammatory disorders. Using a mitochondrial gene knockout [rho(0)] cell line, Desouki *et al*. (19) recently showed that levels of mitochondrial-derived O_2^- positively regulate the expression of Nox1, suggesting a crosstalk between the two O_2^--generating systems (19). LPS-stimulated expression of Nox1 is profoundly suppressed in the presence of antioxidants (Kuwano Y, Kawahara T, Rokutan K, unpublished observations); therefore intracellular redox may also control the Nox1 expression level. Thus, in addition to the factors described earlier, possibly other mediators are able to upregulate the Nox1 expression in different types of cells. Therefore, multiple *trans* factors and *cis* elements may regulate transcription of the Nox1 gene, depending both on the cell type and on the particular agonist used. Further studies will likely reveal multiple pathways for transactivation of the Nox1 gene, paralleling closely the tissue-specific function of Nox1-derived ROS.

Nox1 AND COLON CANCER

Earlier studies showed that injection of NIH 3T3 cells overexpressing Nox1 into nude mice stimulated generation of O_2^- and H_2O_2 for tumorigenic and angiogenic functions in the mice (4, 5, 69), suggesting a possible role of Nox1 in mitogenic regulation and carcinogenesis. However, subsequent studies have revealed that the Nox1-transfected cells also carry a mutation of Ras that may account for the abnormal cell growth and transformation (54). In regard to the expression of the Nox1 mRNA in the normal colon, Geiszt *et al*. (29) reported that the Nox1 transcript expressed mainly within the lower two thirds of mouse colon crypts, where epithelial cells undergo proliferation and differentiation. They also showed colon-specific Nox1 expression, predominantly in differentiated epithelial tumors. Furthermore, differentiation of colon cancer cell lines (Caco2 and HT29 cells) with 1α,25-dihydroxyvitamin D_3 or IFN-γ enhances Nox1 expression and decreases cell proliferation (29), suggesting that

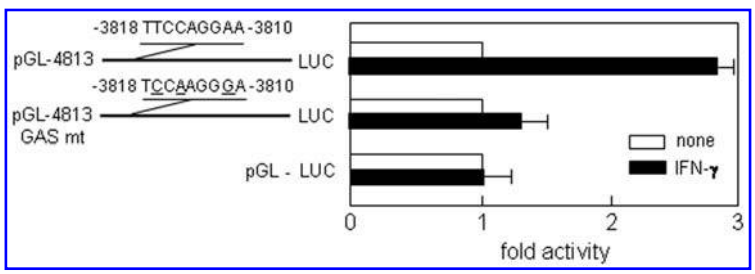

FIG. 4. Involvement of GAS element in IFN-γ–dependent Nox1 promoter activity in T84 cells. T84 cells were transfected with pGL vector alone or with pGL containing the −4831/+195-bp segment of the Nox1 gene (pGL-4831) or the segment carrying the mutated GAS sequence (pGL-4831 GAS mt). Transfection efficacy was standardized by co-transfection with pCMV-β. The mutated nucleotides (AATGGCTCCAAGGGAAACTCC) are underlined. These cells were incubated without (*open bars*) or with 1,000 U/ml IFN-γ (*black bars*) for 6 h and then subjected to the luciferase assay. Values are expressed as mean ± SD in three independent experiments. LUC, Luciferase reporter construct in pGL. [Data published in Kuwano et al. (52) are used with permission.]

Nox1 does not function as a mitogenic oxidase in colonic epithelial cells. According to the immunohistochemical analysis (27), Nox1 protein displays maturation-dependent expression along with upward migration, and surface mucous epithelial cells contain the most abundant Nox1 protein in normal colon tissues. Recently, Szanto *et al*. (71) precisely examined the Nox1 mRNA levels in human colon samples derived from healthy control subjects and patients with colon cancer or inflammatory bowel diseases. In normal tissues, NOX1 expression is low in the ileum, intermediate in the right colon, and high in the left colon. They failed to detect statistical difference in NOX1 expression between samples derived from adenomas, or well-differentiated or poorly differentiated adenocarcinomas. At a cellular level, Nox1 is highly expressed in colon epithelial cells, both within the crypts and on the luminal surface. They also reported the presence of Nox1-positive lymphocytes in the appendix, and lymphocytes in lesions of Crohn disease and ulcerative colitis (71). According to their data, Nox1 constitutively expressed in colon epithelium is an enzyme probably participating in host defense function, but not being associated with tumorigenesis. In addition, they suggest a potential role of Nox1-positive lymphocytes in the pathogenesis of inflammatory bowel diseases. Thus, controversy still exists about the involvement of Nox1 in the pathogenesis of colon cancer.

Colon cancer develops in an orderly fashion from normal mucosa, adenoma, carcinoma-*in-situ*, and frank cancer through the accumulation of several discrete genetic events (26, 43). We have precisely examined the Nox1 protein expression in human colon tissues from patients with adenomas or adenocarcinomas (27). Adenomas and well-differentiated adenocarcinomas upregulate Nox1 expression, whereas poorly differentiated adenocarcinomas scarcely express Nox1. Ki-67–negative, well-differentiated tumor cells contain abundant Nox1, whereas Ki-67–positive, proliferating cells do not express it (27). Thus, overexpression of Nox1 may not be directly linked to mitogenic activity of colon cancer cells. This differentiation-dependent expression in normal as well as tumor tissues suggests distinct roles of Nox1 besides mitogenic function. However, NF-κB is predominantly activated in adenoma and adenocarcinoma cells expressing abundant Nox1 (27), 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 by exerting their genotoxic and proinflammatory properties. Nox1-derived ROS activate crucial components for mitogen signaling, including p38 mitogen-activated protein kinase, Akt/protein kinase B, and tyrosine phosphatase (21, 35). ROS also stimulate expression of NF-κB–linked antiapoptotic proteins (6). Scavenging or inhibition of ROS produced by Nox enzymes increases sensitivity to apoptosis in melanoma cells (15), airway smooth muscle cells (12), prostate cancer cells (11), and gastric pit cells (73). Thus, ROS derived from Nox1 might exert a cancer-promoting effect by increasing resistance to programmed cell death of tumor cells.

Epidemiologic studies as well as gene-manipulation studies in animals provide us with opportunities for early intervention to arrest or retarded the carcinogenic process. Nonsteroidal antiinflammatory drugs, calcium, folic acid, antioxidants, and dietary constituents are applied for this purpose (43). Efficacy of the drugs and antioxidants suggests that Nox1 may be a potential target for chemoprevention of colon cancer.

ABBREVIATIONS

Nox, NADPH oxidase 1; Duox, dual oxidase; TLR, Toll-like receptor; *H. pylori*, *Helicobacter pylori*; *S. enteritidis*, *Salmonella enteritidis*, ROS, reactive oxygen species; O_2^-, superoxide anion; NF-κB, nuclear factor-κB; LPS, lipopolysaccharide; NOXO1, Nox organizer 1; NOXA1, Nox activator 1; PI3K, phosphoinositide 3-kinase; GEF, guanine-nucleotide exchange factor; GAP, GTPase-activating protein; TNF-α, tumor necrosis factor α; LIECs, large intestinal epithelial cells; PMA, phorbol 12-myristate 13-acetate; IFN-γ, interferon-γ; IL, interleukin; STAT1, signal transducers and activators of transcription 1; GAS, γ-activated sequence; NO, nitric oxide.

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