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Nimbolide Inhibits Nuclear Factor-κB Pathway in Intestinal Epithelial Cells and Macrophages, and Alleviates Experimental Colitis in Mice

Nimbolide의 Nuclear Factor-κB 신호 전달 억제 및 대장염 마우스 모델 염증 완화 효과

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Nimbolide Inhibits Nuclear Factor-κB Pathway in Intestinal Epithelial Cells and Macrophages, and Alleviates Experimental Colitis in Mice

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ABSTRACT

Introduction: Nimbolide is a limonoid extracted from neem tree (*Azadirachta indica*) that has antiinflammatory properties. The aim of this study was to investigate the effect of nimbolide on the nuclear factor−kappa B (NF−κB) pathway in intestinal epithelial cells (IECs), macrophages and in murine colitis models.

Methods: The IEC cell−line COLO 205, the murine macrophage cell−line RAW 264.7 and peritoneal macrophages extracted from interleukin (IL)−10 deficient (IL−$10^{-/-}$) mice were preconditioned with nimbolide or vehicle and then stimulated. The secretion of inflammatory cytokines (IL−6, IL−8, IL−12 and tumor necrosis factor−α) was examined by ELISA. Effects on the NF−κB pathway were evaluated by western blot of IκBα phosphorylation and electrophoretic mobility shift assay. Dextran sulfate sodium (DSS)−induced acute colitis model and chronic colitis model in IL−$10^{-/-}$ mice were used for *in vivo* experiments. Colitis was estimated by disease activity index (DAI) and histopathologic evaluation.

Results: Nimbolide significantly suppressed the expression of

inflammatory cytokines, and inhibited the phosphorylation of IκBα and the DNA-binding affinity of NF-κB in IECs and macrophages. Nimbolide was effective in both DSS-induced acute colitis and in IL-$10^{-/-}$ chronic colitis: It ameliorated weight loss, colon shortening, DAI score and histologic scores in DSS colitis. It also improved histopathologic scores in the chronic colitis of IL-$10^{-/-}$ mice. Staining for phosphorylated IκBα was significantly decreased in colon tissue after treatment with nimbolide in both models.

Conclusions: Nimbolide inhibits NF-κB signaling in IECs and macrophages, and ameliorates experimental colitis in mice. Our results suggest nimbolide could be a potentially new treatment for inflammatory bowel disease.

Keywords: Nimbolide, murine colitis, macrophage, NF-κB
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LIST OF ABBREVIATIONS

CD: Crohn's disease

DAI: disease activity index

DMSO: dimethyl sulfoxide

DSS: dextran sulfate sodium

EMSA: electrophoretic mobility shift assay

H&E: hematoxylin and eosin

IBD: inflammatory bowel disease

IEC: intestinal epithelial cell

IHC: immunohistochemical

IL: interleukin

IL-$10^{-/-}$: interleukin-10-deficient

KCLB: Korean Cell Line Bank

LPS: lipopolysaccharide

NF-κB: nuclear factor-kappa B

SPF: specific pathogen-free

TNF-α: tumor necrosis factor-α

UC: ulcerative colitis

INTRODUCTION

The prevalence of inflammatory bowel disease (IBD) varies according to the region, genetic background of the population and its environment. IBD predominantly occurs in Caucasians of North America and Europe, however, its prevalence is steadily increasing in Asia and Middle East.[1] A curative drug for IBD is lacking, thus, IBD patients have been suffered from multiple relapses of the disease and socioeconomic cost for IBD is continuously increasing.[2]

Although the pathogenesis of IBD remains obscure, it has been linked to the mutual interaction between bacteria and a susceptible host. The inappropriate excessive immune reaction toward commensal microbiota is triggered in a genetically susceptible host.[3] Consequently, the balance of proinflammatory and antiinflammatory condition in the intestine is impaired and becomes overly weighted toward a proinflammatory response that eventually leads to IBD. Nuclear factor-κB (NF-κB) plays a salient role in this proinflammatory cascade[4,5] as evidenced by how the activity of NF-κB is increased in the gut of IBD patients, particularly in macrophages and intestinal

epithelial cells (IECs).[6,7] Activation of NF-κB induces a myriad of proinflammatory cytokines, such as interleukin (IL)-8 and tumor necrosis factor (TNF)-α that trigger a proinflammatory cascade. As a result, the NF-κB pathway has been specifically targeted for the treatment of IBD.[5,8]

Limonoid is a highly oxygenated, modified terpenoid with a prototypical structure. It is usually found in plant families of Meliaceae and Rutaceae.[9,10] Limonoid compounds have been showed various medical effects such as antiviral, antibacterial, antifungal, antimalarial, antiinflammatory, and anticancer.[11-15]

The neem tree (*Azadirachta indica*), one of the most abundant source of various limonoids,[9] is a broad-leaved tree found in the tropical regions of South Asia, Southeast Asia and West Africa. This tree has been used as a traditional medicine remedy in India and Africa for thousands of years, and is known as "the tree of 40" because it is used as a treatment for 40 diseases. Various parts of neem tree such as leaf, bark, twig, flower, fruit, seed, oil and gum were used for various medical purposes. The effects were different according to the part of the tree, however, antipyretic, antiviral, antimalarial, antifungal, antiinflammatory, and anticancer effects were reported typically.

Among them, neem leaf was most commonly used: soft leaves were used as bed cover of small pox patients and powdered leaves were applied on wounds traditionally.[16] Many chemical compounds have been extracted from the neem tree, including nimbin, nimbinin, nimbidic acid, and azadirachtin A.[17] Of these, nimbolide ($C_{27}H_{30}O_7$) is a well-known representative neem tree extract with widely studied effects. Previous studies of nimbolide have mainly focused on its anticancer effects,[18-24] with an association between nimbolide and the NF-κB pathway suggested in this regard.[18,19,21] However, the antiinflammatory effect and its mechanism of nimbolide on colitis have not been reported. Therefore, the aim of this study was to examine the antiinflammatory effect of nimbolide in IBD. Specifically, we evaluated the effect of nimbolide on the NF-κB signaling pathway in cultured IECs and macrophages and verified the anti-colitic effect of nimbolide in two different colitis models: a dextran sulfate sodium (DSS)-induced acute colitis model and a chronic colitis model in interleukin-10-deficient (IL-$10^{-/-}$) mice.

MATERIALS AND METHODS

1. Drugs and chemical reagents

Nimbolide was purchased from Biovision (Milpitas, CA, USA, purity ≥98% determined by TLC). Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS, from *Escherichia coli* 0127:B8), and piroxicam were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tumor necrosis factor-α (TNF-α) was obtained from R&D systems (Minneapolis, MN, USA). 4% DSS (molecular weight 36,000 to 50,000) was supplied from MP Biochemicals (Irvine, CA, USA). For immunoblot analysis, anti-human phosphorylated IκBα was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-mouse IκBα, β-actin, and Goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For Immunohistochemical (IHC) analysis, rabbit polyclonal anti-phosphorylated IκBα antibody was supplied by Cell Signaling Technology and mouse anti-rabbit IgG was supplied by Santa Cruz Biotechnology.

2. Cell culture

The human IEC, COLO 205 (KCLB 10222, Korean Cell Line Bank [KCLB], Seoul, Korea), and the mouse macrophage cell line, RAW 264.7 (KCLB 40071, KCLB) were used. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with antibiotics and fetal bovine serum as reported previously.[25,26]

3. Mice

This study protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea, (IACUC No. SNU-140121 and SNU-140526). All procedures performed in studies involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No.80-23, revised in 1978).

Six-week-old C57BL/6 wild type mice grown under specific pathogen-free (SPF) conditions were ordered from Orientbio (Seongnam, Korea). Seven-week-old IL-$10^{-/-}$ mice, in a C57BL/6 background, raised under SPF conditions were provided by the Center for Animal Resource and Development

of Seoul National University (Seoul, Korea).[27] Mice were fed a standard chow diet *ad libitum* and were maintained under a 12h/12h day/night cycle until they reach 7–9 weeks of age and 19–22 g of body weight. Mice were kept in ventilated cages at 24 ± 2° C and in 50 ± 5% relative humidity under SPF conditions.

4. Extraction and culture of peritoneal macrophages

Peritoneal macrophages were extracted from 10– to 12–week–old IL–$10^{-/-}$ mice as previously described.[26,28] Firstly, 2 mL of 4% Brewer's thioglycollate medium (Sigma–Aldrich) in distilled water was injected into the mouse peritoneum. Thioglycollate induces intraperitoneal inflammation and macrophages congregate in the resultant ascites. Four days later, 10 mL of buffer (Hank's balanced salt solution and fetal bovine serum) was injected into the peritoneum and gently extracted with a syringe. Withdrawn peritoneal fluid was centrifuged and pelleted cells were resuspended in RPMI–1640 medium (Invitrogen Corp., Carlsbad, CA, USA). After culturing

for 2 h, cells were washed and any remaining, attached peritoneal macrophages were used.

5. ELISA

COLO 205 cells, RAW 264.7 cells, and IL-10$^{-/-}$ macrophages were pretreated with vehicle (DMSO), or 20 or 200 nM of nimbolide for 24 h. After pretreatment, COLO 205 cells were stimulated with TNF-α and macrophages were stimulated with LPS for 4 h. The secretion of IL-8 protein from COLO 205 cells, and IL-6, IL-12 and TNF-α proteins from RAW 264.7 cells and IL-10$^{-/-}$ peritoneal macrophages was measured with Quantikine® immunoassay kits (R&D Systems).[26,29]

6. Real-time reverse transcription polymerase chain reaction

COLO 205 cells were preconditioned with nimbolide or vehicle for 24 h, and then, cells were stimulated with TNF-α for 4 h. Intracellular RNAs were extracted with TRIzol (Gibco/BRL, Gaithersburg, MD). The mRNAs of IL-8 and β-actin were amplified by real-time reverse transcription polymerase chain

reaction.[25,30] Primers used in real-time reverse transcription polymerase chain reaction were constructed using Primer Express (version 2.0; Applied Biosystems, Foster City, CA, USA).

7. Electrophoretic mobility shift assay

COLO 205, RAW 264.7 cells, and IL-$10^{-/-}$ peritoneal macrophages were preconditioned with nimbolide or vehicle for 24 h, and stimulated with TNF-α for 30 min. Nuclear proteins were extracted and purified as reported previously.[27,29] A LightShift® chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce, Rockford, IL, USA) was used. Biotin-labeled DNA oligonucleotide probes, whose sequences were relevant to the binding site of NF-κB, were added to nuclear extracts.[27,29] An antibody against NF-κB p50 was used for the supershift assay. Bound and unbound DNA samples were loaded on to a 5% polyacrylamide gel. After separating DNAs by electrophoresis, DNA was transferred to a nylon membrane and target DNA labeled with biotin was detected by chemiluminescence.

8. Immunoblot analysis

COLO 205 and RAW 264.7 cells, and IL-$10^{-/-}$ peritoneal macrophages were treated with nimbolide and stimulated as for EMSA. After washing, cells were lysed with lysis buffer as previously described.[26,31] Protein samples were loaded on to a 10% sodium dodecyl sulfate-polyacrylamide gel. After samples were separated by electrophoresis, they were transferred to a 0.1 μm pore size, nitrocellulose membrane. After attachment of primary and secondary antibodies, target protein bands were detected by a chemiluminescent method.

9. DSS-induced acute murine colitis (preventive model)

To induce murine colitis in wild-type C57BL/6 mice, 4% DSS was used. Seven-week-old male mice, approximately 20 g in weight, were used for the preventive model. After checking their body weights, a total of 32 mice were randomly allocated to four groups (negative control, positive control, nimbolide 0.2 mg/kg, and nimbolide 1 mg/kg). Nimbolide was dissolved in DMSO (vehicle), and administered intragastrically once daily

over 7 days for the nimbolide groups. The positive control group received the same volume of vehicle (DMSO) for 7 days. The positive control and nimbolide groups received 4% DSS solution for 5 days (day 0 to day 5) to induce acute colitis after the administration of nimbolide or vehicle up to two days prior (days −2 to −1). For the negative control group, filtered water was administrated for 7 days and artificial administration was not performed. For all mice, body weight, stool form and presence of blood in the stool were checked daily. All mice were sacrificed on day 8. A disease activity index (DAI) was graded as previously described.[32,33] The DAI represents the severity of colitis, which includes weight loss, diarrhea and occult or gross blood in the stool. After sacrifice, entire colons, from the cecum to the anus, were removed. Colons were incised longitudinally and formalin−fixed paraffin−embedded slides were stained with hematoxylin and eosin (H&E). A pathologist, unaware of study details, evaluated the severity of inflammation according to a scoring system.[34] Each score for the extent of crypt damage and the degree and extent of inflammation, was multiplied by a score for the involved area

(scored from 1 to 4) and the sum calculated was used as a histologic score.

10. Chronic colitis in IL−10$^{-/-}$ mice (therapeutic model)

Piroxicam (200 ppm) mixed with chow was fed to 8− to 9−week−old IL−10$^{-/-}$ mice for 14 days to promote colitis.[27,35] After the induction of colitis, chow containing piroxicam was changed to standard chow and a total of 21 mice were randomly allocated to three groups (positive control, nimbolide 0.2 mg/kg, and nimbolide 1 mg/kg). For the nimbolide groups, 0.2 or 1 mg/kg of nimbolide was administered intragastrically, once daily for 14 days. For the positive control group, an equal volume of vehicle (DMSO) was administered for 14 days instead of nimbolide. For the negative control group, filtered water and standard chow were administered without any special treatment including piroxicam (n = 4). Body weight, stool form, and the presence of blood in the stool were monitored for the two weeks of treatment, after which mice were sacrificed. The total lengths of colons were extracted and measured. Proximal

colons, where colitis was most marked, were incised longitudinally and pathology specimens were prepared on slides as detailed above. A pathologist, unaware of study details, reviewed the slides and gave each a score from 0 to 4 according to the degree of inflammation as reported previously.[27,35]

11. Immunohistochemical analysis of IκBα phosphorylation

IκBα phosphorylation was evaluated in colon tissue by IHC analysis as described previously.[36] For microwave antigen retrieval, prepared slides were placed into a clean polyethylene chamber filled with 10 mM of citrate buffer, microwaved, and cooled. After washing, slides were incubated with primary and secondary antibodies. A pathologist, unaware of study details, evaluated the intensity of immunoreactivity, which was scored from 0 to 4+ as described previously.[37]

12. Statistical analysis

Values are expressed as mean ± standard deviation. A Mann-

Whitney test or Student's *t*-test were used to compare differences between groups. To compare serial body weights among groups, a repeated measure one-way analysis of variance followed by Turkey's post-hoc test were used. *P* value under 0.05 was considered to be statistically significant. All statistical analyses in this study were performed using SPSS (version 18.0; Chicago, IL, USA).

RESULTS

Nimbolide inhibits IL-8 expression in TNF-α-stimulated COLO 205 cells

The effect of nimbolide on the production of a proinflammatory cytokine in COLO 205 cells was evaluated. Pretreatment of COLO 205 cells with nimbolide (20, 200 nM) significantly decreased the secretion of IL-8 protein induced by TNF-α ($P < 0.05$, $P < 0.01$, respectively; Figure 1A). Similarly, pretreatment with nimbolide significantly decreased the expression of IL-8 mRNA ($P < 0.01$ for both, Figure 1B).

Nimbolide inhibits inflammatory cytokines in LPS-stimulated RAW 264.7 and IL-$10^{-/-}$ peritoneal macrophages

Secreted inflammatory cytokines (IL-6, IL-12 and TNF-α) were evaluated in RAW 264.7 macrophages and IL-$10^{-/-}$ peritoneal macrophages after stimulation with LPS. In RAW 264.7 macrophages, the expression of IL-6, IL-12, and TNF-α significantly declined in a dose-dependent manner after treatment with nimbolide (20 or 200 nM; $P < 0.05$ and $P < 0.01$,

respectively; Figures 1C, 1D, and 1E). Likewise, levels of IL−6, IL−12, and TNF−α also significantly decreased in IL−$10^{-/-}$ macrophages with nimbolide pretreatment (P < 0.01 for all; Figures 1F, 1G, and 1H).

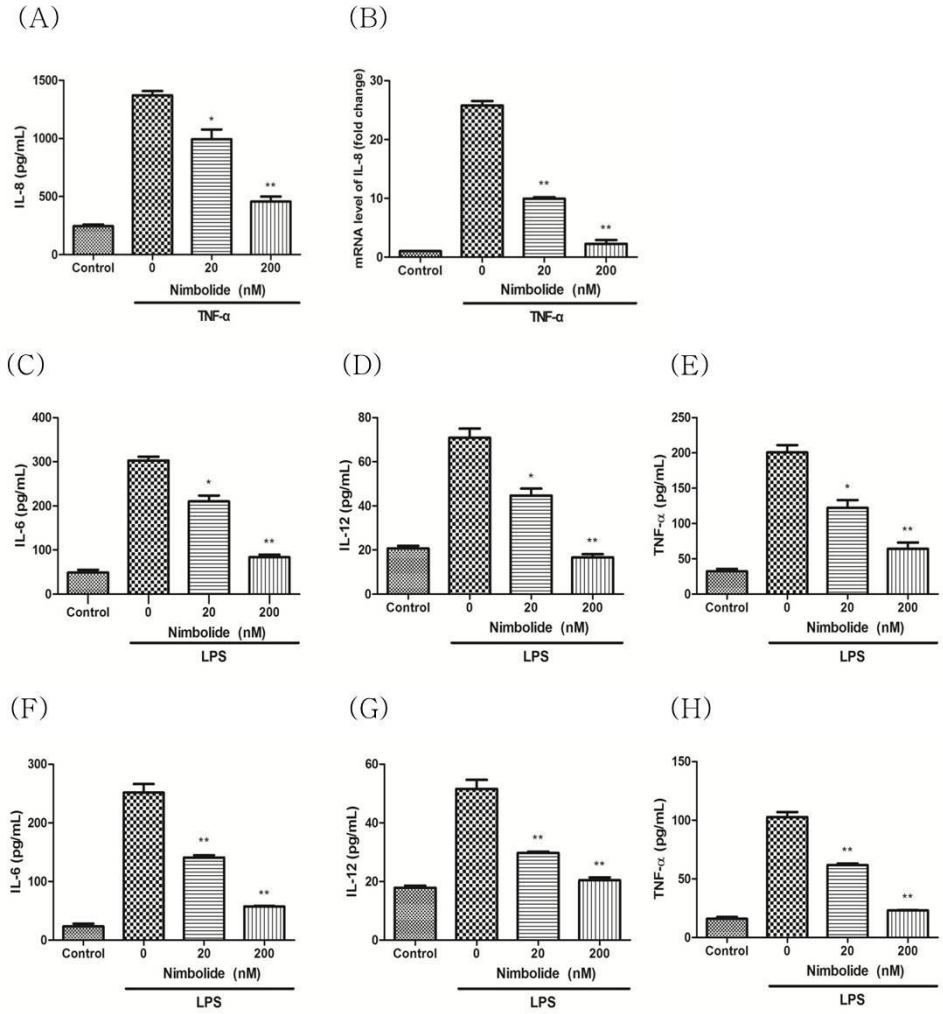


Figure 1. Suppression of proinflammatory cytokines with nimbolide. COLO 205 cells were pretreated with 20 or 200 nM nimbolide or vehicle for 24 h and then stimulated with tumor necrosis factor-α (TNF-α, 10 ng/mL) for 4 h. The production of interleukin (IL)-8 protein and mRNA was detected by ELISA (A) and reverse transcription polymerase

chain reaction (B), respectively. RAW 264.7 and interleukin-10-deficient (IL-$10^{-/-}$) macrophages were treated with nimbolide (20 or 200 nM) or vehicle for 24 h and stimulated with lipopolysaccharide (LPS, 10 μg/mL) for 4 h. The secretion of proinflammatory cytokines: IL-6, IL-12 and TNF-α in RAW 264.7 macrophages (C, D, E) and IL-$10^{-/-}$ peritoneal macrophages (F, G, H) was measured by ELISA. Results are representative of more than three repetitive experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with TNF-α or LPS alone.

Nimbolide reduces DNA-binding activity of NF-κB in TNF-α-stimulated COLO 205 cells, RAW 264.7 macrophages and IL-$10^{-/-}$ macrophages

We postulated that the suppression of inflammatory cytokines by nimbolide might be caused by suppression of the NF-κB pathway. To verify this, the affinity of NF-κB for DNA was assessed by EMSA. In COLO 205 cells, RAW 264.7 macrophages and IL-$10^{-/-}$ macrophages, the DNA-binding affinity of NF-κB greatly increased when cells were treated with TNF-α (Figures 2A, 2B, and 2C). DNA-binding affinity of NF-κB decreased with nimbolide pretreatment, especially at a higher dose (Figures 2A, 2B, and 2C).

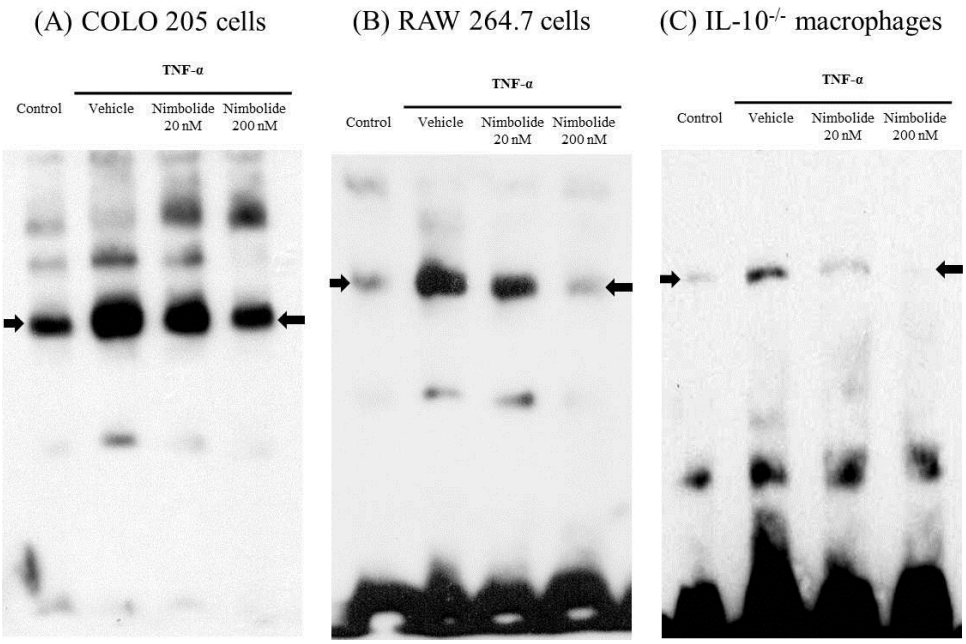


Figure 2. DNA-binding activity of nuclear factor-kappa B (NF-κB) after nimbolide treatment. COLO 205 cells, RAW 264.7 macrophages, and interleukin-10-deficient (IL-$10^{-/-}$) peritoneal macrophages were pretreated with nimbolide (20 or 200 nM) for 24 h, followed by stimulation with tumor necrosis factor-α (TNF-α, 10 ng/mL) for 30 min. Electrophoretic mobility shift assays were performed using anti-NF-κB p50 antibody to detect the DNA-binding activity of NF-κB (arrows) in COLO 205 cells (A), RAW 264.7 macrophages (B) and IL-$10^{-/-}$ peritoneal macrophages (C). The results are representative of more than three repetitive experiments.

Nimbolide suppresses IκBα phosphorylation in TNF-α-stimulated COLO 205 cells, and RAW 264.7 and IL-10$^{-/-}$ macrophages

To establish the precise inhibitory mechanism of nimbolide on NF-κB signaling, levels IκBα and phosphorylated IκBα, which are upstream molecules in NF-κB signaling, were evaluated by western blot analysis, with or without pretreatment with nimbolide. Phosphorylated IκBα decreased in a dose-response manner when nimbolide was used to pretreat COLO 205 cells (Figure 3A), RAW 264.7 macrophages (Figure 3B) and IL-10$^{-/-}$ peritoneal macrophages (Figure 3C), respectively. According to the NF-κB signaling, the inhibition of phosphorylation of IκBα leads to a decline in IκBα degradation and a consequent increase in IκBα, as seen in Figure 3. Consequently, DNA binding by NF-κB would then become suppressed by interfering with the nuclear translocation of NF-κB.

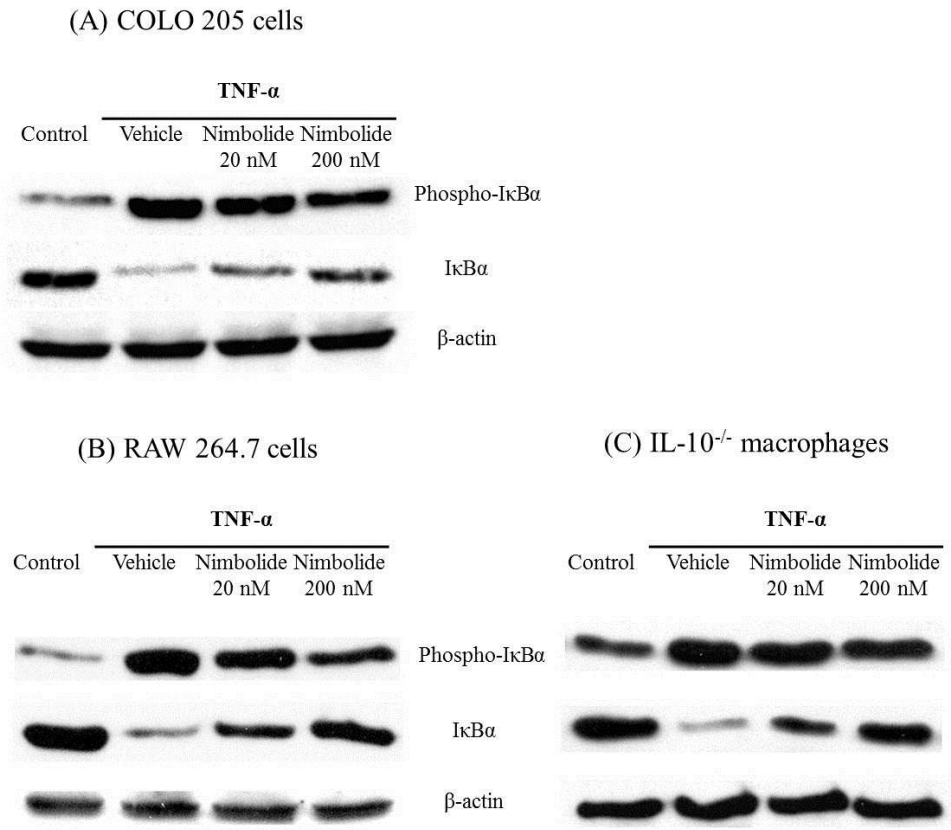


Figure 3. Changes in IκBα phosphorylation and degradation after treatment with nimbolide. Immunoblot analysis of phosphorylated IκBα, IκBα, and β-actin was performed in COLO 205 cells (A), RAW 264.7 macrophages (B) and interleukin-10-deficient (IL-$10^{-/-}$) peritoneal macrophages (C) after pretreatment with nimbolide or vehicle for 24 h and stimulation with tumor necrosis factor-α (TNF-α, 10 ng/mL) for 30 min. β-actin was used as a loading control. The results are representative of more than three repetitive experiments.

Nimbolide alleviates DSS-induced acute colitis

In vivo experiments were conducted to confirm the antiinflammatory effects of nimbolide in colitis using two murine models. The first model was a DSS-induced acute colitis model, applied to elucidate any preventive effect of nimbolide. The other model was a chronic colitis model established in IL-$10^{-/-}$ mice to evaluate the therapeutic effect of nimbolide. The preventive effect of nimbolide on DSS-induced acute colitis is shown in Table 1 and Figure 4. When DSS was administered for 5 days, all mice showed varying degrees of diarrhea, hematochezia, and weight loss. Weight loss was significantly less severe when nimbolide was administered compared to that of the vehicle group (Figure 4A; $P < 0.05$ and $P < 0.01$ for 0.2 and 1 mg/kg nimbolide, respectively). Colon specimens representative of each group are shown in Figure 4B. The whole length of colon was significantly longer in the nimbolide 1 mg/kg group than in the vehicle group (6.9 ± 0.1 vs. 6.2 ± 0.1, $P < 0.01$; Figure 4B). The disease activity index indicated a significant alleviation of colitis with nimbolide treatment compared to the vehicle group ($P < 0.05$ and $P < 0.01$ for 0.2 and 1 mg/kg, respectively; Table 1). In pathologic specimens

from the nimbolide groups, H&E staining revealed that some crypts were retained, and with a small infiltration of inflammatory cells observed (Figure 4C). In contrast, entire crypts were lost, with absent epithelium and mucosal/submucosal layers full of inflammatory cells in pathologic specimens from the vehicle group (Figure 4C). The histologic scores of DSS colitis also decreased significantly in the nimbolide groups compared with the vehicle group ($P < 0.05$ and $P < 0.01$ for 0.2 and 1 mg/kg, respectively; Figure 4D). IHC staining of phosphorylated IκBα indicated the significant inhibition of IκBα phosphorylation in the nimbolide groups compared to the vehicle group ($P < 0.05$ and $P < 0.01$ for 0.2 and 1 mg/kg nimbolide, respectively; Figure 4E, 4F).

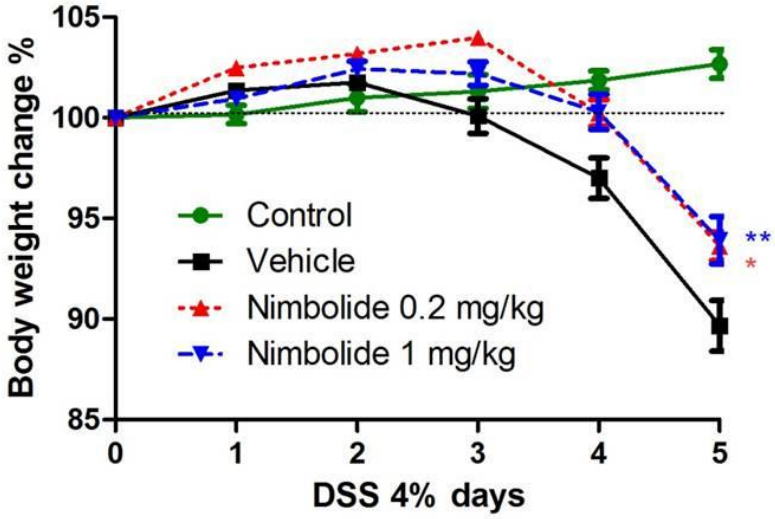
Table 1. Clinical characteristics and histologic evaluations of various treatments in a dextran sulfate sodium (DSS)-induced acute colitis model.

	Control	Vehicle	Nimbolide 0.2 mg/kg	Nimbolide 1 mg/kg
Body weight change (%)	102.7 ± 0.7	89.7 ± 1.3	93.6 ± 0.7*	93.9 ± 1.2*
Colon length (cm)	8.3 ± 0.3	6.2 ± 0.1	6.8 ± 0.2	6.9 ± 0.1**
Disease activity index	—	3.3 ± 0.1	2.5 ± 0.1*	2.4 ± 0.1**

*$P < 0.05$ compared with the vehicle group.

**$P < 0.01$ compared with the vehicle group.

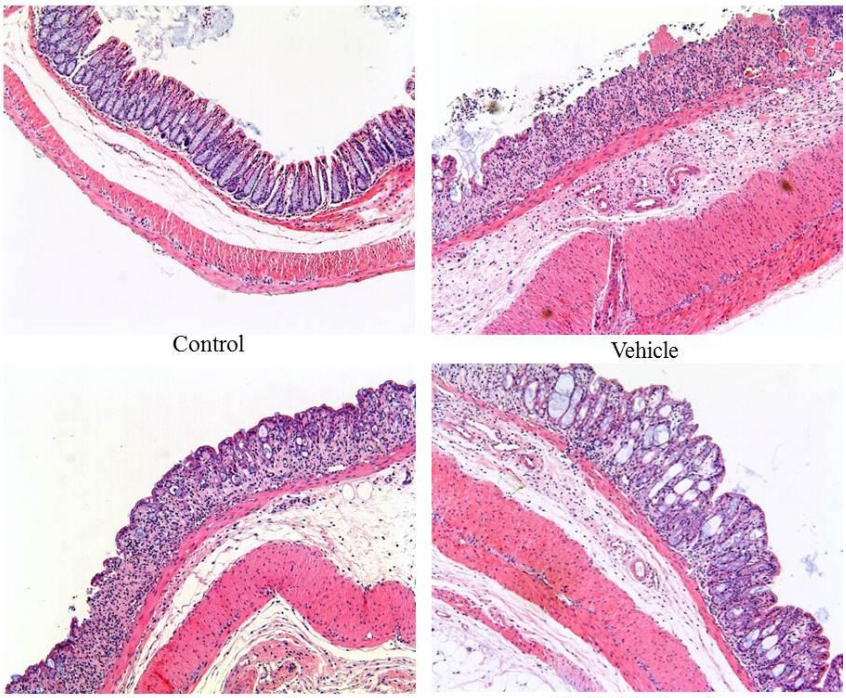
(A)


Body weight change %
105
100
95
90
85
0
1
2
3
4
5
DSS 4% days
Control
Vehicle
Nimbolide 0.2 mg/kg
Nimbolide 1 mg/kg
**
*

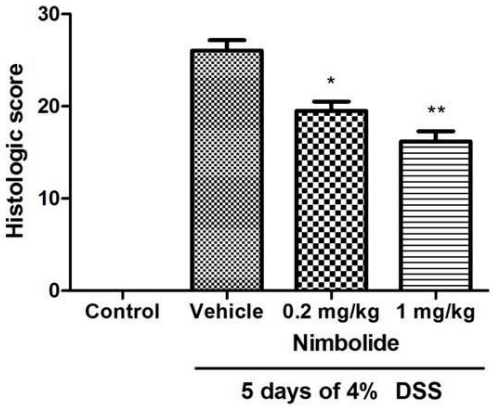
(B)


Control
Vehicle
Nimbolide 0.2 mg/kg
Nimbolide 1 mg/kg

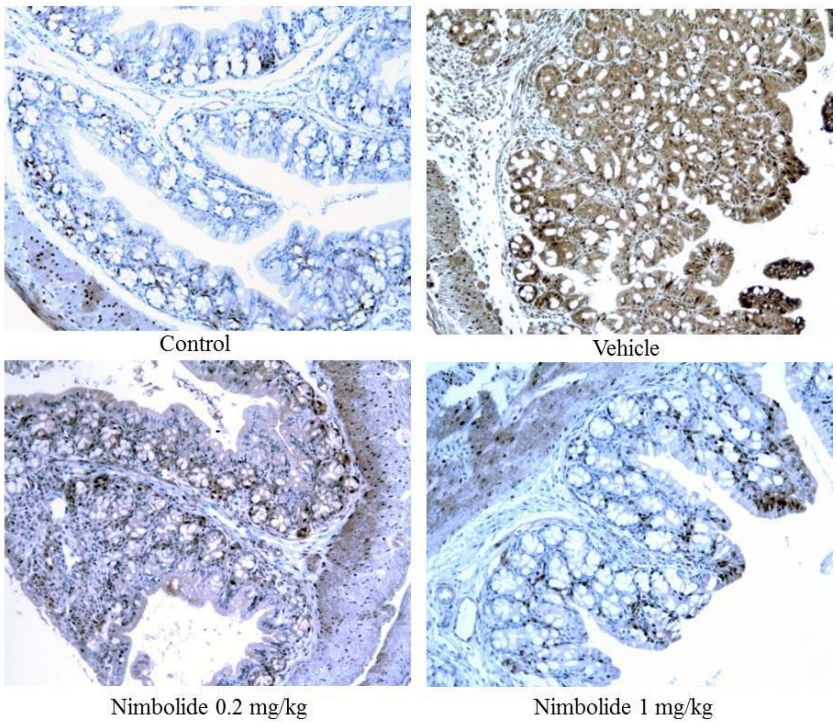
(C)


Control
Vehicle
Nimbolide 0.2 mg/kg
Nimbolide 1 mg/kg

(D)


Histologic score
30
20
10
0
*
**
Control
Vehicle
0.2 mg/kg
1 mg/kg
Nimbolide
5 days of 4% DSS

(E)



(F)

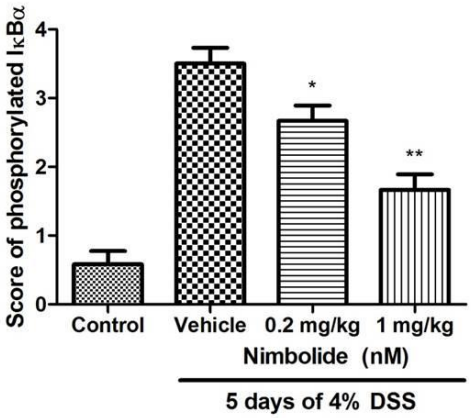


Figure 4. Body weight changes, gross specimens, histopathology and immunohistochemical analysis of a dextran sulfate sodium (DSS)−induced acute colitis model. (A) The effect of nimbolide on weight loss in a preventive model. Dimethyl sulfoxide

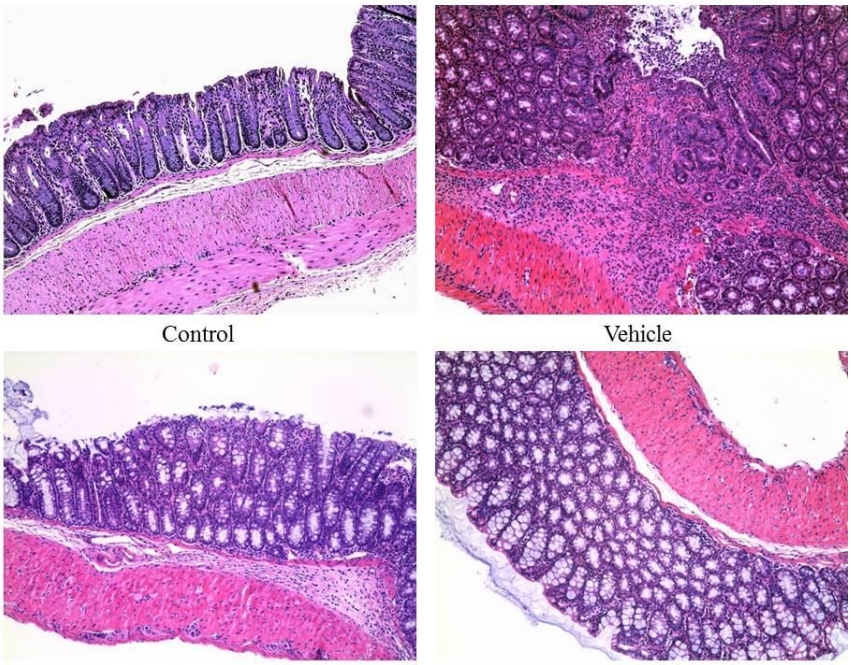
(DMSO) vehicle and nimbolide groups received intragastric treatment for 7 days (from day −2 to day 5) and 4% DSS for 5 days (from day 0 to day 5). Mice were randomly allocated to negative control, positive control, nimbolide 0.2 mg/kg and nimbolide 1 mg/kg groups (n=8, respectively). The negative control group drank filtered water freely. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group. (B) Representative gross mice colon specimens. Colon shortening and hematochezia improved with nimbolide doses compared with the vehicle group. (C) Histopathology of colon (hematoxylin and eosin, ×100). The severe destruction of crypts, damage to the epithelium and infiltration of inflammatory cells were noted in the vehicle group; these improved in the nimbolide groups. (D) Histologic scores of colitis in DSS−induced acute colitis model. Treatment of nimbolide significantly improved histologic scores compared with the vehicle group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group. (E) Immunohistochemical staining of phosphorylated IκBα (×200). The staining of phosphorylated IκBα with anti−phosphorylated IκBα antibody was attenuated in both nimbolide groups compared with the vehicle group. (F) Scores of immunoreactivity in DSS−

induced acute colitis model. Nimbolide significantly inhibited phosphorylation of IκBα. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the vehicle group.

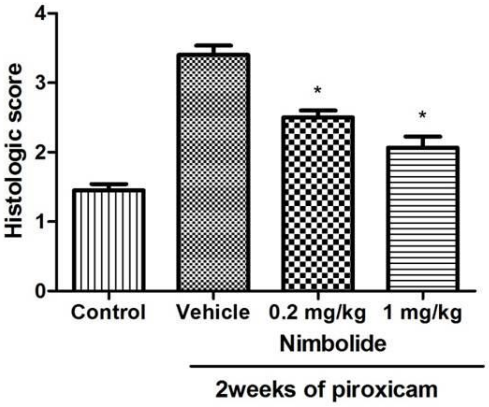
Nimbolide alleviates chronic colitis in IL−10$^{-/-}$ mice

We also examined the effect of nimbolide on chronic colitis in IL−10$^{-/-}$ mice (Figure 5). When we reviewed pathology slides of the proximal colon, there were only a few inflammatory cells in the nimbolide 1 mg/kg group, whereas transmural inflammation with ulcers was detected in the vehicle group (Figure 5A). The histologic scores of chronic colitis also showed significant improvement in the nimbolide groups compared with the vehicle groups ($P < 0.05$ for both, Figure 5B). Staining of phosphorylated IκBα also decreased significantly in the nimbolide groups compared to the vehicle group ($P < 0.01$ for both; Figure 5C, 5D).

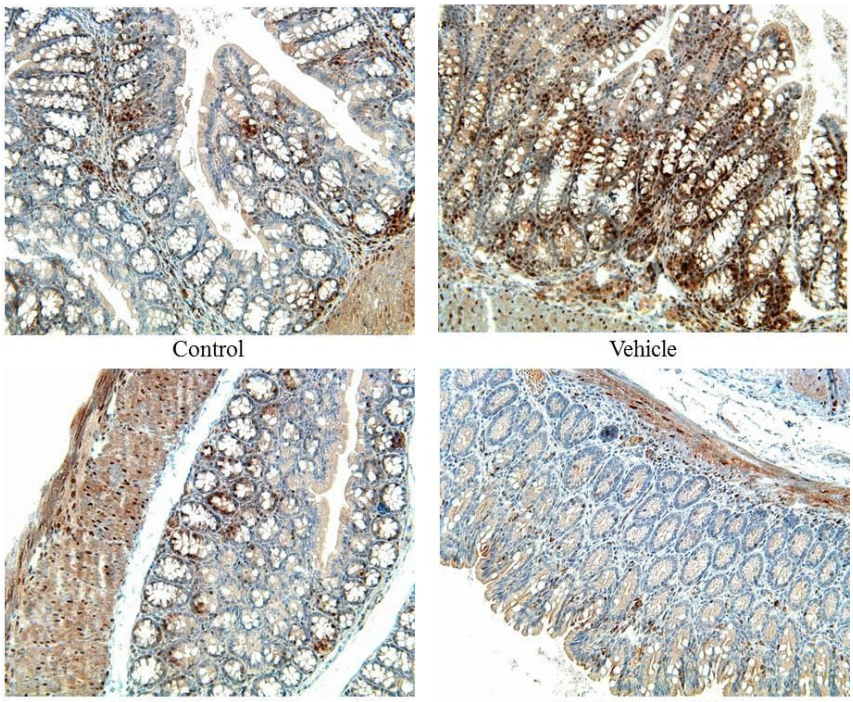
(A)


Control
Vehicle
Nimbolide 0.2 mg/kg
Nimbolide 1 mg/kg

(B)


Histologic score
0
1
2
3
4
*
*
Control
Vehicle
0.2 mg/kg
1 mg/kg
Nimbolide
2weeks of piroxicam

(C)



(D)

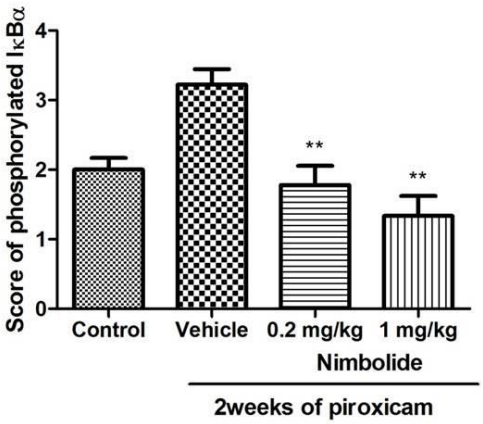


Figure 5. Histopathology and immunohistochemical analysis of chronic colitis in interleukin-10-deficient (IL-$10^{-/-}$) mice.

After the induction of colitis using piroxicam, IL$-10^{-/-}$ mice were randomly allocated to positive control, nimbolide 0.2 mg/kg and nimbolide 1 mg/kg groups (n=7, respectively). (A) Histopathology of the proximal colon (hematoxylin and eosin, ×100). Deep erosions of the epithelium, and a marked infiltration by inflammatory cells were noted in the vehicle group. However, inflammation was mitigated by nimbolide. (B) Histologic scores of chronic colitis in IL$-10^{-/-}$ mice. Nimbolide significantly ameliorated chronic colitis. $^{*}P < 0.05$ compared with the vehicle group. (C) Immunohistochemical evaluation of phosphorylated IκBα (×200) in proximal colon. The staining of phosphorylated IκBα with anti-phosphorylated IκBα antibody decreased with nimbolide treatment. (D) Scores of immunoreactivity in proximal colon. Treatment with nimbolide inhibited phosphorylation of IκBα $^{**}P < 0.01$ compared with the vehicle group.

DISCUSSION

We found that nimbolide strongly reduced the expression of IL-8 protein and mRNA in the IEC. It also significantly decreased protein expression of the inflammatory cytokines, IL-6, IL-12, and TNF-α, in both RAW 264.7 cells and IL-$10^{-/-}$ murine peritoneal macrophages. Phosphorylation of IκBα and the DNA-binding capacity of NF-κB were also significantly inhibited after nimbolide treatment of COLO 205 IECs, RAW 264.7 macrophages and IL-$10^{-/-}$ peritoneal macrophages.

Nimbolide has been the subject of several investigations as an anticancer drug since it is known to suppress the cell cycle in various cancer cell lines and to increase apoptosis through PI3K/Akt signaling.[38] Nimbolide treatment also correlates with an increase in p53 and Bax receptors.[20,21,39] Recently, a relationship between nimbolide and NF-κB has been revealed: the DNA-binding activity of NF-κB in WiDr cells (colon cancer cell line) was suppressed by nimbolide treatment.[18] In contrast, it is also reported that nimbolide inhibited IκBα kinase, which caused the down-regulation of NF-κB signaling in various cancer cell lines.[19]

Activation of NF-κB induces many proinflammatory cytokines that trigger a proinflammatory cascade. Although neem extracts have been used as an antiinflammatory drug for a long time, reported studies targeting the antiinflammatory effect of nimbolide have been lacking. Thus, we chose to evaluate the antiinflammatory effect of nimbolide and used IECs and macrophages as target cells since these play important roles in IBD. Our final aim was to find a new candidate drug for this intractable disease. Additionally, IL-$10^{-/-}$ peritoneal macrophages were used to reconfirm the effect of nimbolide in non-cancer cells and to predict the effect of nimbolide in colitis of IL-$10^{-/-}$ mice. As far as we are aware, this is the first study to investigate the antiinflammatory effect of nimbolide using cultured intestinal cells and macrophages and *in vivo* models of colitis.

According to our study, the antiinflammatory mechanisms involved in the inhibition of NF-κB signaling can be summarized as follows: With nimbolide treatment, the phosphorylation of IκBα is decreased, which leads to the decreased degradation of IκBα. Consequently, NF-κB was not free to bind DNA in both IECs and macrophages. Finally, the NF-κB-induced expression of inflammatory cytokines decreased. Similarly to previous studies,[18,19] this inhibitory mechanism was proved regardless of cell type.

However, comparing with previous studies, the inhibition of NF-κB pathway does not always seem to suppress inflammatory mechanism and it differs according to cell types.[40] In this study, inhibiting NF-κB pathway with nimbolide led to suppression of inflammatory process in particularly IECs and immune cells.

The antiinflammatory effect of nimbolide in cultured IECs and macrophages suggested this would also have an anti-colitic effect. We therefore designed two *in vivo* studies in mice to verify the effectiveness of nimbolide in colitis. We planned preventive and therapeutic model and we also needed to simulate nimbolide treatment in ulcerativc colitis (UC) and Crohn's disease (CD), respectively. We first investigated nimbolide treatment in a preventive model of DSS-induced acute colitis. As explained above, nimbolide was administered prior to the induction of colitis. The DSS induced apoptosis of epithelial cells and breaks in the mucosal barrier, and the activation of macrophages in the colon. In general, DSS-induced colitis is a superficial inflammation of the mucosa and submucosa, with these effects seen from the rectum to the proximal side, similar to that seen in UC. With a progression in colitis, gross diarrhea, hematochezia and weight loss become

apparent.[34,41]

We also investigated a therapeutic model of IL−10$^{-/-}$ mouse colitis. In this model, colitis occurs spontaneously under SPF conditions with age. However, piroxicam was used to accelerate the colitis. Segmental colitis occurs mainly in the proximal colon, with the development of histologically deep, transmural inflammation similar to that seen in CD. Furthermore, the activation of macrophages in IL−10$^{-/-}$ mice plays an important role in developing colitis as this leads to the production of IL−12, which, in turn, activates interferon−γ−producing T cells.[35] With the use of these two complementary models, we verified the effect and a mechanism of nimbolide in animal models of colitis.

In this study, nimbolide was an effective remedy in both DSS−induced acute colitis and an IL−10$^{-/-}$ chronic colitis model: it ameliorated clinical indices of colitis and histologic changes. With IHC staining, we confirmed the decrease in phosphorylated IκBα in resected colon tissue as initially seen in our *in vitro* studies. Others have also reported on the beneficial effects of neem tree extracts on colitis: It is reported that neem tree extract attenuated mucosal damages in a

trinitrobenzenesulfonic acid-induced colitis model.[42] However, Gautam's study had its limitations: a lack of knowledge concerning the specific components of the neem extracts used and various weaknesses in the trinitrobenzenesulfonic acid model itself.[43] We clarified the effectiveness of nimbolide in both UC and CD model, suggesting nimbolide is a potential candidate for the therapeutic agent of IBD.

A major advantage of nimbolide is that it comes from the neem tree, which has been used as a traditional medicine for a long time. In terms of toxicity, nimbolide was not toxic when administered to mice orally and showed a high LD50 when administered through the peritoneum or a vein.[44] Although there is minimal toxicity, nimbolide is basically an anticancer drug that has cytotoxic properties. In both *in vitro*[18,19] and *in vivo*[24] studies, we administered a much lower dose than that previously used in anticancer experiments, highlighting its benefit for antiinflammatory uses. Careful dose adjustments will become necessary if nimbolide is used for its antiinflammatory effects in the clinic.

In conclusion, nimbolide suppresses NF-κB signaling through the inhibition of IκBα phosphorylation in IECs and

macrophages. Nimbolide also ameliorates preventive and therapeutic colitis in mice. These results highlight the potential of nimbolide as a therapeutic medicine for IBD.

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국문 초록

서론: Nimbolide는 neem 나무(*Azadirachta indica*)에서 추출한 limonoid로써 항염증 효과를 가지고 있다. 본 연구에서는 장상피세포, 대식세포에서 nimbolide의 nuclear factor-kappa B (NF-κB) 신호전달에 미치는 영향과 대장염 마우스 모델에서의 항염증 효과에 대해서 알아보고자 하였다.

방법: 장상피세포주인 COLO 205, 대식세포주인 RAW 264.7 그리고 인터루킨-10 결핍(IL-$10^{-/-}$) 마우스에서 추출한 복강 대식세포를 nimbolide 또는 용매로 전처치한 후에 염증 반응을 유발하였다. 이후 염증 반응의 변화를 보기 위해 염증성 사이토카인인 인터루킨 6, 8, 12, 종양괴사인자-α(tumor necrosis factor-α)의 분비를 ELISA로 측정하였다. NF-κB 신호전달에 미치는 영향을 확인하기 위해 IκBα 인산화의 정도를 western blot으로, NF-κB의 DNA 결합 정도를 electrophoretic mobility shift assay로 확인하였다. *In vivo* 실험에서는 dextran sodium sulfate (DSS)로 대장염을 유발시킨 급성 예방 모델과 IL-$10^{-/-}$ 마우스를 이용한 만성 치료 모델을 이용하였다. 대장염의 정도는 질병 활성도(disease activity index)와 적출한 대장의 병리학적 소견으로 평가하였다.

결과: Nimbolide는 장상피세포와 대식세포에서 염증성 사이토카인의 분비를 유의하게 억제하였으며 IκBα의 인산화와 NF-κB의 DNA 결합을 억제하였다. 또한 nimbolide는 급성 예방 모델과 만성 치료 모델 모두에서 대장염의 완화 효과를 보여주었다. DSS를 이용한 급성 예방모델에서 체중 감소, 대장 단축, 질병 활성도 및 병리 소견의 호전을 보여주었고, IL-$10^{-/-}$ 마우스를 이용한 만성 치료 모델에서는 병리 소견이 유의하게 호전되었다. 두 모델 모두 적출한 대장 조직에서 시행한 인산화된 IκBα의 면역화학염색에서, nimbolide를 투약했을 때 인산화된 IκBα의 염색이 유의하게 감소하는 것을 확인할 수 있었다.

결론: Nimbolide는 장상피세포와 대식세포에서 NF-κB의 신호전달을 억제하며 대장염 마우스 모델을 완화시키는 효과를 보였다. 향후 nimbolide가 염증성 장질환의 새로운 잠재적인 치료제로 이용될 수 있을 것으로 기대된다.

주요어: 님볼라이드 (nimbolide), 대장염 마우스 모델, 대식세포, NF-κB
학 번: 2014-30604