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ORIGINAL ARTICLE

NF-E2-Related Factor 2 Suppresses Intestinal Fibrosis by Inhibiting Reactive Oxygen Species-Dependent TGF-β1/SMADs Pathway

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Abstract

Background and Aims This study aimed to evaluate the antifibrotic effects of NF-E2-Related Factor 2 (Nrf2) on intestinal fibrosis. Intestinal fibrosis is a common complication of Crohn's disease; however, its mechanism of intestinal fibrosis is largely unclear.

Methods BALB/c mice received 2,4,6-trinitrobenzene sulfonic acid weekly via intrarectal injections to induce chronic fibrotic colitis. They also diet containing received 1% (w/w) tert-butylhydroquinone (tBHQ), which is an agonist of Nrf2. Human intestinal fibroblasts (CCD-18Co cells) were pretreated with tBHQ or si-Nrf2 followed by stimulation with transforming growth factor-β1 (TGF-β1), which transformed the cells into myofibroblasts. The main fibrosis markers such as α-smooth muscle actin, collagen I, tissue inhibitor of metalloproteinase-1, and TGF-β1/SMADs signaling pathway were detected by quantitative real-time RT-PCR, immunohistochemical analysis, and Western blot analysis. Levels of cellular reactive oxygen species (ROS) were detected by dichlorodihydrofluorescein diacetate.

Results tBHQ suppressed the intestinal fibrosis through the TGF-β1/SMADs signaling pathway in TNBS-induced colitis and CCD-18Co cells. Moreover, Nrf2 knockdown enhanced the TGF-β1-induced differentiation of CCD-18Co cells. ROS significantly increased in TGF-β1-stimulated CCD-18Co cells. Pretreatment with H_2O_2, the primary component of ROS, was demonstrated to block the effect of tBHQ on reducing the expression of TGF-β1. Moreover, scavenging ROS by *N*-acetyl cysteine could inhibit the increasing expression of TGF-β1 promoted by Nrf2 knockdown.

Conclusions The results suggested that Nrf2 suppressed intestinal fibrosis by inhibiting ROS/TGF-β1/SMADs pathway in vivo and in vitro.

Keywords NF-E2-Related Factor 2 · Colitis · Fibrosis · Reactive oxygen species · TGF-β1/SMADs signaling pathway

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Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing disease, including Crohn's disease (CD) and ulcerative colitis (UC). Intestinal fibrosis is a common complication of IBD and occurs in approximately 30% of patients with CD [1]. The mechanism of intestinal fibrosis is complicated, involving excessive extracellular matrix deposition (ECM) and mesenchymal cells proliferation [2, 3]. Intestinal fibrosis can also induce luminal stenosis frequently, which has a serious impact on the life quality of patients, ultimately requiring surgery [4].

NF-E2-Related Factor 2 (Nrf2), a nuclear transcription factor, plays an indispensable role in cellular defense against oxidative stress. Under normal condition, Nrf2 is combined with its negative regulatory protein, Kelch-like ECH-associating protein 1 (Keap1), and retained in the cytoplasm [5].

Nrf2 is released from Keap1 after stimulation by reactive oxygen species (ROS) or inflammatory cytokines and translocates into the nucleus. It combines with antioxidant response element to initiate the expression of various antioxidant-associated genes expression including heme oxygenase 1 (HO-1), glutamate–cysteine ligase catalytic (GCLC) subunit and glutamate–cysteine ligase modifier (GCLM) subunit [6]. The downstream products of Nrf2 can clear ROS and reduce the accumulation of oxidative stress substances.

ROS is reported to induce fibrosis through the TGF-β1/SMADs pathway, which is closely related to fibrosis [7, 8]. Removing ROS can inhibit TGF-β1-induced epithelial-to-mesenchymal transition (EMT) [9]. Transforming growth factor-β1 (TGF-β1) is the most important profibrogenic molecule in intestinal fibrosis. It mainly mediates through the SMADs family to activate the transcription of collagen and fibronectin, the primary component of ECM [10].

The degradation of ECM is also mediated by the matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs). The imbalance between the expression of MMPs and TIMP-1 causes excess ECM deposition [4]. TGF-β1 can promote the expression of TIMP-1 and inhibit the expression of MMPs to prevent the degradation of ECM [11].

Tert-butylhydroquinone (tBHQ), a widely used food additive antioxidant, can induce the expression of Nrf2 and Nrf2-related genes in many organs including the intestine of mice [12]. tBHQs were used to increase the expression of Nrf2, and siRNAs were used to knockdown the expression of Nrf2 to explore whether Nrf2 played a role in intestinal fibrosis. The results of this study demonstrated that overexpressed Nrf2 protected against fibrosis in 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced chronic colitis mouse model and human intestinal fibroblasts. Moreover, the low expression of Nrf2 promoted fibrosis in human colonic fibroblasts. Further, Nrf2 played a protective role through inhibiting the ROS/TGF-β1/SMADs pathway.

Materials and Methods

Animals and Induction of Colitis by TNBS

Female BALB/c mice (7–8 weeks old; weighing 18 ± 1.5 g; Changsheng Biotechnology, Liaoning, China) were maintained in the Animal Center of Shengjing Hospital (Liaoning, China) under conventional conditions. They received TNBS (Sigma-Aldrich, MO, USA) once a week for 6 weeks via intrarectal injections, according to the previously described method, to induce chronic fibrotic colitis [13]. Briefly, after 24-h fasting, the mice were anesthetized with urethane, and then injected with increasing doses of 100 μL of TNBS (1, 1, 1.5, 1.5, 2, and 2 mg in 45% ethanol) per week via an epidural catheter [13]. The catheter was advanced into the rectum until 4 cm proximal to the anus. However, the control group received phosphate-buffered saline (PBS). All mice were killed on the third day after the last TNBS administration. Animal use protocols were approved by the institutional care and animal use committee of the China Medical University and conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Food pellets were mixed with 1% tBHQ (w/w; 97%, Sigma-Aldrich) [14]. The mice were randomly divided into four groups: (1) mice receiving a regular diet (control group, $n = 5$); (2) mice receiving 1% tBHQ diet (tBHQ group, $n = 5$); (3) mice receiving TNBS (TNBS group, $n = 5$); and (4) mice receiving TNBS and 1% tBHQ diet (TNBS + tBHQ group, $n = 5$). The mice were administered diet containing 1% tBHQ at the beginning of the first cycle of TNBS until they were killed.

Evaluation of Colitis

Body weight was routinely measured on the second day after each TNBS administration. The mice were killed on the third day after the sixth injection, and then the colon weight and length were recorded. The distal 4 cm of the colon was collected, 5 mm piece of colon were fixed in 10% buffered formalin, and the rest part of the colon were stored at -80 °C for protein and RNA extraction.

The macroscopic scores were obtained by assessing adhesions, strictures, ulcers, wall thickness, and mucosal edema/hyperemia. Each item was graded from 0 to 2 as follows: adhesions (absent = 0, mild/focal-zonal = 1, severe/diffuse = 2); strictures (absent = 0, mild = 1, severe proximal dilatation = 2); ulcers (absent = 0, one or two linear ulcers <1 cm = 1, more sites of ulceration or one >1 cm = 2); wall thickness (less than 1 mm = 0, 1–3 mm = 1, more than 3 mm = 2); and mucosal edema/hyperemia (absent = 0, mild = 1, severe = 2). The sum of the scores was expressed as a total macroscopic score [15].

Colonic specimens of all mice were washed and immersed in 10% buffered formalin and then embedded in paraffin as the standard procedure. Serial sections (3.5 mm) were stained with hematoxylin and eosin (H&E) to assess the degree of inflammation and with Masson's trichrome to detect tissue fibrosis. The stained sections were observed under a Nikon Eclipse E800 (Nikon Corporation, Tokyo, Japan). Histological scores were determined by ulceration (no ulcers = 0, small ulcers <3 mm = 1, large ulcers >3 mm = 2); inflammation (none = 0, mild = 1, moderate = 2, severe = 3);

depth of lesion (none = 0, submucosa = 1, muscularis propria = 2, serosa = 3); and fibrosis (none = 0, mild = 1, severe = 2) [15].

Cell Culture and Treatment

Human intestinal CCD-18Co fibroblasts cell lines (from 2.5 months age old female) obtained from the American Type Culture Collection (ATCC; Manassas, VA), were cultured in Dulbecco's modified Eagle medium (high glucose) (HyClone, UT, USA) containing 10% fetal bovine serum (Biological Industries, Beit-Haemek, Israel) and incubated at 37 °C with 5% CO_2. After pretreating with 25 μmol/L tBHQ for 24 h, CCD-18Co cells were treated with or without 100 mM hydrogen peroxide (H_2O_2) (Sigma-Aldrich) for 1 h. Then, the cells were stimulated with 10 ng/mL human transforming growth factor (hTGF-β1; Cell Signaling Technology, MA, USA) for 12 h, which could induce the expression of fibrosis-associated molecules.

Small Interfering RNA Transfection

Nrf2 small interfering RNA (siRNA) and negative control siRNA were purchased from Sangon Biotech (Shanghai, China). The target sequence for Nrf2 siRNAs was sense: 5′-GCCUGUAAGUCCUGGUCAUTT-3′, antisense: 5′-AUGACCAGGACUUACAGGCTT-3′. After starving for 4 h, the cells were treated with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h of transfection, the cells were treated with or without 5 mM N-acetyl cysteine (NAC) (Sigma-Aldrich) for 1 h and then cells were stimulated with 10 ng/mL TGF-β1.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted with Trizol Reagent (Invitrogen) following the manufacturer's instructions and then reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Bio, Dalian, China). Quantitative real-time PCR (qPCR) was run in LightCycler 480 II (Roche, Basel, Switzerland) using an SYBR Premix Ex Taq II kit (Takara Bio). The sequences of primers (Sangon Biotech, Shanghai, China) are listed in Table 1.

Immunohistochemical Analysis

The 3.5-mm-thick sections were rehydrated in xylene and down-graded ethanol. The sections were blocked with 5% bovine serum albumin in PBS for 30 min and then incubated with goat polyclonal collagen I antibody (Santa Cruz Biotechnology, CA, USA) at a 1:100 dilution overnight at 4 °C. The samples were washed for 15 min with PBS, incubated with rabbit anti-goat immunoglobulin G (IgG) for 30 min at room temperature, and stained with DAB detection kit (Maixin Biotechnology, Fuzhou, China) for 1–3 min, then counterstained with hematoxylin. The slides were photographed under a Nikon Eclipse E800 (Nikon Corporation).

Western Blot Analysis

Colon samples and cells were homogenized in RIPA lysis buffer (Beyotime Biotechnology, Beijing, China) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Nuclear protein was obtained using a nuclear isolation kit (Beyotime Biotechnology). Equal amounts of protein were loaded onto 10% polyacrylamide gels, electrophoresed, and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk [dissolved in Tris-buffered saline with Tween 20 (TBST)]. Then, the membranes were incubated with antibodies against Nrf2, collagen I and TIMP-1 (Santa Cruz Biotechnology) at a 1:500 dilution; antibodies against α-SMA, MMP3, SMAD3, phospho-SMAD3 (Ser 423/425) and LaminB1 (Abcam, MA, USA) at a 1:1000 dilution; antibodies against TNF-α, IL-1β (Proteintech Group, PA, USA) at a 1:2000 dilution; antibodies against TGF-β1, SMAD2/3, phospho-Smad2(Ser465/467)/Smad3(Ser423/425) and GAPDH (Cell Signaling Technology) at a 1:2000 dilution at 4 °C overnight. The membranes were washed with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were visualized using an Amersham Imager 600 Luminescent image analyzer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Myeloperoxidase (MPO) Activity Assay

The activity of MPO was used to quantify neutrophil infiltration in the colonic tissues. The MPO activity was measured by a testing kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocols. The results of the MPO activity were shown as U/mg protein.

Measurement of ROS

Cellular ROS levels were detected using dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime Biotechnology). Briefly, the cells were cultured in 96-well plates and pretreated with or without NAC (5 mM) or H_2O_2 (100μM) for 1 h before TGFβ1 (10 ng/μl) for 12 h. Then,

Table 1 Primer sequences used for qRT-PCR analysis

Gene	Source	Forward (5′–3′)	Reverse sequences (5′–3′)
Nrf2	Human	CGG TAT GCA ACA GGA CAT TG	ACT GGT TGG GGT CTT CTG TG
HO-1	Human	GGG TGA TAG AAG AGG CCA AGA CT	CAG CTC CTG CAA CTC CTC AAA
GCLC	Human	TGA AGG GAC ACC AGG ACA GCC	GCA GTG TGA ACC CAG GAC AGC
GCLM	Human	AAT CTT GCC TCC TGC TGT GTG A	TGC GCT TGA ATG TCA GGA ATG C
α-SMA	Human	TGT GGC TAT CCA GGC GGT GC	TCT CGG CCA GCC AGA TCC AGA C
COL1α1	Human	GGA ATG AAG GGA CAC AGA GG	CTT AGC ACC AAC AG CAC CAG
MMP3	Human	TGC TTT GTC CTT TGA TGC TG	GAT TTT CCT CAC GGT TGG AG
TIMP-1	Human	CTG TTG TTG CTG TGG CTG AT	GTT GTG GGA CCT GTG GAA GT
GAPDH	Human	GCA CCG TCA AGG CTG AGA AC	TGG TGA AGA CGC CAG TGG A
α-SMA	Mouse	ATG AAG CCC AGA GCA AGA GA	TCC AGA GTC CAG CAC AAT ACC
COL1α1	Mouse	GAA CCC CAA GGA AAA GAA GC	TGC TGT AGG TGA AGC GAC TG
MMP-3	Mouse	CGA TGC TGC CAT TTC TAA TAA A	GAT TTT CCT CAC GGT TGG AG
TIMP-1	Mouse	TCC CCA GAA ATC AAC GAG AC	ACC CCA CAG CCA GCA CTA T
GAPDH	Mouse	ATG TTT GTG ATG GGT GTG AA	ATG CCA AAG TTG TCA TGG AT

they were washed with Dulbecco's modified Eagle's medium (serum-free) three times and incubated with DCFH-DA for 30 min at 37 °C. After washing with PBS thrice, the fluorescence intensity of ROS was detected with a fluorescence microplate reader (BioTek Instruments, VT, USA,) at 488 nm excitation wavelength and 520 nm emission wavelength.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by analysis of variance (ANOVA) using GraphPad Prism version 6 (GraphPad, CA, USA). A value of $P < 0.05$ was considered statistically significant. The experiment shown was replicated at least 3 times.

Results

Effect of tBHQ on TNBS-Induced Colitis

The colon of the TNBS group showed more severe intestinal inflammation, obvious hyperemia, edema, and thickened colonic wall. Moreover, the length of colon was significantly shorter compared with the normal group. Adhesion, strictures, dilation, wall thickness, mucosal edema/hyperemia, and mucosal ulcerations were improved by tBHQ treatment, and the macroscopic scores of the tBHQ + TNBS group were reduced than that of the TNBS-treated group, as shown in Fig. 1a, c. The colon weight/length ratio was representative of inflammation and fibrosis. The colon weight/length ratio increased in the TNBS group, and tBHQ treatment slightly reduced the ratio compared with the TNBS group (Fig. 1b).

The TNBS group had a high colonic histological score and exhibited obvious inflammatory cell infiltration, and reduction in crypt and goblet cell number. ECM deposition was also observed in the subepithelial and serosal areas. The mice treated with tBHQ had a significant reduction in the colonic histological score and ECM deposition compared with the mice in the TNBS group (Fig. 1d–f).

tBHQ Attenuates TNBS-Induced Chronic Intestinal Inflammation

MPO is produced by neutrophils and serves as a biomarker of inflammation. As shown in Fig. 2a, compared with the control group, MPO activity was significantly increased in TNBS group. The oral administration of tBHQ effectively reduced MPO activity in the TNBS-treated mice. We next detected the expression of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1β), which are major cytokines in IBD. TNF-α and IL-1β were expressed at relatively high levels in TNBS-treated mice, however, tBHQ suppressed the expression of TNF-α and IL-1β (Fig. 2b–d).

tBHQ Suppressed the Increased Expression of Fibrosis Markers in TNBS-Induced Colitis

α-SMA and collagen I were considered as the main intestinal fibrosis markers. The expression of Col1a1 and α-SMA mRNA increased 1.84-fold and 2.28-fold, respectively, in the TNBS group compared with control group. In contrast, the expression of Col1a1 and α-SMA mRNA increased only

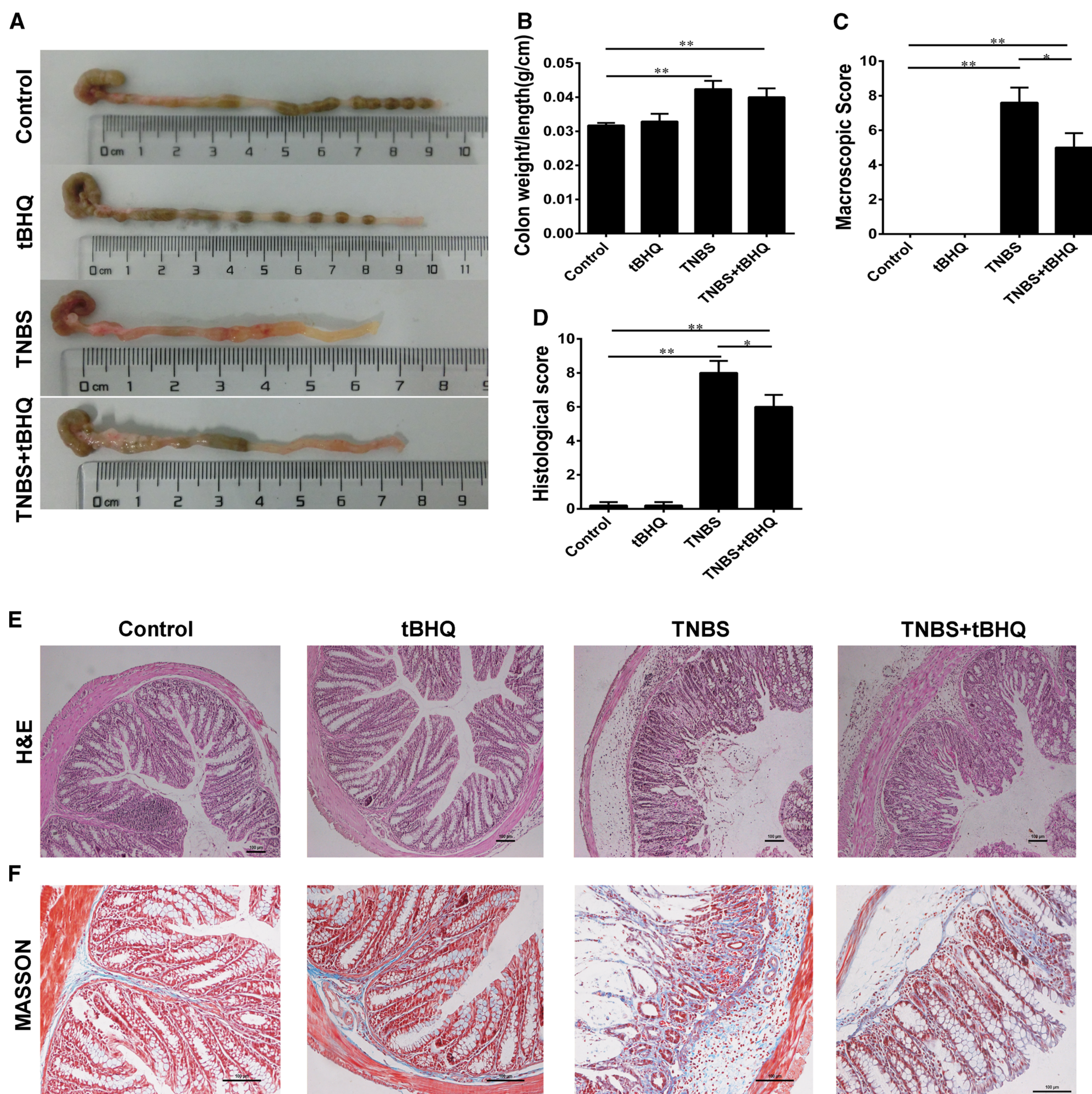

Fig. 1 Effect of tBHQ on TNBS-Induced colitis. **a** Macroscopic appearance of colons from the mice in four different groups. The colon length of the TNBS group was shorter compared with the other three groups. Adhesion, strictures, dilation, and wall thickness induced by TNBS are improved by tBHQ treatment. **b** Colon weight/length ratio. **c** Macroscopic score. **d** Histological score. **e** Representative H&E staining (×100). The TNBS group exhibited obvious inflammatory cell infiltration, and reduction in crypt and goblet cells. **f** Representative Masson trichrome staining: ECM deposition (*blue area*) decreased with tBHQ treatment (×200). Data were presented as mean ± SD, $n = 5$ per group. $*P < 0.05$ and $**P < 0.01$

1.15-fold and 1.34-fold, respectively, in the TNBS + tBHQ group compared with the control group (Fig. 3b, c). Moreover, the Western blot analysis demonstrated that the protein expression levels of α-SMA and collagen I were higher in the TNBS group than in the control group. tBHQ treatment decreased the high protein levels of α-SMA and collagen I (Fig. 3f–h). Also, the immunohistochemical analysis of collagen I also confirmed that tBHQ reduced the high expression of collagen I (Fig. 3a).

MMP3, an ECM-degrading protease, and TIMP-1, a specific inhibitor of MMPs, were also detected to prove the effect of tBHQ on ECM degradation. As shown in Fig. 3d,

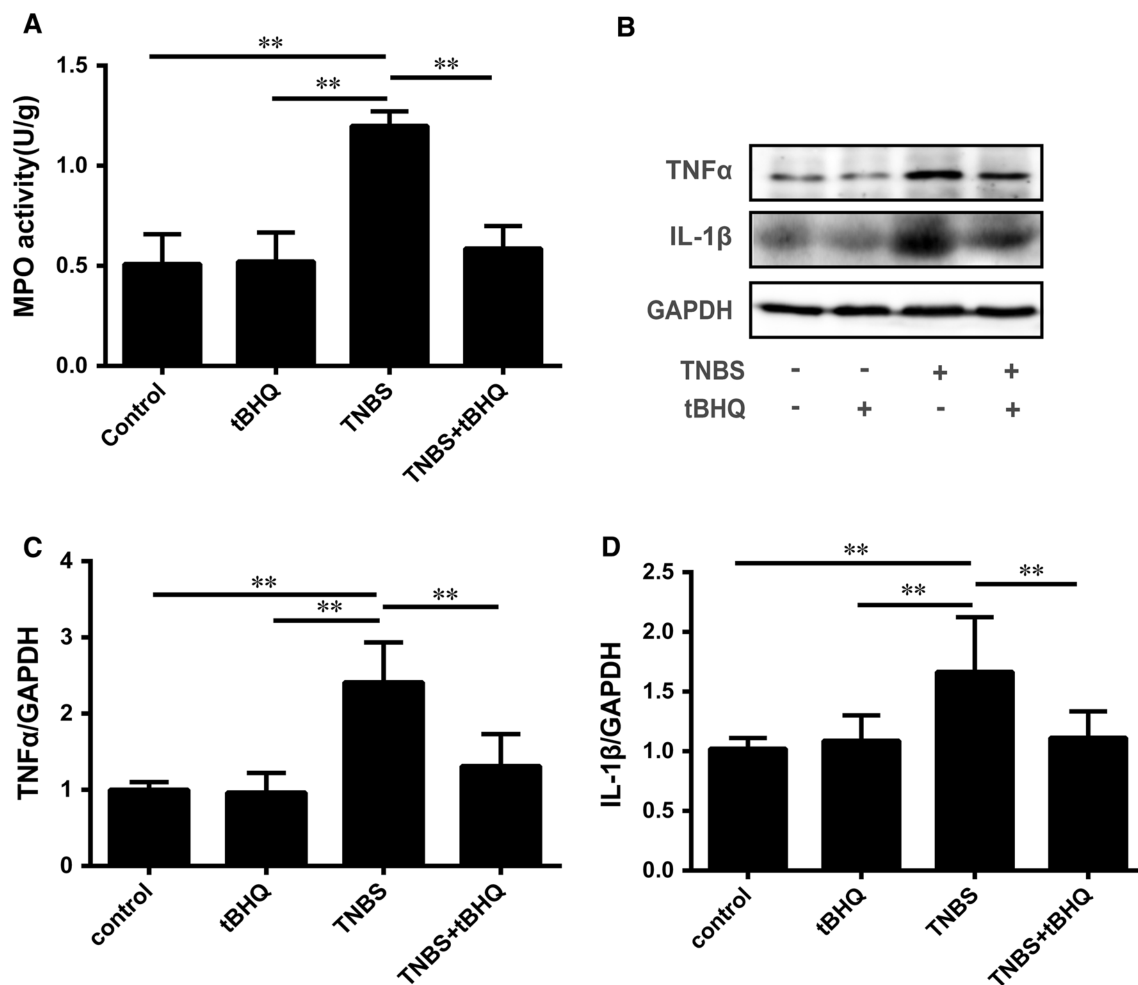

Fig. 2 tBHQ suppressed the increased expression of MPO and inflammation markers in TNBS-induced colitis. **a** MPO activity in colonic tissues from each group. Data were presented as mean ± SD, $n = 4$ per group, $*P < 0.05$ and $**P < 0.01$. **b–d** Western blot analysis for the expression of TNF-α and IL-1β and GAPDH in colonic tissues from each group. Data were presented as mean ± SD, $n = 5$ per group, $*P < 0.05$ and $**P < 0.01$

the expression of TIMP-1 mRNA in the TNBS-treated mice was 8.73-fold compared with the control mice. The expression of TIMP-1 in the TNBS + tBHQ group was 3.61-fold compared with the control group (Fig. 3d). The Western blot analysis of TIMP-1 had similar results as gene expression (Fig. 3f, i). The expression of MMP3 mRNA in the TNBS group showed no significant difference compared with the control group. The expression of MMP3 mRNA gene increased 1.12-fold in the TNBS + tBHQ group compared with the control group (Fig. 3e). Nevertheless, the Western blot result showed that the levels of MMP3 decreased significantly in the mice treated with TNBS (Fig. 3f, j). The expression of MMP3 protein in TNBS + tBHQ group elevated slightly compared with the TNBS group, but with no significant difference ($P > 0.05$).

tBHQ Induced Nrf2 and Nrf2-Related Antioxidant Expression in Intestinal Fibroblast Cells

The expression levels of Nrf2, HO-1, GCLC, and GCLM were examined by qRT-PCR and Western blot analysis to investigate the effect of tBHQ and TGF-β1 on Nrf2 and Nrf2-related antioxidant expression. As shown in Fig. 4a, pretreatment of CCD-18Co cells with tBHQ(25 μmol/L) for 24 h then treatment with TGFβ1(10 ng/mL) for 12 h elevated the mRNA expression of Nrf2, HO-1, GCLC and GCLM.

The other experimental data illustrated that TGF-β1 could induce the nuclear protein expression of Nrf2 (Fig. 4b). Moreover, treatment of CCD-18Co cells with tBHQ + TGF-β1 increased the nuclear protein expression of Nrf2 compared with TGF-β1 alone.

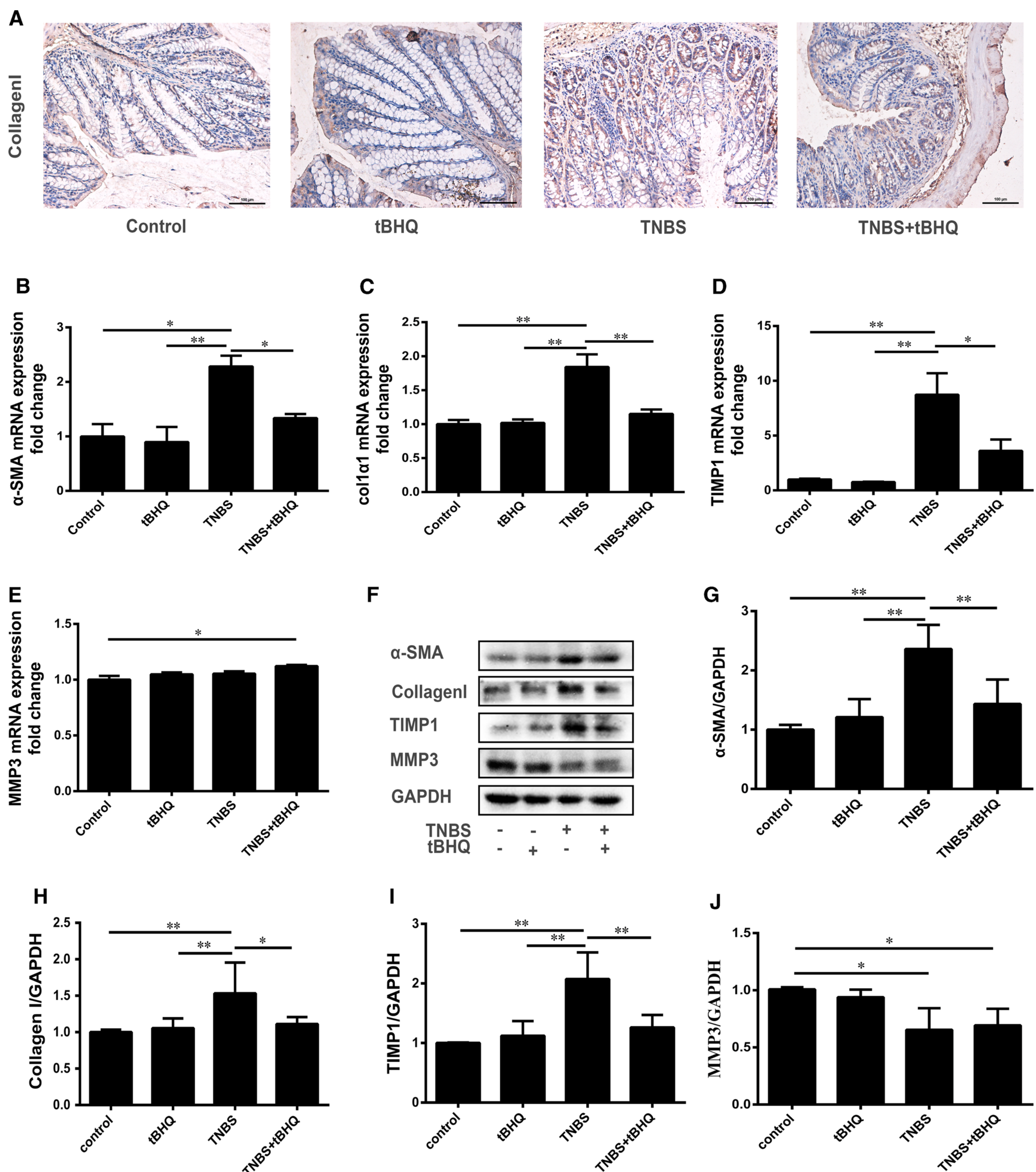

Fig. 3 tBHQ suppressed the increased expression of fibrosis markers in TNBS-induced colitis. **a** Immunohistochemical analysis of collagen I (×200). **b–e** Expression of α-SMA, Col1a1, MMP3, and TIMP-1 mRNA was measured by qRT-PCR and normalized to GAPDH mRNA. **f–j** Representative Western blot analysis for α-SMA, collagen I, MMP3, TIMP-1, and GAPDH in colonic tissues from each group. Data were presented as mean ± SD, $n = 5$ per group, $*P < 0.05$ and $**P < 0.01$

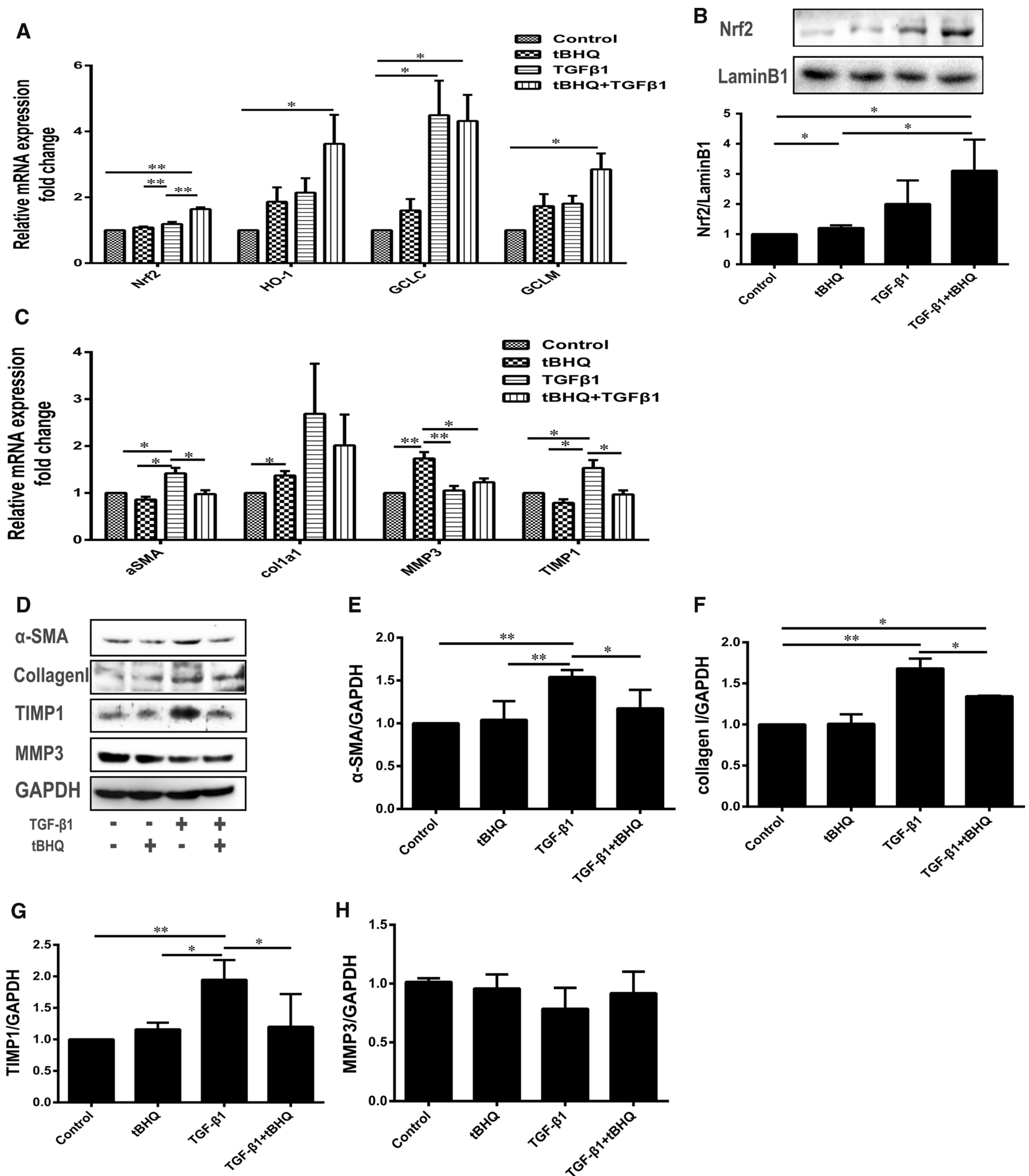

Fig. 4 Nrf2 inhibits fibrosis in CCD-18Co cells. **a** Effect of tBHQ and TGF-β1 on the Nrf2 and Nrf2-related antioxidant expression in CCD-18Co cells. Nrf2, HO-1, GCLC, and GCLM were examined by qRT-PCR and normalized to GAPDH mRNA. **b** Nuclear protein expression of Nrf2 was measured by Western blot analysis. **c** Treatment with tBHQ prevented the TGF-β1–induced differentiation of CCD-18co cells. The expression of α-SMA, Col1a1, MMP3, TIMP-1 mRNA was measured by qRT-PCR after pretreatment of CCD-18Co cells with tBHQ (25 μmol/L) for 24 h followed by treatment with TGFβ1 (10 ng/mL) for 12 h, **d**–**h** protein expression was measured by Western blot analysis. Data were presented as mean ± SD, $n = 3$–4 experiments, $*P < 0.05$ and $**P < 0.01$

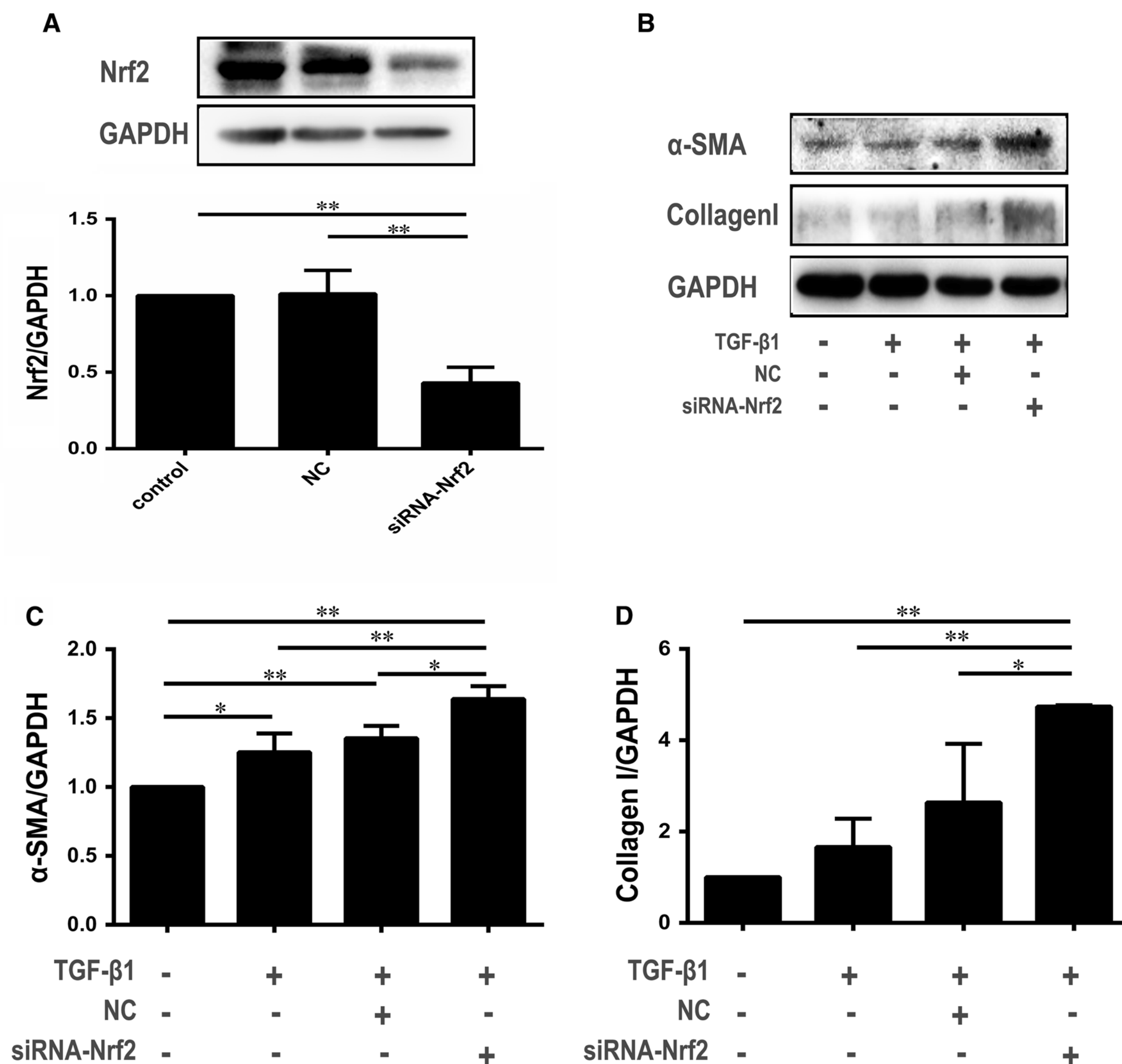

Fig. 5 Nrf2-siRNA enhanced fibrosis in CCD-18Co cells. **a** Western blot analysis showed that the expression of Nrf2 protein in CCD-18Co cells was reduced by siRNA transfection. **b–d** CCD-18Co cells were transfected with Nrf2 siRNA before TGF-β1 (10 ng/mL, 12 h) treatment, and the protein expression levels of α-SMA and collagen I were assessed by Western blot analysis. Data were presented as mean ± SD, $n = 3$–4 experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$

Treatment with tBHQ Prevented the TGF-β1-Induced Differentiation of CCD-18Co Cells

TGF-β1 could induce the differentiation of CCD-18Co fibroblasts transforming them into ECM-producing myofibroblasts. The results of Western blot and qPCR analyses revealed that the CCD-18Co cells stimulated with TGF-β1 indicated a significantly increase in the expression of fibrosis markers, α-SMA, collagen I, and TIMP-1. However, pretreatment with tBHQ significantly inhibited the TGF-β1-induced increase in the expression levels of α-SMA, collagen I, and TIMP-1 (Fig. 4c–g).

tBHQ treatment resulted in significantly higher expression of MMP3 mRNA compared with the control group, and the cells treated with TGF-β1 just increased slightly. Nevertheless, no differences were found in the expression of MMP3 between TGF-β1 and TGF-β1 + tBHQ treatment (Fig. 4c). The Western blot analysis also showed that the expression of MMP3 had no difference in the TGF-β1 and TGF-β1 + tBHQ groups (Fig. 4d, h).

Nrf2 Knockdown Enhanced TGF-β1-Induced Differentiation of CCD-18Co Cells

CCD-18Co cells were transfected with Nrf2 siRNA before TGF-β1 (10 ng/mL, 12 h) treatment, and fibrosis-related proteins were assessed by Western blot analysis to determine whether Nrf2 knockdown enhanced TGF-β1-induced fibrosis. The expression of Nrf2 significantly decreased by transfection with Nrf2 siRNA compared with negative control siRNA-transfected cells after 24 h (Fig. 5a). The protein expression levels of α-SMA and collagen I

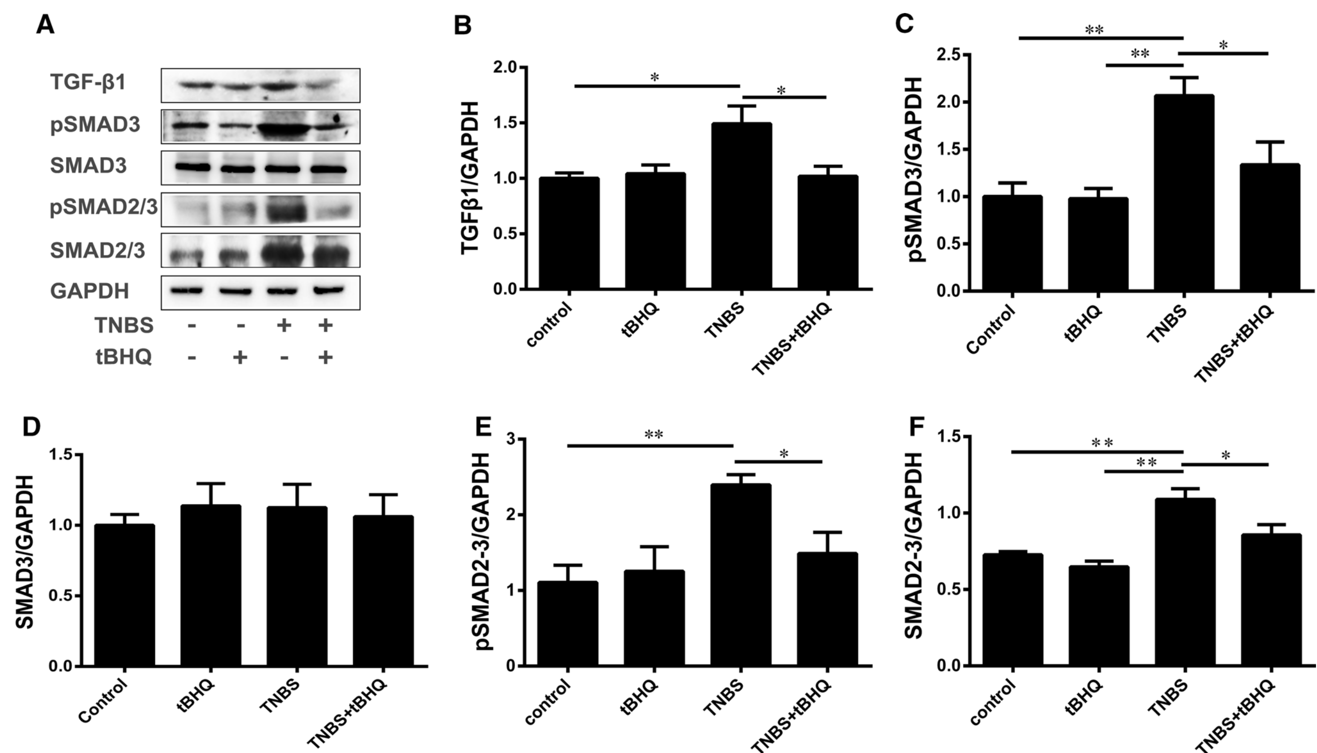

Fig. 6 Effect of tBHQ on the TGF-β1/Smads signaling pathway in TNBS-induced colitis. **a–f** Western blot analysis for TGF-β1, SMAD3, phosphorylation SMAD3, SMAD2/3, phosphorylation Smad2/3, and GAPDH in colonic tissues from each groups. Data were presented as mean ± SD, $n = 5$ per group, $^{*}P < 0.05$ and $^{**}P < 0.01$

markedly increased in the Nrf2 knockdown group compared with the negative control siRNA group (Fig. 5b–d), indicating that the downregulation of Nrf2 could enhance TGFβ1-induced differentiation of CCD-18Co cells.

Effect of tBHQ Treatment on the TGF-β1/SMADs Signaling Pathway in TNBS-Induced Colitis and CCD-18Co Cells

The TGF-β1/SMADs signaling pathway was considered to be closely involved in fibrosis and extracellular matrix production. The expression of TGF-β1 protein and its downstream effector molecules SMAD3, SMAD2/3, phosphorylated SMAD3, and phosphorylated Smad2/3 were examined by Western blot analysis to study the effect of tBHQ treatment on the TGF-β1/SMADs signaling pathway in TNBS-induced colitis. As shown in Fig. 6, TNBS administration led to a significant increase in the expression of TGF-β1, phosphorylated SMAD3, SMAD2/3, and phosphorylated SMAD2/3, whereas the expression of SMAD3 was not significantly altered. Daily oral tBHQ treatment decreased the expression of TGF-β1, phosphorylated SMAD3, SMAD2/3, and phosphorylated SMAD2/3.

The same results were observed in the in vitro study. The Western blot results showed that pretreatment with 25 μmol/L tBHQ for 24 h significantly blocked the increased expression of TGF-β1, phosphorylated SMAD3, SMAD2/3, and phosphorylated SMAD2/3 induced by treatment with 10 ng/mL TGF-β1 for 12 h in CCD-18co cell (Fig. 7b–h).

Effect of Nrf2 Knockdown on the TGF-β1/SMADs Signaling Pathway in CCD-18Co Cells

The levels of TGF-β1, phosphorylated SMAD3, and phosphorylated SMAD2/3 increased in CCD-18Co cells with negative control siRNA transfection and TGF-β1 treatment. Also, Nrf2-siRNA transfection significantly increased TGF-β1-induced proteins expression of phosphorylated SMAD3, phosphorylated SMAD2/3, and TGF-β1 compared with the negative control (Fig. 8a–d).

Nrf2 Inhibited Intestinal Fibrosis by Inhibiting the ROS-Dependent TGF-β1 Signaling Pathway in CCD-18Co Cells

The level of ROS was assayed with a fluorescence probe after 12-h incubation with TGF-β1 in CCD-18Co cells to determine whether ROS played a role in intestinal fibrosis. The result showed that ROS significantly increased with TGF-β1

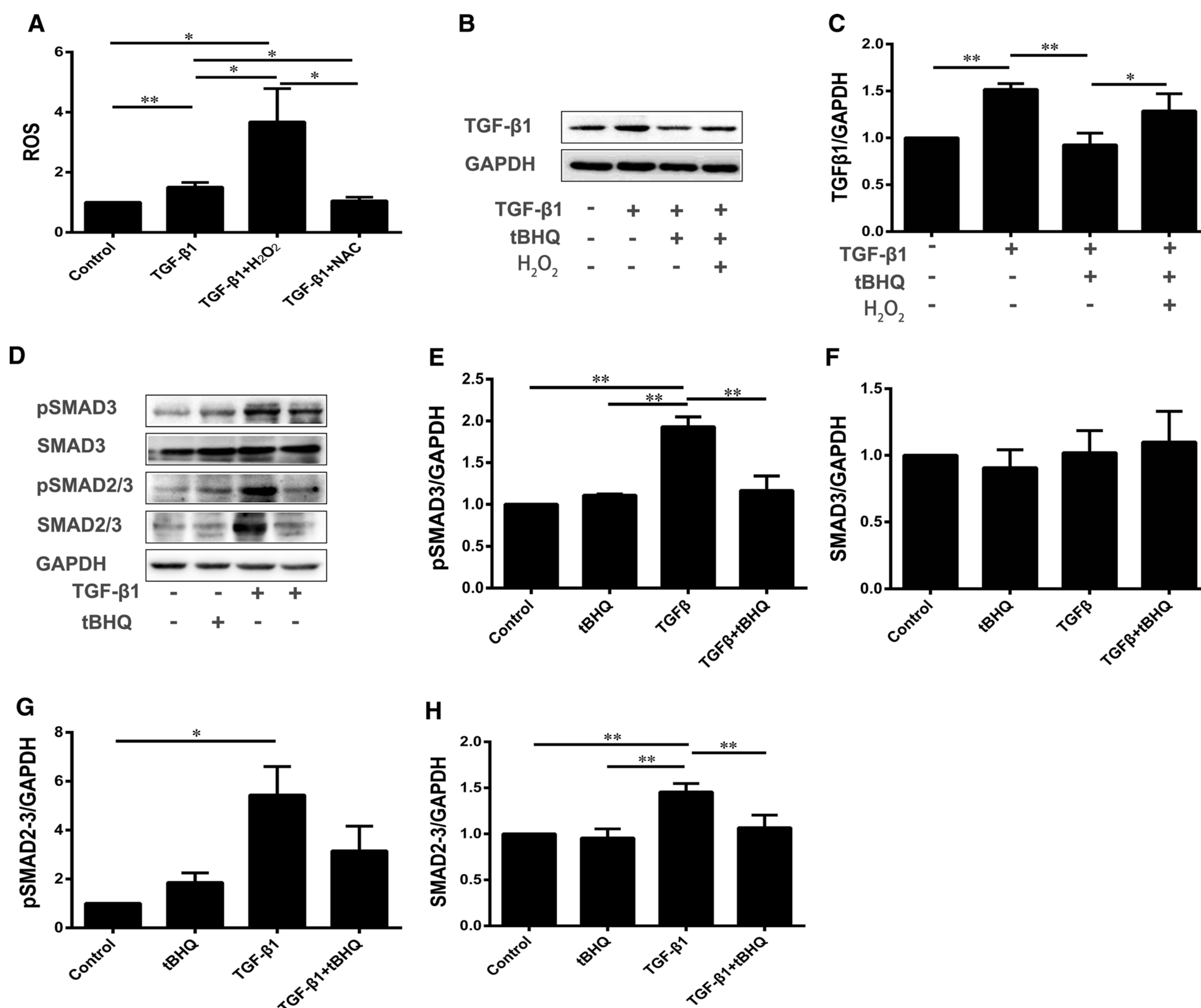

Fig. 7 Nrf2 inhibits ROS/TGF-β1/SMADs signaling pathway. **a** Expression of ROS generation was increased by TGF-β1. **b–c** Effect of tBHQ treatment on the expression of TGF-β1 in CCD-18Co cells. The protein level of was determined by Western blot analysis. Co-treatment with 200 mM H_2O_2 could block the effect of tBHQ on reducing the expression of TGF-β1 in CCD-18Co cells **d–h** effect of tBHQ treatment on the TGF-β1/SMADs signaling pathway in CCD-18Co cells. The protein levels of SMAD3, phosphorylation SMAD3, SMAD2/3, and phosphorylation Smad2/3 were determined by Western blot analysis. Data were presented as mean ± SD, $n = 3$–4 experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$

treatment (Fig. 7a). Cells pretreated with TGF-β1 and 100 mM hydrogen peroxide (H_2O_2), which is the main component of ROS, had a higher level of ROS compared with TGF-β1 only. The results of this study also indicated that pretreatment with 5 mM NAC, an ROS scavenger, could partially abolish the expression of TGF-β1-induced ROS.

H_2O_2 and NAC were used to confirm whether Nrf2 could inhibit intestinal fibrosis by inhibiting the ROS-dependent TGF-β1 signaling pathway in CCD-18Co cells. The Western blot results demonstrated that treatment with 200 mM H_2O_2 could block the effect of tBHQ on reducing the expression of TGF-β1 in CCD-18Co cells (Fig. 7b, c). Moreover, treatment with 5 mM NAC partially abolished the effect of Nrf2 siRNA on the activation of TGF-β1 expression (Fig. 8a). Altogether, all these results indicated that Nrf2 inhibited the expression of TGF-β1 signaling pathway via scavenging ROS in CCD-18Co cells (Fig. 9).

Discussion

Nrf2 plays a major role in regulating genes encoding many antioxidants and defending against oxidative stress [16]. The immunohistochemical analysis in a recent study revealed that the expression of Nrf2 was upregulated at inflammatory sites of IBD tissues [17]. Also, heme

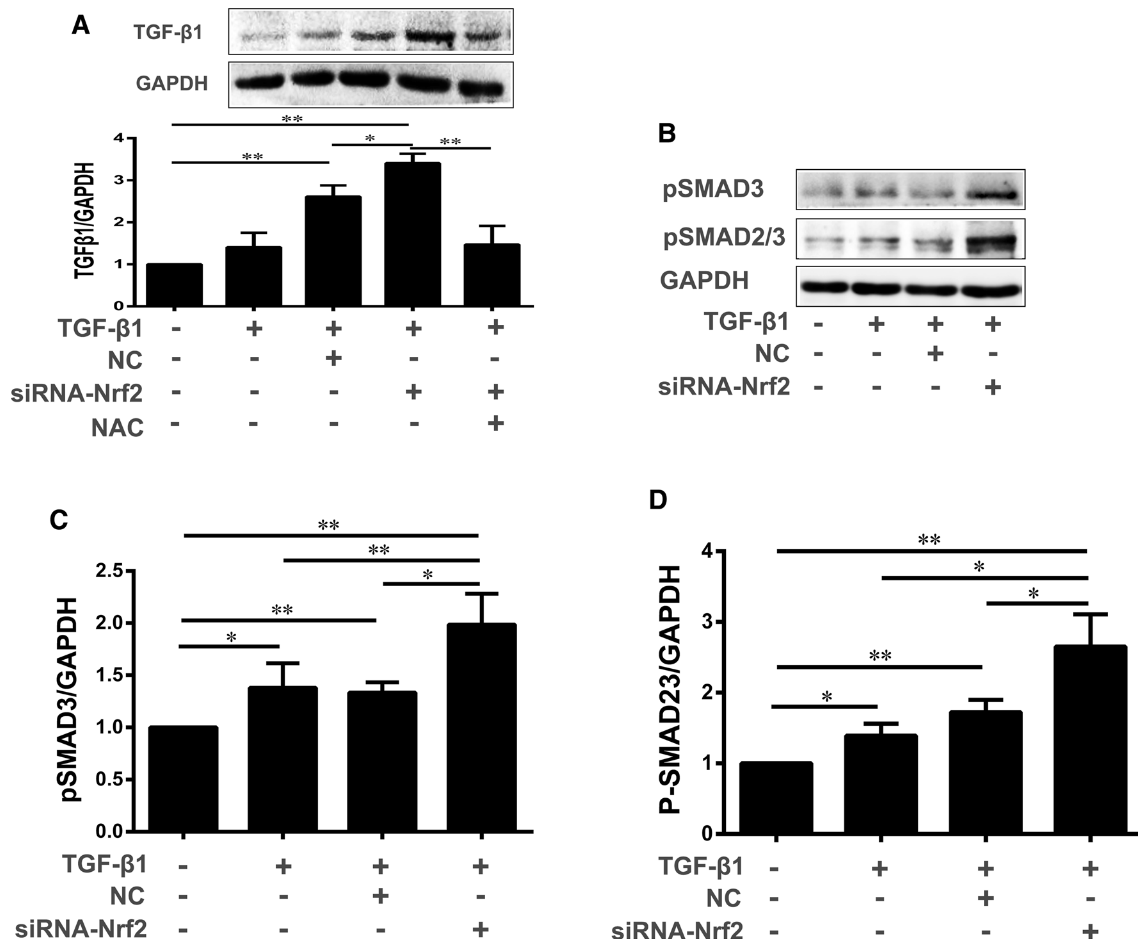

Fig. 8 Nrf2 inhibits ROS/TGF-β1/SMADs signaling pathway. **a** Effect of Nrf2-siRNA transfection on the expression of TGF-β1 in CCD-18Co cells. Co-treatment with 5 mM NAC partially abolished the effect of Nrf2-siRNA on the activation of TGF-β1 expression. **b–d** Effects of Nrf2 knockdown on TGF-β-stimulated phosphorylation SMAD3 and phosphorylation SMAD2/3 in CCD-18Co cells. Data were presented as mean ± SD, $n = 3$–4 experiments, $^*P < 0.05$ and $^{**}P < 0.01$

oxygenase-1 (HO-1), one of the Nrf2 target genes, also significantly increased in the colonic mucosa of patients with active UC [18]. In a preliminary experiment, Nrf2 nuclear transfer was increased in intestinal mesenchymal cells of chronic colitis-associated fibrosis mouse model.

Some reports revealed that sulforaphane, gallic acid, 3-(3-pyridylmethylidene)-2-indolinone, and cocoa could reduce the severity of colonic injury in dextran sulfate sodium (DSS)-induced colitis and protect against colitis-associated cancer by activating the Nrf2 pathway [19–21]. Moreover, Nrf2-deficient mice were more sensitive to DSS-induced colitis compared with wild mice [22]. It suggested that the Nrf2 pathway possessed the ability to alleviate acute colitis, chronic colitis, and associated colorectal cancer in the mouse model, but whether Nrf2 can protect against colitis-associated fibrosis is unexplored. Meanwhile, it has been confirmed that the Nrf2 pathway is a negative regulator of fibrosis in other organs [23, 24].

Nrf2 agonist tBHQ was used to investigate the antifibrotic role of Nrf2 in intestinal fibrosis. H&E and Masson's trichrome staining showed that the oral administration of tBHQ significantly alleviated high levels of inflammation and fibrosis in the colon of TNBS-induced chronic colitis mouse model.

The expression of MPO and proinflammatory cytokines, including TNF-α and IL-1β, was measured to evaluate the degree of inflammation in mouse model. This study demonstrated that administration of tBHQ decreased the high expression of MPO, TNF-α and IL-1β in TNBS-induced colitis. It appears to be generally accepted that intestinal fibrosis is a consequence of chronic inflammation [1]. TNF-α and IL-1β are not only proinflammatory cytokines but also profibrotic cytokines [25]. And decreasing the expression of TNF-α and IL-1β can inhibit the progression of fibrosis [26].

Collagen is a major constituent of ECM in the colon. Its deposition in the intestinal subepithelial layer increased

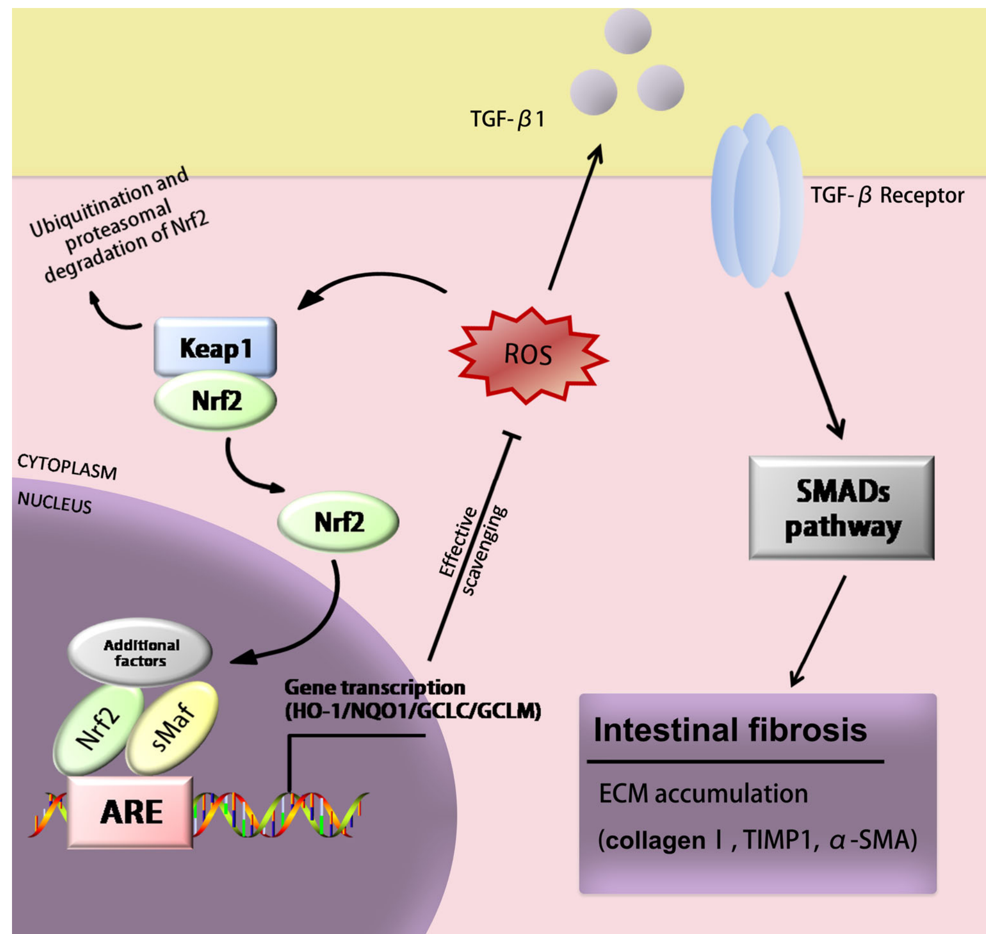

Fig. 9 Model illustrating how Nrf2 suppresses intestinal fibrosis by inhibiting the ROS-dependent TGF-β1/SMADs signaling pathway. ROS induce intestinal fibrosis through profibrotic TGF-β1/SMADs pathway. Nrf2 is released from Keap1 after stimulation by ROS, and translocates into the nucleus, then initiates the expression of various antioxidant-associated genes expression. The downstream products of Nrf2 scavenge the ROS, thereby inhibiting the profibrotic TGF-β1/SMADs pathway

after the repeated administration of TNBS [27]. α-smooth muscle actin (α-SMA), another major constituent of ECM and the marker of the activated myofibroblasts, also increased after the repeated administration of TNBS [28]. The results of this study showed that the high expression of collagen I and α-SMA in TNBS-induced colitis was attenuated by tBHQ treatment. The antifibrotic effect of tBHQ was also tested in vitro. This study demonstrated that the overexpression of collagen I and α-SMA stimulated by TGF-β1 was reduced due to pretreatment with tBHQ. It was also found that the downregulation of Nrf2 by siRNA transfection could enhance TGFβ1-induced overexpression of α-SMA and collagen I.

Intestinal fibrosis and deposition of ECM are also regulated by the balance between MMPs and TIMPs. The balance of MMPs/TIMPs is lost in intestinal tissues of patients with IBD [4]. Also, the mucosa overlying the strictured gut of fibrotic CD had lower expression of MMP3 compared with the mucosa overlying the non-strictured gut [29]. This study showed that the expression of MMP3 reduced in the TNBS group. Previous studies reported that the expression of TIMP1 increased in colonic tissues from DSS-induced murine models and myofibroblasts from fibrotic CD [30, 31]. And TIMP1 deficiency attenuated the development of intestinal fibrosis in DSS-induced murine models of colitis [10]. This study showed that tBHQ could reduce the expression of TIMP-1 in the mouse fibrosis model or human intestinal fibroblast cells. Both in vivo and in vitro experiments suggested that Nrf2 was a protective factor against intestinal fibrosis.

TGF-β1 is the most potent fibrogenic cytokine in the colon. It mediates mainly through SMADs signal transduction path ways to promote fibrosis. When TGF-β1 binds to its transmembrane receptor, SMAD2 and SMAD3 are phosphorylated and bind to SMAD4 to form a complex. This complex moves into the nucleus and induces the expression of target genes such as collagen and fibronectin, which are the main ECM components [29]. The expression of pSMAD2/3, TGF-β1, and its receptor TGF-β1R was significantly higher in the fibrotic colon tissue of patients with IBD and intestinal fibrosis animal model compared with the normal tissue [29, 32]. Vallance et al. [33] reported that the overexpression of TGF-β1 in the murine colon led to colonic fibrosis. Moreover, compared with wild mice, SMAD3-deficient mice had a lower susceptibility to trinitrobenzene sulfonic acid, which could induce

chronic colitis and intestinal fibrosis [34]. The other studies also demonstrated that SMAD3 deficiency prevented the development of fibrosis in various organs, such as the kidney, lungs, and liver [35].

The findings of this study demonstrated that tBHQ treatments in mice with TNBS-induced intestinal fibrosis downregulated the expression of TGF-β1, phosphorylation SMAD3, phosphorylation SMAD2/3 and SMAD2/3 in the Nrf2-dependent manner. The same result was obtained in CCD-18Co fibroblast cells stimulated with TGF-β1. Moreover, this study also demonstrated that Nrf2 knockdown increased the expression of TGF-β1/SMADs signaling pathway in CCD-18Co fibroblasts cells. This evidence indicated that TGF-β1/SMADs signaling pathway was inhibited by Nrf2 activation in intestinal fibrosis.

Available evidence shows that ROS contribute to the development of fibrosis in various organs including the liver, lung, heart, and kidney, and the reduction in ROS has a beneficial impact [36–38]. The results of this study showed that the production of ROS increased when TGF-β1 induced human intestinal fibroblasts to differentiate into activated ECM-producing myofibroblasts. The result was consistent with previous studies about lung fibroblasts and hepatic satellite cells in a fibrotic situation [39, 40]. Moreover, the accumulation of ROS could be reduced by the downstream production of Nrf2, such as HO-1 and NQO1, both in vivo and in vitro [36, 41]. This study showed that tBHQ, the inducer of Nrf2, decreased ROS production in TGF-β1-mediated intestinal fibroblasts. Therefore, it was conjectured that Nrf2 attenuated intestinal fibrosis by scavenging ROS.

Furthermore, it has been shown that ROS can influence the expression of TGF-β1 and mediate the TGF-β1-induced development of fibrosis in various fibrotic organs [42–44]. tBHQ can reduce the expression of TGF-β1, which is increased when intestinal fibroblasts differentiate into myofibroblasts. This study also demonstrated that pretreatment with H_2O_2, the primary component of ROS, could block the effect of tBHQ on reducing the expression of TGF-β1. Moreover, scavenging ROS by NAC could inhibit the increasing expression of TGF-β1 promoted by Nrf2 knockdown. It suggested that Nrf2 ameliorated intestinal fibrosis by inhibiting the ROS-dependent TGF-β1 pathway in human intestinal fibroblasts.

In conclusion, the findings of this study suggested that the activation of Nrf2 could decrease intestinal fibrosis both in vivo and in vitro by scavenging ROS and modulating TGF-β1/SMADs signaling. Therefore, it might provide a new possible target for treating IBD-related intestinal fibrosis.

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Author's contribution CZ and YG conceived and designed the experiments; YG, DP and JY performed the experiments; YS, YG and DW analyzed the data and prepared figures; YG, YT and WL wrote the paper; CZ revised the manuscript for important intellectual content; all authors approval of the final version to be published.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. The founding sponsors had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Ethical approval All animal experiments were approved by the institutional care and animal use committee of the China Medical University and conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals.

References

1. Fiocchi C, Lund PK. Themes in fibrosis and gastrointestinal inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2011; 300:G677–G683.
2. Latella G, Di Gregorio J, Flati V, Rieder F, Lawrance IC. Mechanisms of initiation and progression of intestinal fibrosis in IBD. *Scand J Gastroenterol*. 2015;50:53–65.
3. Rieder F, Fiocchi C, Rogler G. Mechanisms, management, and treatment of fibrosis in patients with inflammatory bowel diseases. *Gastroenterology*. 2017;152:340–350.
4. Lakatos G, Hritz I, Varga MZ, et al. The impact of matrix metalloproteinases and their tissue inhibitors in inflammatory bowel diseases. *Dig Dis*. 2012;30:289–295.
5. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med*. 2004;10:549–557.
6. Liebler DC, Guengerich FP. Elucidating mechanisms of drug-induced toxicity. *Nat Rev Drug Discov*. 2005;4:410–420.
7. Latella G, Rogler G, Bamias G, et al. Results of the 4th scientific workshop of the ECCO(I): pathophysiology of intestinal fibrosis in IBD. *J Crohns Colitis*. 2014;8:1147–1165.
8. Krstic J, Trivanovic D, Mojsilovic S, Santibanez JF. Transforming growth factor-beta and oxidative stress interplay: implications in tumorigenesis and cancer progression. *Oxid Med Cell Longev*. 2015;2015:654594.
9. Ge A, Ma Y, Liu YN, et al. Diosmetin prevents TGF-beta1-induced epithelial-mesenchymal transition via ROS/MAPK signaling pathways. *Life Sci*. 2016;153:1–8.
10. Kashima S, Fujiya M, Konishi H, et al. Polyphosphate, an active molecule derived from probiotic *Lactobacillus brevis*, improves the fibrosis in murine colitis. *Transl Res*. 2015;166:163–175.
11. Meng XM, Tang PM, Li J, Lan HY. TGF-beta/Smad signaling in renal fibrosis. *Front Physiol*. 2015;6:82.
12. Jin W, Ni H, Dai Y, et al. Effects of tert-butylhydroquinone on intestinal inflammatory response and apoptosis following traumatic brain injury in mice. *Mediators Inflamm*. 2010;2010: 502564.
13. Tao Q, Wang B, Zheng Y, Jiang X, Pan Z, Ren J. Vitamin D prevents the intestinal fibrosis via induction of vitamin D receptor and inhibition of transforming growth factor-beta1/Smad3 pathway. *Dig Dis Sci*. 2015;60:868–875.

14. Shih AY, Imbeault S, Barakauskas V, et al. Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. *J Biol Chem*. 2005;280: 22925–22936.
15. Videla S, Vilaseca J, Medina C, et al. Selective inhibition of phosphodiesterase-4 ameliorates chronic colitis and prevents intestinal fibrosis. *J Pharmacol Exp Ther*. 2006;316:940–945.
16. Aminzadeh MA, Nicholas SB, Norris KC, Vaziri ND. Role of impaired Nrf2 activation in the pathogenesis of oxidative stress and inflammation in chronic tubulo-interstitial nephropathy. *Nephrol Dial Transplant*. 2013;28:2038–2045.
17. Kruse ML, Friedrich M, Arlt A, et al. Colonic lamina propria inflammatory cells from patients with IBD induce the nuclear Factor-E2 Related Factor-2 thereby leading to greater proteasome activity and apoptosis protection in human colonocytes. *Inflamm Bowel Dis*. 2016;22:2593–2606.
18. Takagi T, Naito Y, Mizushima K, et al. Increased intestinal expression of heme oxygenase-1 and its localization in patients with ulcerative colitis. *J Gastroenterol Hepatol*. 2008;23:S229–S233.
19. Wagner AE, Will O, Sturm C, Lipinski S, Rosenstiel P, Rimbach G. DSS-induced acute colitis in C57BL/6 mice is mitigated by sulforaphane pre-treatment. *J Nutr Biochem*. 2013;24:2085–2091.
20. Pandurangan AK, Mohebali N, Norhaizan ME, Looi CY. Gallic acid attenuates dextran sulfate sodium-induced experimental colitis in BALB/c mice. *Drug Des Devel Ther*. 2015;9: 3923–3934.
21. Wang KP, Zhang C, Zhang SG, et al. 3-(3-pyridylmethylidene)-2-indolinone reduces the severity of colonic injury in a murine model of experimental colitis. *Oxid Med Cell Longev*. 2015;2015: 959253.
22. Khor TO, Huang MT, Kwon KH, Chan JY, Reddy BS, Kong AN. Nrf2-deficient mice have an increased susceptibility to dextran sulfate sodium-induced colitis. *Cancer Res*. 2006;66: 11580–11584.
23. Koo YC, Pyo MC, Nam MH, Hong CO, Yang SY, Lee KW. Chebulic acid prevents hepatic fibrosis induced by advanced glycation end-products in LX-2 cell by modulating Nrf2 translocation via ERK pathway. *Toxicol In Vitro*. 2016;34:8–15.
24. Divya T, Dineshbabu V, Soumyakrishnan S, Sureshkumar A, Sudhandiran G. Celastrol enhances Nrf2 mediated antioxidant enzymes and exhibits anti-fibrotic effect through regulation of collagen production against bleomycin-induced pulmonary fibrosis. *Chem Biol Interact*. 2016;246:52–62.
25. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med*. 2012;18:1028–1040.
26. Robert S, Gicquel T, Bodin A, Lagente V, Boichot E. Characterization of the MMP/TIMP imbalance and collagen production induced by IL-1β or TNF-α release from human hepatic stellate cells. *PLoS ONE*. 2016;11:e0153118.
27. Zhu MY, Lu YM, Ou YX, Zhang HZ, Chen WX. Dynamic progress of 2,4,6-trinitrobenzene sulfonic acid induced chronic colitis and fibrosis in rat model. *J Dig Dis*. 2012;13:421–429.
28. Melchior C, Loeuillard E, Marion-Letellier R, et al. Magnetic resonance colonography for fibrosis assessment in rats with chronic colitis. *PLoS ONE*. 2014;9:e100921.
29. Di Sabatino A, Jackson CL, Pickard KM, et al. Transforming growth factor beta signalling and matrix metalloproteinases in the mucosa overlying Crohn's disease strictures. *Gut*. 2009;58:777–789.
30. McKaig BC, McWilliams D, Watson SA, Mahida YR. Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. *Am J Pathol*. 2003;162:1355–1360.
31. Breynaert C, de Bruyn M, Arijs I, et al. Genetic deletion of tissue inhibitor of metalloproteinase-1/TIMP-1 alters inflammation and attenuates fibrosis in dextran sodium sulphate-induced murine models of colitis. *J Crohns Colitis*. 2016;10:1336–1350.
32. Rieder F, Kessler S, Sans M, Fiocchi C. Animal models of intestinal fibrosis: new tools for the understanding of pathogenesis and therapy of human disease. *Am J Physiol Gastrointest Liver Physiol*. 2012;303:G786–G801.
33. Vallance BA, Gunawan MI, Hewlett B, et al. TGF-beta1 gene transfer to the mouse colon leads to intestinal fibrosis. *Am J Physiol Gastrointest Liver Physiol*. 2005;289:G116–G128.
34. Latella G, Vetuschi A, Sferra R, et al. Smad3 loss confers resistance to the development of trinitrobenzene sulfonic acid-induced colorectal fibrosis. *Eur J Clin Invest*. 2009;39:145–156.
35. Speca S, Giusti I, Rieder F, Latella G. Cellular and molecular mechanisms of intestinal fibrosis. *World J Gastroenterol*. 2012;18:3635–3661.
36. Wang G, Yeung CK, Wong WY, et al. Liver fibrosis can be induced by high salt intake through excess reactive oxygen species (ROS) production. *J Agric Food Chem*. 2016;64:1610–1617.
37. Maimaiti R, Zhang Y, Pan K, Wubuli M, Andersson R. Frequent coinfection with hepatitis among HIV-positive patients in Urumqi China. *J Int Assoc Provid AIDS Care*. 2013;12:58–61.
38. Shen Y, Miao NJ, Xu JL, et al. *N*-acetylcysteine alleviates angiotensin II-mediated renal fibrosis in mouse obstructed kidneys. *Acta Pharmacol Sin*. 2016;37:637–644.
39. Jain M, Rivera S, Monclus EA, et al. Mitochondrial reactive oxygen species regulate transforming growth factor-beta signaling. *J Biol Chem*. 2013;288:770–777.
40. Yang Y, Kim B, Park YK, Koo SI, Lee JY. Astaxanthin prevents TGFbeta1-induced pro-fibrogenic gene expression by inhibiting Smad3 activation in hepatic stellate cells. *Biochim Biophys Acta*. 2015;1850:178–185.
41. Nie H, Xue X, Liu G, et al. Nitro-oleic acid ameliorates oxygen and glucose deprivation/re-oxygenation triggered oxidative stress in renal tubular cells via activation of Nrf2 and suppression of NADPH oxidase. *Free Radic Res*. 2016;50:1200–1213.
42. Liu RM, Desai LP. Reciprocal regulation of TGF-beta and reactive oxygen species: a perverse cycle for fibrosis. *Redox Biol*. 2015;6:565–577.
43. Wu H, Li GN, Xie J, et al. Resveratrol ameliorates myocardial fibrosis by inhibiting ROS/ERK/TGF-beta/periostin pathway in STZ-induced diabetic mice. *BMC Cardiovasc Disord*. 2016;16:5.
44. Manoury B, Nenan S, Leclerc O, et al. The absence of reactive oxygen species production protects mice against bleomycin-induced pulmonary fibrosis. *Respir Res*. 2005;6:11.