Role of NADH: quinone oxidoreductase-1 in the tight junctions of colonic epithelial cells

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INTRODUCTION

NADH:quinone oxidoreductase 1 (NQO1) is known to be involved in the regulation of energy synthesis and metabolism, and the functional studies of NQO1 have largely focused on metabolic disorders. Here, we show for the first time that compared to NQO1-WT mice, NQO1-KO mice exhibited a marked increase of permeability and spontaneous inflammation in the gut. In the DSS-induced colitis model, NQO1-KO mice showed more severe inflammatory responses than NQO1-WT mice. Interestingly, the transcript levels of claudin and occludin, the major tight junction molecules of gut epithelial cells, were significantly decreased in NQO1-KO mice. The colons of NQO1-KO mice also showed high levels of reactive oxygen species (ROS) and histone deacetylase (HDAC) activity, which are known to affect transcriptional regulation. Taken together, these novel findings indicate that NQO1 contributes to the barrier function of gut epithelial cells by regulating the transcription of tight junction molecules. [BMB Reports 2014; 47(9): 494-499]

RESULTS AND DISCUSSION

NQO1 deficiency markedly increases gut permeability and inflammation

In the present study, we observed that the expression levels of NQO1 in the intestine and colon were relatively higher than those in other organs, including brain, spleen, liver and lung in NQO1-WT mice (Fig. 1A). Marked expression of NQO1 was also detected in the kidneys of NQO1-WT mice. This is con-
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**Fig. 1.** NQO1 is abundant in gut epithelial cells, and NQO1 knockdown causes increased epithelial permeability in the mouse gut. (A) The organ distribution of NQO1 was elucidated by immunoblot analysis with tissue extracts of NQO1-WT mice. (B) The organ distribution of NQO1 was elucidated by RT-PCR with NQO1-specific primers. (C) Left panel: Ileal loops of NQO1-WT and NQO1-KO mice were lumenally injected with fluorescein-labeled dextran, and blood fluorescence was determined. *P < 0.05 vs. NQO1-WT mice (n=12 per group). Right panel: Colon tissues from near the cecum were isolated from NQO1-WT and NQO1-KO mice, total protein extracts were resolved on polyacrylamide gels, and the blotted membrane was probed with antibodies against NQO1, Cox2, tubulin, and β-actin. (D) The concentrations of TNF-α were measured in the intestine (left panel) and colon (right panel). The bars represent the mean ± SEM of three independent experiments (*P < 0.005 vs. NQO1-WT mice).

**Fig. 2.** Decreased expression of the tight junction molecules, occludin and claudin, in the colons of NQO1-KO mice. (A) Colon tissue extracts were obtained from NQO1-WT and NQO1-KO mice respectively (n=8 mice per group). The presented results are representative of three independent experiments. (B) The mRNA expressions levels of claudin-1, claudin-6 and occludin were evaluated by real-time reverse transcription-PCR (RT-PCR). (C) Light micrographs of mouse colon samples subjected to immunohistochemistry for occludin (IHC stating; original magnification, ×200). Data are representative of three independent samples (arrows indicate occludin proteins in the apical area of colonocytes).
ability, we next assessed whether NQO1 deficiency affects the expression levels of tight junction molecules, such as claudin and occludin. Protein extracts were isolated from the colons of NQO1-WT and NQO1-KO mice, and the expression levels of claudin and occludin were determined by immunoblot analysis. As shown in Fig. 2A, the expression levels of claudin-1 and occludin in the colons of NQO1-KO mice were significantly lower than those in NQO1-WT colons. In contrast, the expression of E-cadherin, a major protein component of desmosomal junctions for cell-cell adhesion (12), did not differ between NQO-WT and NQO1-KO mice. RT-PCR experiments showed that the colons of NQO1-KO mice had reduced transcript levels of claudin-1, claudin-6 and occludin compared to those of NQO1-WT mice (Fig. 2B). These results suggest that colonic NQO1 may be associated with the transcriptional regulation of tight junction molecules, thereby affecting epithelial barrier function. Immunohistochemistry confirmed that the protein levels of occludin were lower in the colonic epithelial cells of NQO1-KO mice versus NQO1-WT mice (Fig. 2C).

**NQO1 deficiency increases various symptoms of inflammation in the DSS-induced colitis model**

Next, we evaluated the potential pathophysiological role of NQO1 in compromised colons. To induce colitis in mice, NQO1-WT and NQO1-KO mice were treated with DSS (3%) in their drinking water, and several clinical parameters of colitis (e.g., mortality and weight loss) were measured daily for the indicated durations. As shown in Fig. 3A, on day 14 of treatment, DSS-treated NQO1-KO mice showed significantly reduced survival rates, whereas DSS-treated NQO1-WT mice showed relatively weak reductions in survival by day 14 (NQO1-WT, 20% vs. NQO1-KO, 80%; n = 12 per group). Similarly, we observed marked decreases in body weight among NQO1-KO mice, but saw only a relatively small decrease in body weight among NQO1-WT mice (Fig. 3B). The levels of TNF-α (Fig. 3C) and IL-6 (Fig. 3D) were also higher in the colons of NQO1-KO mice compared to NQO1-WT mice. H&E staining of colonic sections revealed that DSS-induced neutrophil infiltration were relatively higher in NQO1-KO mice compared to NQO1-WT mice (Fig. 3E). Next, we evaluated several histopathological parameters of colonic inflammation (13). Colonic tissue sections of NQO1-KO mice treated with DSS showed much higher histopathologic scores of colonic inflammation compared to NQO1-WT mice (Fig. 3F).

Given that DSS is known to cause severe paracellular permeability followed by massive exposure of luminal pathogens to the human body, leading to gut inflammation and weight loss (14), the NQO1 deficiency (NQO1-KO)-induced increase in paracellular permeability and the DSS-induced mucosal damage may act synergistically to aggravate the inflammatory responses. Therefore, our results strongly support the physiological role of NQO1 in mediating tight junction integrity in the gut.

**Molecular mechanism for the transcriptional down-regulation of claudin and occludin in NQO1-KO mice**

Our findings suggest that the function of NQO1 in colonic epithelial cells may be associated with transcriptional regulation of the claudin and occludin genes. Having previously shown that Sp1 is a major transcriptional factor for claudin and occludin (15, 16), we assessed whether NQO1 knockdown affected the expression levels of Sp1. However, the expression level of Sp1 did not differ between NQO1-WT and NQO1-KO mice (Fig. 4A). The levels of acetylated histone-3 were significantly lower in the colons of NQO1-KO mice compared to NQO1-WT mice (Fig. 4A). However, total histone-3 levels did not differ between NQO1-WT and NQO1-KO mice, suggesting that the reduced acetylation of histone-3 could be associated with the transcrip-
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Fig. 4. In NQO1-KO mice, ROS accumulation sequentially causes HDAC activation, histone deacetylation, and transcriptional inhibition. (A) Colon samples taken from near the cecum were isolated from NQO1-WT and NQO1-KO mice (n=8 per group), total protein extracts were resolved on polyacrylamide gels, and blots were probed with antibodies against Sp1, acetylated histone-3, histone-3, and β-actin. (B) Primary colonic epithelial cells were isolated from NQO1-WT and NQO1-KO mice, nuclear extracts were isolated and incubated with an acetylated fluorogenic substrate for 30 min at 37 oC, and fluorescence was measured (*P < 0.05 vs. NQO1-WT mice). (C) Intracellular ROS was determined by DCFH-DA. (D) Human active recombinant HDAC-1 proteins were incubated with an acetylated fluorogenic substrate in the presence of H2O2 for 30 min (*P < 0.005 vs. untreated control).

Consistent with previous reports showing that NQO1 largely functions as an antioxidant to decrease the formation of ROS, including H2O2 (21, 22), we found that H2O2 levels were significantly higher in CECs from NQO1-KO mice compared to those of NQO1-WT mice (Fig. 4C). Next, we assessed whether these increased ROS levels could affect HDAC activity. To explore this, we incubated 1 μg of active human recombinant HDAC-1 and substrate in the presence of different concentrations of H2O2, and then measured the activity of HDAC-1. As shown in Fig. 4D, H2O2 treatment significantly increased the activity of HDAC-1. Indeed, ROS (including H2O2) have been shown to increase the expression/activity of HDACs or their association with target proteins (e.g., p53 and FOXO3), leading to transcriptional regulation of target genes (23-26). These results suggest that HDACs are highly sensitive to NQO1-regulated ROS levels, and HDAC activity may affect the transcriptional regulation of certain genes. Thus, our present findings and the previous reports collectively suggest that knockdown of the well-known antioxidant, NQO1, sequentially triggers ROS accumulation, HDAC activation, histone deacetylation and chromatin compaction, and decreased transcription of target genes, such as claudin and occludin.

MATERIALS AND METHODS

Mice and reagents
NQO1-WT and NQO1-KO mice were kindly provided by Dr. Shong (Chungnam University, Daejeon, Korea). All mice were bred and maintained in conventional mouse facilities at Daejin University (Pochen, Korea), housed four per cage in a room maintained at a constant temperature (25°C). All protocols conformed to the Animal Care and Use Committee guidelines. The polyclonal antibody against claudin-1 was obtained from Cell Signaling Technology (Beverly, MA, USA). The polyclonal antibodies against NQO1, occludin, E-cadherin, acetylated histone-3, histone-3, Sp1, Cox-2 and tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β-actin antibody, dextran sulfate sodium salt (DSS) and 2, 7-dichlorofluorescin-diacetate (DCFH-DA) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Measurement of intestinal 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 also 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, Oakville, ON, Canada) using a 0.5 ml U-100 insulin syringe. To keep the animal warm and protect the dye from light exposure, each mouse was covered with an aluminum foil blanket. After 3 h, 0.5 ml of blood was collected by cardiac stab. The blood was centrifuged at 5,000 rpm for 10 min, and the supernatant was diluted 1:2 in PBS.

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BMB containing 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, 0.5 mM in Buffer A [10 mM HEPES-potassium hydroxide (KOH), pH 7.9, suspended in five volumes of 0.3 M sucrose and 2% Tween-40

HDAC activity was measured using a fluorescence activity assay kit (Cayman Chemical, Ann Arbor, MI, USA) (28). Briefly, nuclear proteins (1 μg) obtained from the primary CECs of NQO1-WT or NQO1-KO mice were incubated at 37°C with 100 μM acetylated fluorogenic substrate in HDAC assay buffer. Fluorescence was measured using a Spectra Max M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation set at 360 nm and emission set at 460 nm. Active human recombinant HDAC-1 (Biomol, Palatine House, Matford Court, UK) was also used to measure deacetylation activity in the presence and absence of H2O2.

**Mouse colitis and clinical assessment of symptoms**

Acute colitis was induced by administration of dextran sodium sulfate (DSS, 40-50 kDa; ICN, Costa Mesa, CA, USA). NQO1-WT and NQO1-KO mice were given 3% DSS dissolved in regular tap water throughout the experimental period (up to 14 days). For the duration of the experiment, animals were observed three times per day for morbidity, mortality and weight (14).

**Measurement of mouse IL-6 and TNF-α**

Mouse colons were homogenized (40 seconds) in PBS and centrifuged (11,000 × g, 10 min at 4°C), and supernatants were collected. Mouse IL-6 and TNF-α were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) (14).

**Immunoblot analysis**

Mouse tissues were washed with cold PBS and lysed in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 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, MA, USA) (14).

**Statistical analysis**

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

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