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NQO1-Knockout Mice Are Highly Sensitive to *Clostridium Difficile* Toxin A-Induced Enteritis

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Clostridium difficile toxin A causes acute gut inflammation in animals and humans. It is known to downregulate the tight junctions between colonic epithelial cells, allowing luminal contents to access body tissues and trigger acute immune responses. However, it is not yet known whether this loss of the barrier function is a critical factor in the progression of toxin A-induced pseudomembranous colitis. We previously showed that NADH:quinone oxidoreductase 1 (NQO1) KO (knockout) mice spontaneously display weak gut inflammation and a marked loss of colonic epithelial tight junctions. Moreover, NQO1 KO mice exhibited highly increased inflammatory responses compared with NQO1 WT (wild-type) control mice when subjected to DSS-induced experimental colitis. Here, we tested whether toxin A could also trigger more severe inflammatory responses in NQO1 KO mice compared with NQO1 WT mice. Indeed, our results show that *C. difficile* toxin A-mediated enteritis is significantly enhanced in NQO1 KO mice compared with NQO1 WT mice. The levels of fluid secretion, villus disruption, and epithelial cell apoptosis were also higher in toxin A-treated NQO1 KO mice compared with WT mice. The previous and present results collectively show that NQO1 is involved in the formation of tight junctions in the small intestine, and that defects in NQO1 enhance *C. difficile* toxin A-induced acute inflammatory responses, presumably via the loss of epithelial cell tight junctions.

Keywords: *Clostridium difficile*, bacterial toxins, enteritis, NQO1, gut epithelial cell tight junction, barrier function

Introduction

Clostridium difficile is the major cause of pseudomembranous colitis, which is characterized by inflammation and severe diarrhea [9, 24]. Studies have shown that in recent years, *C. difficile* infection rates have risen greatly, and the disease has gradually become more difficult to treat [12–14, 20]. Half the infections occur in those older than 65 years, but they account for 90% of the deaths [10, 12–14, 20]. However, few therapeutics are available to treat the disease.

The use of antibiotics is associated with *C. difficile* infection-induced pseudomembranous colitis because oral antibiotics kill normal flora, making room for the colonization of *C. difficile* [19, 20]. This leads to the release of toxins (toxin A and toxin B) that display cytotoxicity against various mucosal cells (*e.g.*, epithelial and immune cells) [12–14, 16, 24]. These toxins trigger the loss of epithelial cell junctions; this is believed to promote disease progression, as these tight junctions in the gut prevent tissues from being exposed to luminal contents, and their loss triggers immune responses [19, 20]. However, the importance of mucosal barrier function in toxin-induced gut inflammation has not yet been fully elucidated.

NADH:quinone oxidoreductase 1 (NQO1), which catalyzes

the reduction of quinone metabolites by using nicotinamide adenine dinucleotide (NADH) as an electron donor, is known to regulate the intracellular ratio of NAD and NADH (two fundamental mediators of energy metabolism) in various cell systems [3, 5, 18]. NQO1 is an antioxidant flavoprotein that scavenges reactive oxygen species (ROS) [1, 8, 15, 21], and it has been associated with cancer [25], diabetes [4, 26], and obesity [6]. We recently reported that a defect in NQO1 triggered a severe loss of tight junctions in the gut epithelial cells of mice [17]. Moreover, in the chronic mouse colitis model induced by DSS treatment, NQO1 knockout (KO) mice exhibited highly increased colonic inflammation compared with NQO1 wild-type (WT) mice [17].

Here, we tested whether *C. difficile* toxin A triggers greater inflammatory responses in NQO1 KO mice compared with NQO1 WT mice. Our findings support the notion that NQO1 contributes to the regulating epithelial cell tight junctions, and promotes the ability of the mucosal barrier to protect against toxin-mediated inflammation.

Materials and Methods

C. difficile Toxin A

Toxin A was purified from culture supernatants of *C. difficile* strain VPI 10463 (American Type Culture Collection, USA) using anion-exchange chromatography and fast protein liquid chromatography as previously described [12].

Reagents

The polyclonal antibody against caspase-3 was obtained from Cell Signaling Technology (USA). The polyclonal antibody against NQO1 was obtained from Santa Cruz Biotechnology (USA). The β-actin antibody was purchased from Sigma Aldrich (USA).

C. difficile Toxin A-Induced Acute Mouse Enteritis

NQO1 WT and NQO1 KO mice were kindly provided by Dr. Shong (Chungnam National University, Korea). All mice were bred and maintained in conventional mouse facilities at Daejin University (Korea), housed four per cage in a room maintained at a constant temperature (25°C). All protocols conformed to the guidelines of the institute's Animal Care and Use Committee. NQO1 WT and NQO1 KO mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and ileal loops (2 cm) were prepared and injected with control buffer or *C. difficile* toxin A (3 μg) in a volume of 100 μl PBS. After 4 h, animals were sacrificed and ileal loop tissues were collected. The ileal loops were weighed and their lengths were measured. Fluid secretion was expressed as the loop weight-to-length ratio (mg/cm), as previously reported [13].

Immunohistochemistry

Small intestines were isolated from NQO1 WT mice and were fixed for 12 h at 4°C in 4% buffered formalin and routinely processed for paraffin embedding at 56°C. Paraffin sections were cut and stretched at 45°C, allowed to dry, and stored at 4°C until use. Sections were deparaffinized and rehydrated via xylene and a graded series of ethyl alcohol, and treated with 0.1% trypsin (Sigma Chemical Co.) in distilled water for 5–10 min at 37°C. Sections were incubated for 30 min with 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidases and then for 30 min at room temperature with non-immune serum. Afterwards, the sections were incubated overnight at 4°C with antibody against NQO1 diluted 1:200 (v/v). After washing with PBS, the bound antibody was visualized by the peroxidase ABC method. Non-immune serum was used as a negative control [17].

Measurement of Mucosal Macromolecular Permeability

NQO1 WT and NQO1 KO mice were starved for 36 h prior to experiments, to reduce the luminal contents of their intestines. Each mouse was anesthetized with an intraperitoneal injection of Avertin (250 mg/kg; Sigma Aldrich). Both renal pedicles were ligated with 5-0 silk to prevent urinary excretion of the fluorescent probe. Ileal loops (3–4 cm) were prepared by silk ligation, and then lumenally injected with normal saline (0.3 ml, PBS) containing fluorescein-labeled dextran (MW 4,000; 25 mg/ml; Sigma, Canada) using a 0.5 ml U-100 insulin syringe. To keep the animals warm and protect the dye from light exposure, we covered each mouse with an aluminum foil blanket. After 3 h, 0.5 ml of blood was collected by cardiac puncture. The blood was centrifuged at 5,000 rpm for 10 min, and the supernatant was diluted 1:2 in PBS (pH 7.3). The concentration of fluorescein-labeled dextran was determined with GloMax 20/20 (Promega, USA), as previously described [2].

Histopathology Assessment

H&E-stained ileal sections were coded for blind microscopic assessment of inflammation. Histological scoring was based on two parameters. Severity of inflammation was scored as follows: 0, rare inflammatory cells in the lamina propria; 1, increased numbers of granulocytes in the lamina propria; 2, confluence of inflammatory cells extending into the submucosa; 3, transmural extension of the inflammatory infiltrate. Epithelial damage was scored as follows: 0, intact crypts; 1, loss of the basal one-third; 2, loss of the basal two-thirds; 3, entire crypt loss. The histological severity of colitis was graded in a "blinded" fashion [22].

Measurement of Mouse IL-6 and TNF-α

Ileal loops were homogenized (40 sec) in PBS and then centrifuged (11,000 ×*g*, 10 min at 4°C), and the supernatant was collected for protein concentration determination. Mouse IL-6 and TNF-α were measured by ELISA kits (R&D Systems, USA), and standardized with protein concentrations of each sample [22].

Immunoblot Analysis

Mouse tissues were washed with cold PBS and lysed in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Nonidet P-40), and equal amounts of protein were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The appropriate antibodies were applied, and antigen-antibody complexes were detected with the LumiGlo reagent (New England Biolabs, USA) [11].

Statistical Analysis

The results are presented as mean values ± SEM. Data were analyzed using the SIGMA-STAT professional statistics software program (Jandel Scientific Software, USA). Analyses of variance with protected *t* tests were used for intergroup comparisons [11].

Results and Discussion

NQO1 KO Mice Show Increased Mucosal Permeability and Inflammation in the Small Intestine

Given our previous findings that NQO1 expression is relatively high in various regions of the gut (*e.g.*, the small intestine and colon), and that NQO1 contributes to regulating epithelial cell tight junction formation [17], we first used immunohistochemistry with an antibody against NQO1 to identify the specific cells that mainly express this protein in the mouse small intestine. As shown in Fig. 1A, the NQO1 protein was mainly detected in epithelial cells of the small intestine (ileum), but not in lamina propria cells (immune cells, fibroblasts, and other connective tissue cells) or muscle cells. This indicates that the main NQO1-expressing cells are the epithelial cells that form the mucosal barrier through cell-cell tight junctions. A macromolecular permeability test revealed that the ileums of NQO1 KO mice were much more permeable than those of NQO1 WT mice (Fig. 1B). Moreover, the concentrations of the proinflammatory cytokine IL-6 [14] were significantly higher in the ileums of NQO1 KO mice compared with those of NQO1 WT mice (Fig. 1C). These results suggest that the defect in NQO1 markedly reduced epithelial cell tight junctions and slightly increased inflammation in the small intestine.

C. difficile Toxin A-Induced Fluid Secretion in the Small Intestine Is Higher in NQO1 KO Mice than in NQO1 WT Mice

We previously observed that, when subjected to DSS-induced colitis, NQO1 KO mice showed significantly higher lethality rates, inflammatory responses (TNF-α and IL-6 production), and mucosal damage in the colon compared

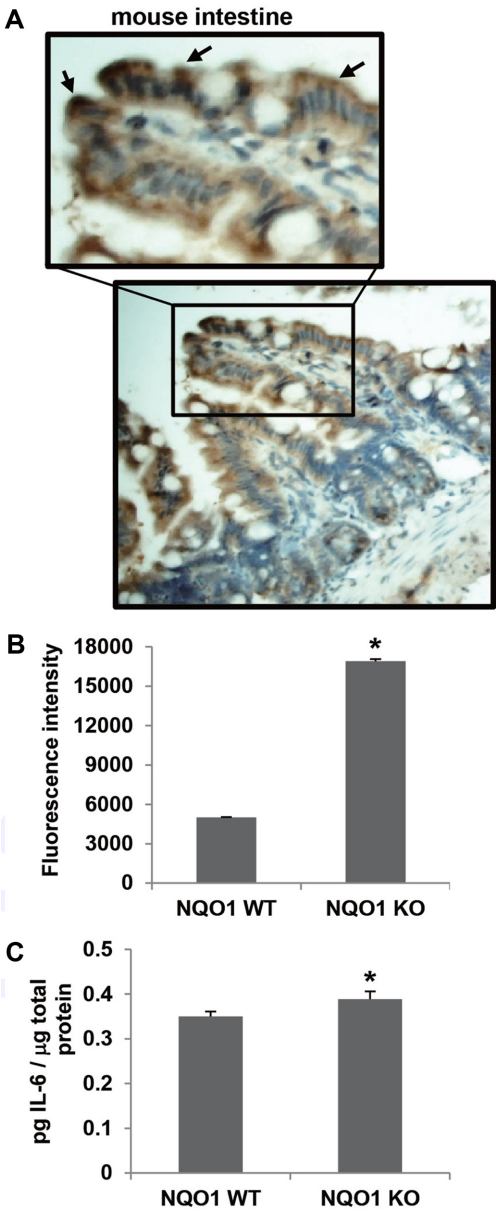

Fig. 1. NQO1 KO increases mucosal permeability and inflammation in the mouse small intestine.

(**A**) Immunohistochemistry with an antibody against NQO1 was used to elucidate the distribution of this protein in the small intestine (ileum) of NQO1 WT mice. The presented results are representative of those obtained from three independent experiments (arrows indicate cells expressing NQO1). (**B**) Ileal loops of NQO1 WT and NQO1 KO (deficient) mice were lumenally injected with fluorescein-labeled dextran, and blood fluorescence was determined. The results shown are representative of three independent experiments; *, $p < 0.05$ vs. NQO1 WT mice ($n = 12$ per group). (**C**) The concentrations of IL-6 were measured in the small intestines. The bars represent the mean ± SEM of three independent experiments, each with triplicate determinations; *, $p < 0.005$ vs. NQO1 WT mice.

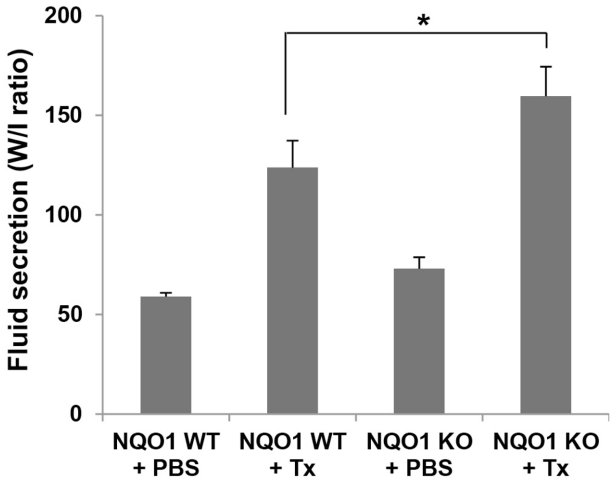

Fig. 2. *C. difficile* toxin A-induced fluid secretion is higher in NQO1 KO mice than in NQO1 WT mice.

NQO1 WT and NQO1 KO mice were anesthetized and ileal loops (2 cm) were prepared and injected with PBS buffer containing toxin A (Tx, 3 μg). After 4 h, the ileal loops were collected, weighed, and measured for their lengths. Fluid secretion is expressed as the loop weight-to-length ratio (mg/cm). The bars represent the mean ± SEM of three independent experiments, each with triplicate determinations; *, $p < 0.05$ ($n = 8$ per group).

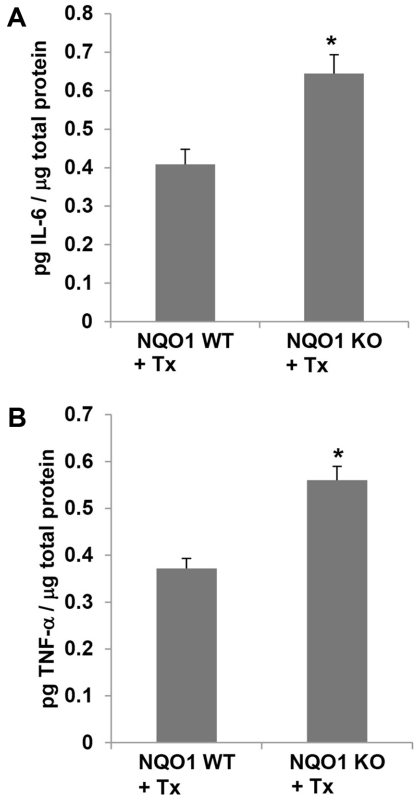

Fig. 3. *C. difficile* toxin A-induced inflammatory cytokine levels in the ileum are higher in NQO1 KO mice than in NQO1 WT mice.

NQO1 WT and NQO1 KO mice were anesthetized, and ileal loops were prepared and injected with toxin A (Tx). The ileal loops were collected, and the concentrations of IL-6 (**A**) and TNF-α (**B**) were measured. The bars represent the mean ± SEM of three independent experiments, each with triplicate determinations; *, $p < 0.005$ vs. NQO1 WT mice ($n = 8$ per group).

with NQO1 WT mice [17]. In the present study, we assessed whether the small intestines of NQO1 KO mice are also more sensitive to *C. difficile* toxin A-induced inflammation, which is a model of acute enteritis in which a massive amount of fluid is secreted to the lumen [16]. Briefly, NQO1 WT and NQO1 KO mice were anesthetized, and ileal loops were prepared and injected with *C. difficile* toxin A (3 μg) [16]. After 4 h, the mice were sacrificed, and fluid secretion levels in the ileal loop tissues were measured. As shown in Fig. 2, the level of toxin A-induced fluid secretion was significantly higher in NQO1 KO mice than in NQO1 WT mice. This suggests that the defect in NQO1 enhances *C. difficile* toxin A-induced fluid secretion, presumably via the loss of tight junctions.

Toxin A is known to cause severe paracellular permeability, followed by massive exposure of lumenal pathogens to the human body, leading to gut inflammation and weight loss [12, 13, 16, 20]. Thus, the increase in paracellular permeability that is associated with NQO1 KO and the well-known mucosal damage induced by toxin A may synergistically aggravate inflammatory responses in the tested NQO1 KO mice. Our results also strongly support the physiological role of NQO1 in mediating tight junction integrity in the gut.

C. difficile Toxin A-Mediated Inflammation Is Higher in NQO1 KO Mice than in NQO1 WT Mice

Next, we assessed whether the NQO1 defect aggravates inflammatory responses in the *C. difficile* toxin A-induced acute enteritis model. To test this, ileal loops were homogenized, supernatants were collected, and the concentrations of IL-6 and TNF-α (proinflammatory cytokines) [16] were measured by ELISA. As shown in Fig. 3A, NQO1 KO mice exhibited highly increased IL-6 production compared with NQO1 WT mice. Very similar results were obtained for TNF-α (Fig. 3B). These results indicate that the tight junction loss caused by NQO1 KO is also highly sensitive to *C. difficile* toxin A-induced inflammatory responses.

NQO1 is known to be involved in innate and adaptive

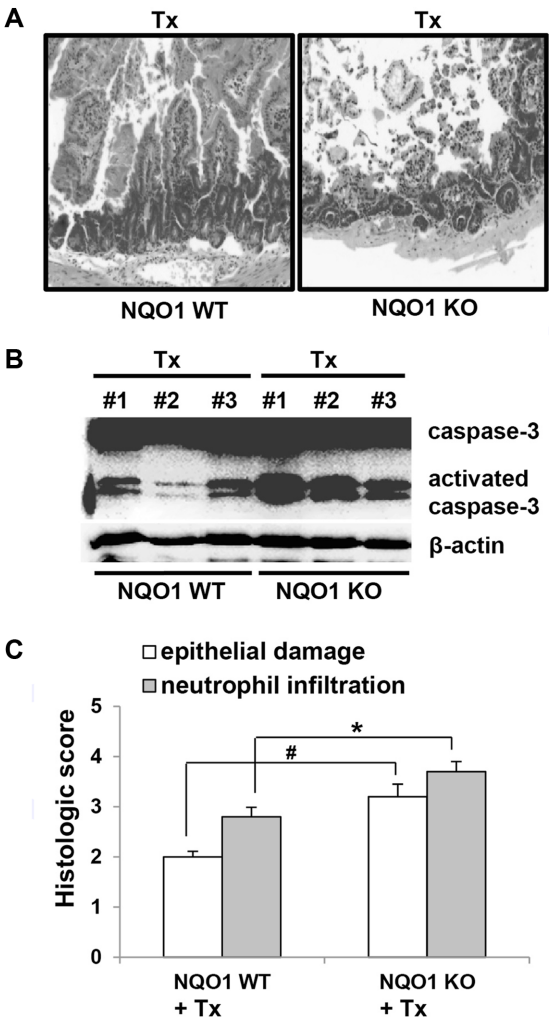

Fig. 4. *C. difficile* toxin A-induced gut mucosal damage in the ileum is higher in NQO1 KO mice than in NQO1 WT mice.
NQO1 WT and NQO1 KO mice (n = 8 per group) were anesthetized, and ileal loops were prepared and injected with PBS buffer containing *C. difficile* toxin A (Tx). After 4 h, the ileal loops were collected and inflammatory parameters were evaluated. (**A**) Light micrographs of mouse ileums (n = 8 per group; H&E staining, ×200). (**B**) Epithelial cell extracts (extracts of ileal scrapings) were isolated from ileums of NQO1 WT and NQO1 KO mice and resolved on polyacrylamide gels. Blots were probed with antibodies against caspase-3 and β-actin. The presented results are representative of three independent experiments. (**C**) Histological scores (n = 8 per group; *, $p < 0.005$; #, $p < 0.01$).

immune responses. For example, the LPS-induced activation of monocytes is highly dependent on the upregulation of NQO1 [7]. In addition, NQO1 null mice exhibit decreased levels of B cells and a high susceptibility to autoimmune disease [23], suggesting that NQO1 may play a pivotal role in immune regulation. Thus, the increased inflammation we observed in toxin A-treated NQO1 KO mice may reflect the ability of NQO1 to regulate immune responses. If this is the case, such finding would suggest that the function of NQO1 in the gut mucus is anti-inflammatory in nature. However, we conclude that gut-expressed NQO1 also critically regulates epithelial cell tight junctions (and thus the mucosal barrier), as shown by the following results: NQO1 levels are lower in the spleen (an organ containing numerous immune cells) than in other gut organs [17]; NQO1 KO causes loss of mucosal barrier function [17]; and, in the gut, NQO1 is mainly expressed by epithelial cells, not immune cells (this study). Taken together, the previous and present findings suggest that the NQO1 KO-dependent reduction of epithelial cell tight junctions can enhance toxin A-induced inflammation in the mouse small intestine.

C. difficile Toxin A-Mediated Villus Disruption and Apoptosis Are Increased in NQO1 KO Mice Compared with NQO1 WT Mice

We also tested whether NQO1 KO highly increased villus disruption and gut epithelial cell apoptosis, which are two markers of mucosal damage that have been associated with *C. difficile* toxin A-induced acute enteritis in mice [13, 14, 16, 20]. As shown in Fig. 4A, toxin A exposure triggered villus disruption in NQO1 WT mice, and this disruption was greatly enhanced in NQO1 KO mice. As shown in Fig. 4B, our assessment of apoptosis in ileal scrapings revealed that toxin A-induced mucosal apoptosis was much higher in NQO1 KO mice than in NQO1 WT mice. These results indicate that the expression of NQO1 in epithelial cells may help protect against harmful agents. Many of the existing reports have indicated that the main function of NQO1 is to decrease ROS production as an antioxidant [1, 8, 15, 21]. Conversely, toxin A is known to cause rapid ROS generation and subsequent toxicity in various mammalian cells [13]. We thus speculate that the lack of NQO1-mediated antioxidant activity in NQO1 KO mice may result in the massive accumulation of ROS, potentially accelerating the toxin A-induced damage to epithelial cells.

Finally, the toxin A-induced inflammation score (in terms of epithelial damage and neutrophil infiltration) was higher in NQO1 KO mice than in NQO1 WT mice (Fig. 4C). Similar to our previous findings in the DSS-induced colitis model, this indicates that NQO1 KO mice are very sensitive to *C. difficile* toxin A-induced acute enteritis, likely due to their lack of mucosal barrier function. Our present results also strongly support the notion that disruption of mucosal integrity could be a critical factor in the disease progression

of *C. difficile* toxin A-induced gut inflammation.

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