Nrf2-Deficient Mice Have an Increased Susceptibility to Dextran Sulfate Sodium–Induced Colitis

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Abstract

Inflammatory bowel diseases, chronic inflammatory disorders, have been strongly linked with an increased risk of the development of colorectal cancer. Understanding the etiology of these diseases is pivotal for the improvement of currently available strategies to fight against inflammatory bowel disease, and more importantly, to prevent colorectal cancer. Nuclear factor-erythroid 2–related factor 2 (Nrf2) has been known to be a transcriptional factor which plays a crucial role in cytoprotection against inflammation, as well as oxidative and electrophilic stresses. The aim of this study is to investigate the role of Nrf2 in the regulation of dextran sulfate sodium (DSS)–induced experimental colitis in mice. Nrf2-deficient mice were found to be more susceptible to DSS-induced colitis as shown by the increased severity of colitis following 1 week of oral administration of 1% DSS. The increased severity of colitis in Nrf2(−/−) mice was found to be associated with decreased expression of antioxidant/phase II detoxifying enzymes including heme-oxygenase-1, NAD(P)H-quinone reductase-1, UDP-glucurosyltransferase 1A1, and glutathione *S*-transferase Mu-1. In addition, proinflammatory mediators/cytokines such as COX-2, inducible nitric oxide, interleukin 1β, interleukin 6, and tumor necrosis factor α were significantly increased in the colonic tissues of Nrf2(−/−) mice compared with their wild-type (Nrf2+/+) counterparts. In summary, we show for the first time that mice lacking Nrf2 are more susceptible to DSS-induced colitis. Our data suggests that Nrf2 could play an important role in protecting intestinal integrity, through regulation of proinflammatory cytokines and induction of phase II detoxifying enzymes. (Cancer Res 2006; 66(24): 11580-4)

Introduction

Rapidly accumulating evidence from epidemiologic and clinical studies indicate that chronic inflammatory disorders harbor an increased risk of cancer development (1, 2). Indeed, inflammation has been linked with cancers for over a century since Rudolph Virchow discovered a dramatic increment of infiltrating inflammatory cells in neoplastic tissues. Inflammation occurs when tissues are wounded by microbial infection or noninfective physical or chemical irritants (3). It is believed that the generation of the progrowth/survival microenvironment, as part of the wound healing process by activated inflammatory cells, promotes neoplastic risk (4).

Inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, have been strongly linked with an increased risk of colorectal cancer (5–7). The emergence of experimental model systems of IBD has tremendously increased our understanding of the etiology and the role of IBD in colorectal carcinogenesis. The dextran sulfate sodium (DSS)–induced mouse model of colitis is one of the most widely used models that mimics ulcerative colitis–like disease in humans (8). This model system has been used to reveal important events leading to IBD and colorectal carcinogenesis. Moreover, administration of DSS to gene-knockout mice becomes a very useful approach for the identification of genes that could be important in maintaining the integrity of intestinal tissue (8).

In this study, the role of nuclear factor-erythroid 2–related factor 2 (Nrf2), a basic leucine zipper redox-sensitive transcriptional factor in the DSS-induced mouse model of colitis was investigated. Nrf2 plays a central role in the transcriptional regulation of antioxidant and/or detoxifying genes. *In vivo* studies show that Nrf2 may also be important in the regulation of inflammation (9–12). For the first time, we show that mice lacking Nrf2 are more susceptible to DSS-induced colitis. Our data suggests that Nrf2 is essential in protecting intestinal integrity through the regulation of proinflammatory cytokines and induction of phase II detoxifying enzymes.

Materials and Methods

Reagents. DSS was purchased from MP Biomedicals (Aurora, OH). Rabbit polyclonal antibodies against nitric oxide synthase 2 (NOS2), NAD(P)H-quinone reductase-1 (NQO1), UDP-glucurosyltransferase 1A1 (UGT1A1), goat polyclonal antibodies against actin, heme oxygenase-1 (HO-1), and glutathione *S*-transferase Mu-1 (GSTM-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 and nitrotyrosine were purchased from Cayman Chemical (Ann Arbor, MI) and Chemicon International (Temecula, CA), respectively.

Animal care and treatment. Nrf2(−/−) mice (C57BL/SV129) have been described previously (13). Nrf2(−/−) mice were backcrossed with C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor, ME). To confirm the genotype from each animal, DNA was extracted from the tail and analyzed by PCR using the following primers: 3′-primer, 5′-GGAATGGAAAATAGCTCCTGCC-3′; 5′-primer, 5′-GCCTGAGAGCTGTAGGCCC-3′; lacZ primer, 5′-GGGTTTTCCCAGTCACGAC-3′. Nrf2(−/−) and Nrf2(+/+) mice exhibited one band at 200 and 300 bp, respectively. The eighth generation (F8) of male Nrf2 knockout mice were used in this study. Age-matched male C57BL/6J mice were purchased from The Jackson Laboratory. Nine- to 12-week-old mice were used and housed at Rutgers Animal Facility and maintained under 12-hour light/dark cycles. All animals were allowed water and food *ad libitum*. After 1 week of acclimatization, mice were divided into four groups: group I, wild-type + 1% DSS in drinking water for 1 week; group II, Nrf2(−/−) + 1% DSS in drinking water for 1 week; group III,

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doi:10.1158/0008-5472.CAN-06-3562

control wild-type; group IV, control Nrf2(−/−). All animal use procedures were in accordance with the NIH Guide for the Care and Use for Laboratory Animals, and were approved by the Rutgers Institutional Animal Care and Use Committee.

Histopathologic examinations. At the end of the experiment (1 week DSS exposure in drinking water), all the mice were killed by carbon dioxide asphyxiation. At autopsy, the large bowel was flushed with saline and excised. After measuring the length and weight of the large bowel, it was cut longitudinally and fixed in 10% formalin before being embedded in paraffin. Sections (3 μm) were stained with H&E as previously described (13).

Immunohistochemistry. Four-micrometer-thick sections were cut from formalin-fixed, paraffin-embedded blocks and mounted on glass slides. After deparaffinization, antigen retrieval was done by immersing the sections in 10 mmol/L of sodium citrate buffer (pH 6.0) and heated to below the boiling point for 20 minutes in a microwave oven. The sections were left to cool for ~20 minutes. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 10 minutes. The sections were then washed twice in PBS (pH 7.4) for 5 minutes each. Nonspecific binding was blocked by incubating with 3% bovine serum albumin for 10 minutes. Incubation with a primary rabbit polyclonal anti-nitrotyrosine (1:100 dilution) took place at room temperature for 30 minutes. For the subsequent reaction, a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) was used. The peroxidase activity was developed with the substrate, 3,3′-diaminobenzidine tetrahydrochloride (DAB Substrate Kit, Vector Laboratories) by incubating the sections for 2 minutes. The slides were then rinsed gently with distilled water and counterstained with hematoxylin for 30 seconds. The slides were dehydrated in alcohol prior to mounting. Negative controls were carried out by omitting the primary antibodies from duplicate sections and substituted with PBS.

Western blotting. Large bowel tissues dissected from mice of each treatment group were weighed, pooled, and treated with radioimmunoprecipitation assay buffer [50 mmol/L NaCl, 0.5% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaF, 20 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na_3VO_4, and protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] at a concentration of 10 μg/mL for 40 minutes on ice, followed by centrifugation at 14,800 × *g* for 15 minutes. The protein concentrations of the supernatant were measured by using the bicinchoninic acid solution (Pierce, Rockford, IL). Protein (20 μg) was loaded onto Criterion 4% to 15% electrophoresis gel (Bio-rad, Hercules, CA), and after electrophoresis, transferred onto polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in 0.1% Tween 20 in PBS (PBST) for 1 hour and incubated with primary antibody in 5% bovine serum albumin and 0.1% PBST overnight at 4°C. After three 5-minute washes with PBST, the membrane was then incubated with horseradish peroxidase–conjugated secondary antibody in 5% nonfat dry milk-PBST for 1 hour at room temperature, and then washed thrice with PBST. The transferred proteins were visualized with the SuperSignal chemiluminescent substrate (Pierce).

RNA isolation and reverse transcription-PCR. Total RNA was extracted from the frozen tissues using the Qiashredder and RNeasy Mini Kit (Qiagen, Valencia, CA). Steady-state mRNA levels of interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α), inducible NOS (iNOS), COX-2, HO-1, NQO1, and UGT1A1 were detected by reverse transcription-PCR. First-strand cDNA was synthesized from 2 μg of total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. PCR reactions were done by using 1 μL of reverse transcription product, 2 μL of primer mixture (final concentration, 10 μmol/L), and 17 μL of Invitrogen Platinum Super Mixture. Primers to specifically amplify the genes involved were as follows: forward, 5-GAG TGT GGA TCC CAA GCA AT-3 and reverse, 5-CTC AGT GCA GGC TAT GAC CA-3 for the IL-1β gene (543 bp); forward, 5-AGT TGC CTT CTT GGG ACT GA-3 and reverse, 5-GCC ACT CCT TCT GTG ACT CC-3 for the IL-6 gene (521 bp); forward, 5-ACG GCA TGG ATC TCA AAG AC-3 and reverse, 5-GGT CAC TGT CCC AGC ATC TT-3 for the TNF-α gene (550 bp); forward, 5-GTG GTG ACA AGC ACA TTT GG-3 and reverse, 5-GGC TGG ACT TTT CAC TCT GC-3 for the iNOS gene (487 bp); forward, 5-TCC TCC TGG AAC ATG GAC TC-3 and reverse, 5-TGA TGG TGG CTG TTT TGG TA-3 for the COX-2 gene (543 bp); forward, 5-AAG AGG CTA AGA CCG CCT TC-3 and reverse, 5-GTC GTG GTC AGT CAA CAT GG-3 for the HO-1 gene (591 bp); forward, 5-CAG ATC CTG GAA GGA TGG AA-3 and reverse, 5-AAG TTA GTC CCT CGG CCA TT-3 for the NQO1 gene (597 bp); forward, 5-GTG GCC CAG TAC CTG ACT GT-3 and reverse, 5-CGA TGG TCT AGT TCC GGT GT-3 for the UGT1A1 gene (552 bp). For the internal control gene GAPDH (522 bp), forward, 5-TGC TCG AGA TGT CAT GAA GG-3 and reverse, 5-TTG CGC TCA TCT TAG GCT TT-3 were used. The PCR conditions consisted of 94°C for 2 minutes followed by 35 cycles of 94°C denaturation for 30 seconds, 55°C annealing for 30 seconds, 72°C extension for 1 minute, and a final extension for 10 minutes at 72°C. The number of cycles used was optimized for each series of PCR to ensure that the amounts of amplification products increased proportionally and that the results were semiquantitative. Eight microliters of the amplified products were run on a 1.0% agarose gel followed by visualization using ethidium bromide staining.

Statistical analysis. Colon weights were represented as mean ± SE. Student's *t* test was used for the statistical analysis. $P < 0.05$ were considered significant.

Results

General observation. After 1 week of DSS exposure, the mean length of colons from C57/B6J (Nrf2+/+) and Nrf2(−/−) mice were significantly shorter than colons from the control mice without DSS exposure ($P < 0.01$; Fig. 1). The mean length of colons in Nrf2(−/−) mice was significantly shorter than C57/B6J (Nrf2+/+) mice after 1 week of DSS exposure. Rectal bleeding was evident in two out of five (40%) of DSS-exposed Nrf2(−/−) mice.

Histologic assessment of DSS-induced colitis in C57/B6J (Nrf2+/+) and Nrf2(−/−) mice. Oral administration of 1% DSS for 1 week resulted in colitis-associated histologic alterations, including shortening of colonic crypts, infiltration of inflammatory cells into the lamina propria, and separation of the crypt base from the muscularis mucosae (Fig. 2*C*). Interestingly, increased DSS-induced colitis severity was found in Nrf2(−/−) mice (Fig. 2*A* and *B*) as compared with their wild-type C57/B6J (Nrf2+/+) counterparts. Loss of crypts with severe inflammation in the

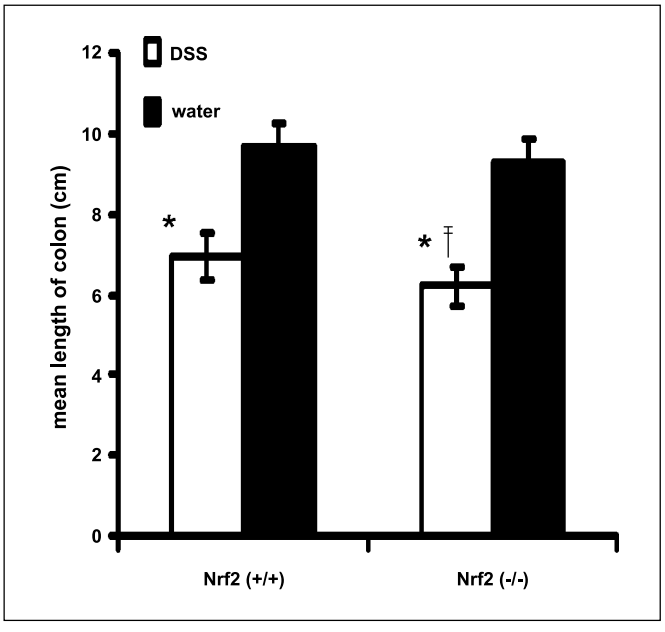

Figure 1. Effect dextran sodium sulfate (DSS)-induced colitis on the length of colon of C57BL/6J (Nrf2+/+) and Nrf2(−/−) mice. Male C57BL/6J (Nrf2+/+) and Nrf2(−/−) mice were given water or 1% DSS as sole source of drinking fluid for 1 week. The length of colon was measured at the end of study. Data represents mean ± SE. *, $P < 0.01$, differs from water-treated control of the same genotype; ‡, $P < 0.05$, differs from DSS-treated wild-type mice.

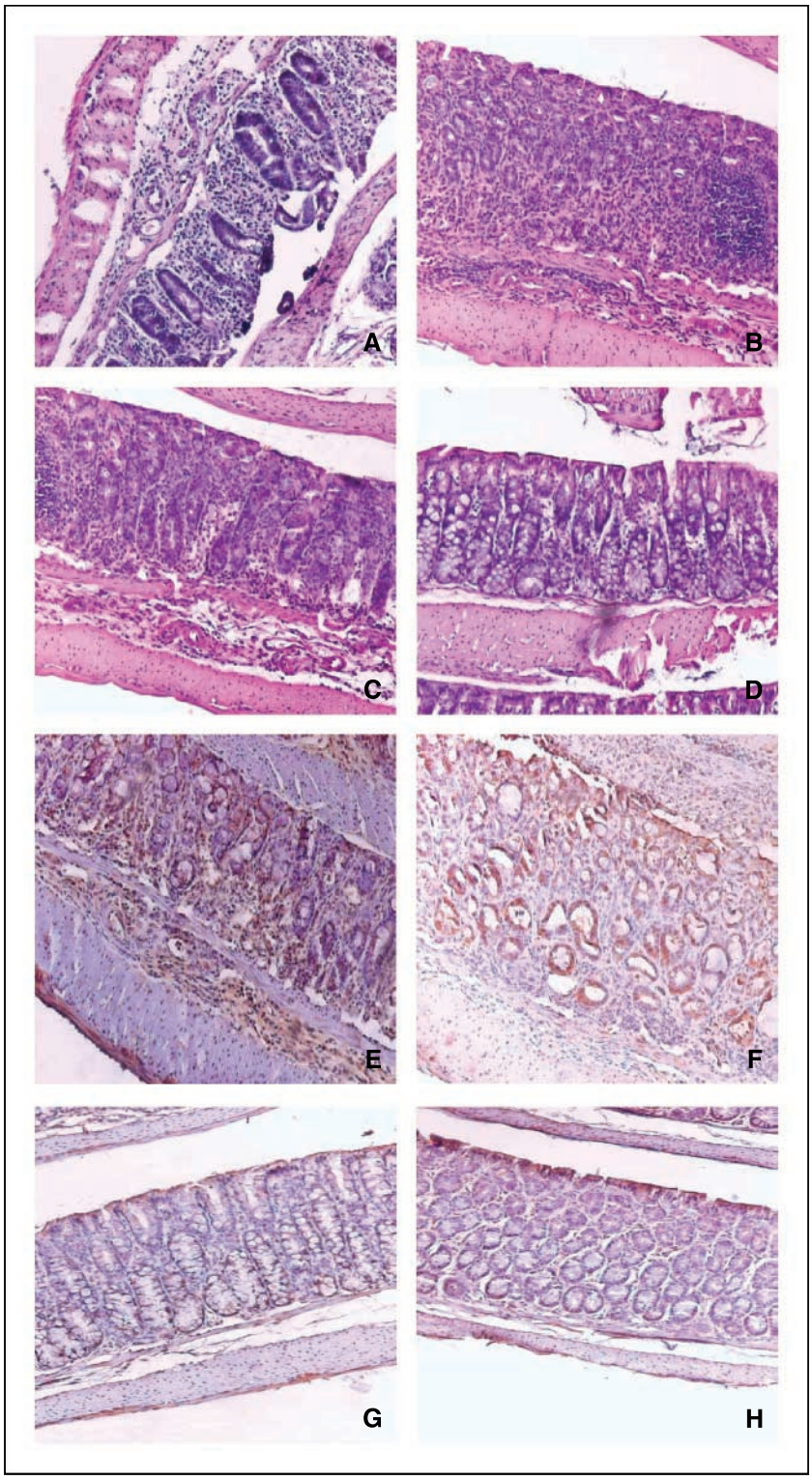

Figure 2. Histopathology and nitrotyrosine immunohistochemical staining of colonic tissues in DSS-treated and untreated control C57BL/6J (Nrf2+/+) and Nrf2(−/−) mice. Severe to moderate inflammation was observed in the colonic tissues of DSS-treated Nrf2(−/−) mice. *A,* loss of colonic crypts and severe infiltration of proinflammatory cells into the lamina propria of DSS-treated Nrf2(−/−) mice. *B,* loss of the basal two-thirds of crypts with moderate inflammation in colonic tissues of DSS-treated Nrf2(−/−) mice. *C,* most of the DSS-treated wild-type (Nrf2+/+) mice experienced loss of one third of crypts with moderate inflammation. *D,* intact colonic tissues of untreated control mice. Strong to moderate nitrotyrosine immunoreactivity was observed in DSS-treated (*E*) Nrf2(−/−) mice compared with their (*F*) wild-type counterpart which showed a reduced immunoreactivity. *G* and *H,* untreated controls showed a minimal immunoreactivity for nitrotyrosine.

lamina propria (Fig. 2*A*) and loss of the basal two-thirds of crypts with moderate inflammation (Fig. 2*B*) were observed in DSS-administered Nrf2(−/−) mice.

Immunohistochemical staining of nitrotyrosine. Strong to moderate nitrotyrosine immunoreactivity was observed in the colonic crypt cells of DSS-administered C57/B6J (Nrf2+/+) and Nrf2(−/−) mice. The immunoreactivity was found to be relatively weaker in C57/B6J (Nrf2+/+; Fig. 2*F*) as compared with Nrf2(−/−) mice (Fig. 2*E*), and was almost undetectable in the untreated control mice (Fig. 2*G* and *H*).

The mRNA and protein expression of some proinflammatory mediators and antioxidant/phase II detoxifying enzymes in the colon of 1% DSS-administered C57/B6J (Nrf2+/+) and Nrf2(−/−) mice. Administration of 1% DSS induced mRNA

expression of proinflammatory mediators (IL-1β, IL-6, TNF-α, iNOS, and COX-2) and antioxidant/phase II detoxifying enzymes (HO-1, NQO-1, and UGT1A1) in the colonic tissues of C57/B6J (Nrf2+/+) and Nrf2(−/−) mice (Fig. 3*A*). The protein expression levels of iNOS, COX-2, HO-1, and GSTM1 were also induced by DSS administration (Fig. 3*B*). Interestingly, Nrf2-deficient mice (Nrf2−/−) showed an increased susceptibility to DSS-induced proinflammatory mediators but showed decreased expression levels of DSS-triggered antioxidant/phase II detoxifying enzymes compared with their wild-type C57/B6J (Nrf2+/+) counterparts.

Discussion

It has been generally accepted that patients with long-standing IBDs such as ulcerative colitis and Crohn's disease have an increased lifetime risk of developing colorectal cancer (5). Despite tremendous efforts, the etiology of IBD remains unclear. Nrf2 is the key transcription factor regulating the antioxidant response which is crucial for cytoprotection against extracellular stresses. Upon oxidative or electrophilic insults, Nrf2 will translocate into the nucleus where it binds with antioxidant response elements, and transactivates phase II detoxifying and antioxidant genes. Numerous *in vivo* studies indicate that Nrf2 may play a role in the regulation of inflammation (9–12, 14). Nrf2 protects against chemical-induced pulmonary injury and inflammation (9, 12), whereas genetic ablation of Nrf2 enhances the susceptibility to cigarette smoke–induced emphysema and to severe airway inflammation and asthma in mice (10, 14). In addition, Nrf2 was found to be a crucial regulator of the innate immune response and survival during experimental sepsis (11). The present study aimed to investigate the role of Nrf2 in the regulation of the DSS-induced experimental colitis model, which mimics ulcerative colitis–like disease found in humans. Oral administration of 1% DSS for 1 week induces colitis-associated histologic alterations such as shortening of crypts and inflammatory cell infiltration in the colonic tissues. Interestingly, Nrf2-deficient mice were found to

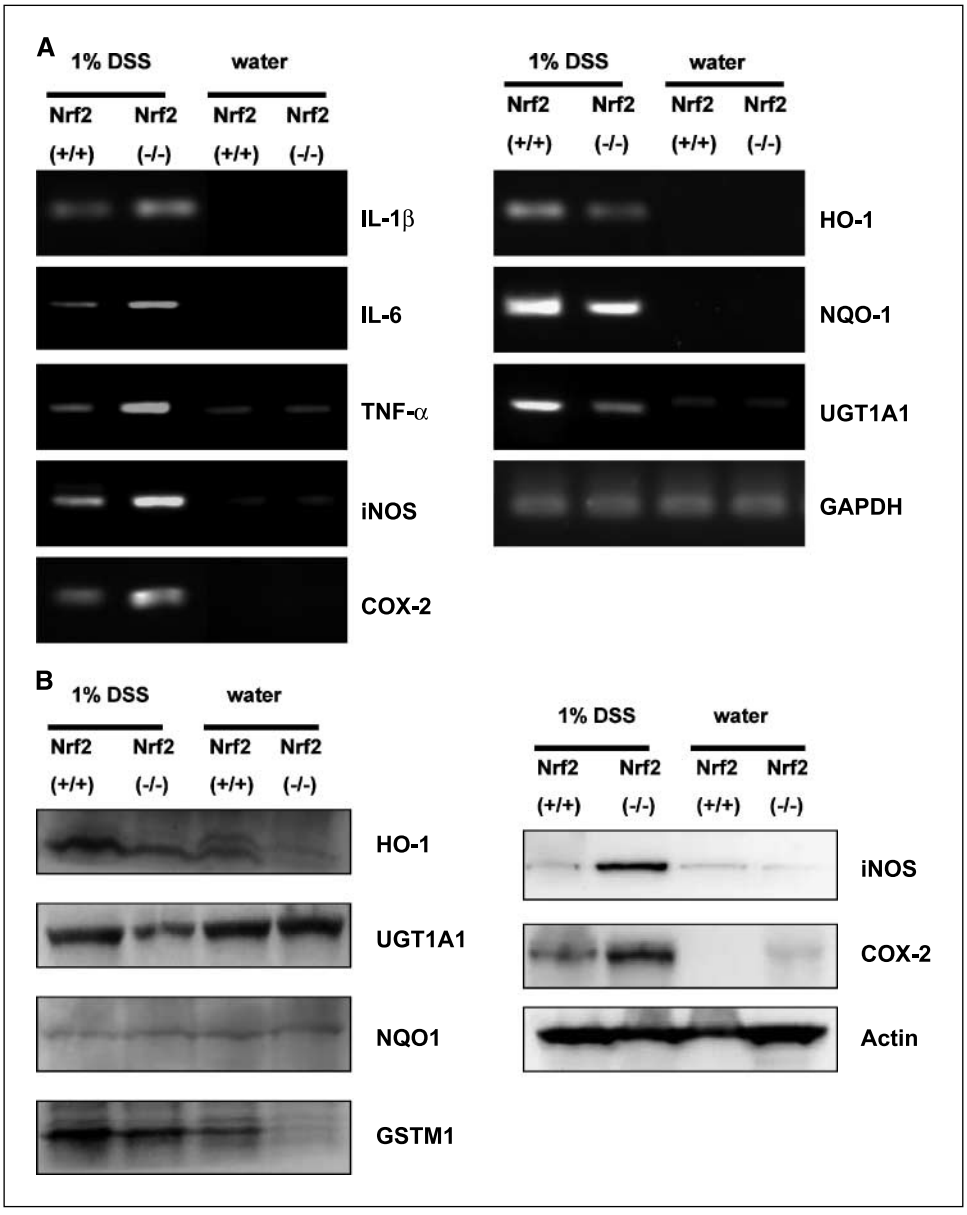

Figure 3. mRNA (*A*) and protein expression profiles (*B*) of proinflammatory mediators and antioxidant/phase II detoxifying enzymes in the colonic tissues of DSS-treated and untreated C57BL/6J (Nrf2+/+) and Nrf2(−/−) mice. Total RNA and protein were extracted and pooled from colonic tissues of at least three mice treated either with DSS or water.

be more susceptible to DSS-induced colitis as shown by the following observations: (*a*) the mean length of colons in Nrf2(−/−) mice were significantly shorter than in C57/B6J (Nrf2+/+) mice after 1 week of DSS exposure and rectal bleeding was only observed in 40% of Nrf2(−/−) mice, but not in C57/B6J (Nrf2+/+) mice. (*b*) Increased severity of histologic changes were observed in the Nrf2(−/−) mice compared with their wild-type C57/B6J (Nrf2+/+) counterparts. The cytoprotective function of Nrf2 has been linked to its ability to induce the expression of diverse antioxidant and phase II detoxifying enzymes. Therefore, we postulated that increased susceptibility of Nrf2(−/−) mice to DSS-induced colitis may at least in part be due to defects in regulating antioxidant and the phase II detoxifying enzymes. A previous report showed that HO-1, an important antioxidant and anti-inflammatory enzyme regulated by Nrf2, plays a protective role in trinitrobenzene sulfonic acid–induced colitis in rats (15). To test our hypothesis, the expression levels of several antioxidant and phase II detoxifying enzymes were evaluated. We found that DSS-induced colitis was associated with increased expression of Nrf2-regulated enzymes such as HO-1, NQO1, UGT1A1, and GSTM1. Interestingly, ablation of Nrf2 significantly reduced the mRNA and/or protein expression levels of these enzymes. Furthermore, Nrf2 deficiency also contributed to increased expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and proinflammatory mediators such as iNOS and COX-2 in the colonic tissues. It is still not clear how Nrf2 regulates the expression of these proinflammatory cytokines/mediators. Two previous studies indicate that there is a reciprocal cross-talk between Nrf2 and iNOS/COX-2 (16, 17). Healy et al. found that stimulation of the Nrf2/antioxidant response element pathway abrogates the expression of shear-induced COX-2 (16). On the other hand, induction of HO-1 by overexpression of nitric oxide was found to suppress the expression of iNOS through a negative feedback loop (17). Although the underlying mechanisms are still ambiguous, it is believed that Nrf2 could directly interfere with c-Jun NH_2-terminal kinase 2 activity upstream of COX-2 and iNOS (16). Another study by Jun et al. suggests that HO-1 expression has a direct effect on the activation of the proinflammatory nuclear factor-κB pathway. They found that induction of HO-1 by gliotoxin resulted in blockage of the nuclear factor-κB signaling pathway, and conversely, inhibition of HO-1 by zinc protoporphysin IX reversed the inhibitory effect (18). Future experiments will be necessary to elucidate these complicated networks in the regulation of DSS-induced colitis. In conclusion, our results show that Nrf2-deficient mice have an increased susceptibility to DSS-induced colitis, possibly due to reduced expression of antioxidant and phase II detoxifying enzymes, with a concomitant increased expression of proinflammatory mediators. To the best of our knowledge, this is the first study that dissects the role of Nrf2 in DSS-induced colitis. The present study also supported the rationale of targeting Nrf2 as an important cancer chemoprevention strategy, particularly in the prevention of colorectal cancer.

Acknowledgments

Received 9/26/2006; accepted 11/1/2006.

Grant support: NIH grants R01 CA076374 and R01 CA094828.

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