Fluoxetine inhibits NF-κB signaling in intestinal epithelial cells and ameliorates experimental colitis and colitis-associated colon cancer in mice

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Fluoxetine inhibits NF-κB signaling in intestinal epithelial cells and ameliorates experimental colitis and colitis-associated colon cancer in mice. Am J Physiol Gastrointest Liver Physiol 301: G9–G19, 2011. First published March 24, 2010; doi:10.1152/ajpgi.00267.2010.—Although fluoxetine, a selective serotonin reuptake inhibitor, is known to demonstrate anti-inflammatory activity, little information is available on the effect of fluoxetine regarding intestinal inflammation. This study investigates the role of fluoxetine in the attenuation of acute murine colitis by suppression of the NF-κB pathway in intestinal epithelial cells (IEC). Fluoxetine significantly inhibited activated NF-κB signals and the upregulated expression of interleukin-8 (IL-8) in COLO 205 colon epithelial cells stimulated with tumor necrosis factor-α (TNF-α). Pretreatment with fluoxetine attenuated the increased IkB kinase (IKK) and IkBα phosphorylation induced by TNF-α. In a murine model, administration of fluoxetine significantly reduced the severity of dextran sulfate sodium (DSS)-induced colitis, as assessed by the disease activity index, colon length, and histology. In addition, the DSS-induced phospho-IKK activation, myeloperoxidase activity, a parameter of neutrophil accumulation, and the secretion of macrophage-inflammatory protein-2, a mouse homolog of IL-8, were significantly decreased in fluoxetine-pretreated mice. Moreover, fluoxetine significantly attenuated the development of colon cancer in mice inoculated with azoxymethane and DSS. These results indicate that fluoxetine inhibits NF-κB activation in IEC and that it ameliorates DSS-induced acute murine colitis and colitis-associated tumorigenesis, suggesting that fluoxetine is a potential therapeutic agent for the treatment of inflammatory bowel disease.

INFLAMMATORY BOWEL DISEASE (IBD) is defined as a chronic and relapsing gut inflammatory disorder caused by the dysregulation of the gastrointestinal immune system. It has been proposed that intestinal inflammation in IBD is attributable to the interplay between microbial, genetic, environmental, and immunological factors (59). Although updated therapeutic strategies such as biological agents have been developed and have been shown to be effective for the treatment of IBD, patients with IBD frequently experience flare-ups and occasionally must undergo a surgical treatment. Patients with long-standing IBD are at great risk of developing colitis-associated colon cancer (CAC) (52). According to a meta-analysis, the prevalence of CAC was ~3.7% in patients with ulcerative colitis (18). A few agents such as 5-aminosalicylate, folate, and corticosteroids are known to decrease the risk of colon cancer associated with chronic IBD (8). However, there is no effective treatment for CAC that is safe, efficacious, and well-tolerated.

The intestinal epithelium serves as a barrier between the luminal triggers and the host. Impaired barrier function may lead to increased uptake of luminal antigens that promote mucosal inflammation. In addition, activation of the proinflammatory gene transcriptional program in intestinal epithelial cells (IEC), when challenged by bacterial products, including lipopolysaccharide (LPS), or various inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, is associated with acute or chronic intestinal inflammation (32). Activation of transcription factor NF-κB leads to upregulation of the expression of these proinflammatory genes (2). In addition, the increase in intestinal epithelial tight-junction permeability requires NF-κB activation (48). Therefore, NF-κB activation in IECs appears to be strongly associated with IBD, suggesting that modulation of NF-κB signaling could be a potential target for the treatment of IBD (4, 32, 34).

Selective serotonin reuptake inhibitors (SSRIs) have been widely prescribed for patients with depression, anxiety, or insomnia (55). Beside their antidepressive effects, there is increasing evidence that SSRIs have anti-inflammatory and antitumor effects in vitro and in vivo (1, 53, 54). Fluoxetine was the first SSRI described, and it remains extremely popular despite the availability of new antidepressants (58). Fluoxetine has an active metabolite, norfluoxetine, which is more potent than its parent compound (28). The very slow elimination of fluoxetine and norfluoxetine differentiates it from other SSRIs (28). Compared with other antidepressants, it is safer in terms of overdose and causes fewer and less severe side effects, which has enhanced patient compliance (56). Fluoxetine has been proposed to have an anti-inflammatory effect. It was also found to reduce Th1 cytokine IFN-γ in human whole blood cells (15). In addition, it has been demonstrated that fluoxetine reduces the inflammatory response in the septic shock animal model (53). Although these studies suggest that fluoxetine has an anti-inflammatory effect, it has not been determined whether it also attenuates inflammation in IEC and in the dextran sulfate sodium (DSS)-induced colitis model. In addition, little information is available on the effect of fluoxetine on CAC despite the fact that SSRIs showed an antitumor effect in mice xenografted with a colon cancer cell line (24) and inhibited the growth of colorectal tumors (13, 60). This study reports an investigation of the effect of fluoxetine on TNF-α-induced NF-κB signaling in IEC and a murine model for DSS-induced acute colitis and colitis-associated tumorigenesis.

MATERIALS AND METHODS

Cell culture. The human colon cancer cell line COLO 205 [KCLB 10222; Korean Cell Line Bank (KCLB), Seoul, Korea] was used between passages 15 and 30. Cells were grown in DMEM (Sigma, St

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Louis, MO) supplemented with 10% FBS, 2 mM glutamine, and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin (31, 41). Cells were seeded at ∼0.5–2.0 × 10^6 cells per well onto six-well plates and allowed to attach overnight. After 12 h of serum starvation, cells were pretreated for 24 h with fluoxetine (Sigma) in the presence or absence of 5-HT (10^{-10} mol/l) (Sigma), after which they were stimulated with TNF-α (10 ng/ml).

Real-time RT-PCR and ELISA. Total cellular RNA was extracted from COLO 205 cells using Trizol (GIBCO, Gaithersburg, MD). Real-time PCR for IL-8 and β-actin mRNA was performed as described previously (39). Briefly, 1 µg of extracted RNA was reverse-transcribed and amplified using the SYBR green PCR Master Mix and an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers designed using Primer Express v2.0 were purchased from Applied Biosystems. Amplifications were performed in triplicate, and the data were normalized vs. β-actin for human IL-8.

The amounts of human IL-8 and mouse macrophage-inflammatory protein (MIP)-2 were measured using a commercially available ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Colonic tissues were homogenized with 0.3 ml PBS containing a complete protease inhibitor mixture (Roche, Tokyo, Japan), as previously described (37). To measure the concentration of MIP-2, homogenates were centrifuged at 12,000 g for 15 min, and the supernatants were filtered through a 0.22-µm filter to remove any contaminants. The myeloperoxidase (MPO) assay was performed as previously described (29). Briefly, tissue samples were suspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. This was followed by sonication on ice for 15 s. Suspensions were then freeze-thawed three times, and the supernatant was separated from the solid phase by centrifugation at 16,000 g for 20 min. A total of 10 µl of the supernatant was mixed with 140 µl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.005% hydrogen peroxide.

MPO activity was derived from the observed change in the absorbance measured by spectrophotometry at 450 nm and normalized to the total protein content of the supernatant.

EMSA. Cells were harvested, and nuclear extracts were prepared as described previously (40). The concentrations of proteins in the extracts were determined using a Bradford assay (Bio-Rad, Hercules, CA). EMSA was performed using an assay kit (Promega, Madison, WI) as described previously (40). In brief, 5 µg of nuclear extract was incubated for 30 min at room temperature with a 32P-labeled oligonucleotide probe (5’-AGT TGA GGG GAC TTT CCC AGG C-3’) corresponding to a consensus NF-kB binding site. After incubation, both bound and free DNA was resolved on 5% polyacrylamide gels.

Transfection and reporter assay. Reporter plasmids, including pLL-8-luciferase, p2x NF-κB-luciferase, pβ-actin- and pRSV-β-galactosidase-luciferase transcriptional reporters, were provided by Dr. Kagnoff of the University of California, San Diego, CA (20). Cells in six-well dishes were transfected with 1.5 µg of plasmid DNA using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA). Transfected cells were incubated for 24 h at 37°C in a 5% CO2 incubator. Cells were then harvested, and whole cell lysates were prepared as described previously (38). Luciferase activity was determined in accordance with the manufacturer’s instructions (Tropix, Bedford, MA), and luminescence was quantitated for 10 s using a luminometer (MicroLumat Plus; Berthold, Bad Wildbad, Germany). Luciferase activity was determined and normalized relative to β-galactosidase expression.

Immunoblot analysis. Cells were washed with ice-cold PBS and lysed in 0.5 ml/well lysis buffer (150 mM NaCl, 20 mM Tris at pH 7.5, 0.1% Triton X-100, 1 mM PMSF, and 10 µg/ml aprotenin). Twenty micrograms of protein per lane was size-fractionated on a 10% polyacrylamide minigel and electrophoretically transferred to a nitrocellulose membrane (0.1-µm pore size). Specific proteins were detected using mouse IκBα, anti-human phospho-IκBα, and actin (Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies and peroxidase-conjugated anti-mouse IgG (Biosource, Camarillo, CA) as a secondary antibody. Specifically bound peroxidase was detected by an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, England) and exposure to Kodak X-OMAT film.

IkB kinase assay. IκB kinase (IKK) activity on IκBα phosphorylation was determined using a commercially available HTScan IKK-β kinase assay kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer’s instructions (44). Briefly, cells were washed with ice-cold PBS. Whole cell extracts were prepared using lysis buffer (150 mM NaCl, 20 mM Tris at pH 7.5, 0.1% Triton X-100, 1 mM PMSF, and 10 µg/ml aprotinin), after which lysates were cleared by centrifugation at 14,000 revolution/min for 10 min at 4°C. Levels of IKK activity were measured by colorimetric ELISA per the manufacturer’s instructions.

Preventive and therapeutic models of DSS-induced acute murine colitis. Specific pathogen-free mice (C57BL/6NarlJ) male mice, 7–8 wk) were purchased from Orient (Seongnam, Korea). Mice were given ad libitum access to water and standard rodent food until they reached the desired age (8–9 wk) or weight (18–20 g). Mice were maintained on a 12-h:12-h light/dark cycle under specific pathogen-free conditions. All procedures using the mice were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

In the preventive model, colitis was induced by 4% DSS (MP Biochemicals, Irvine, CA) as described previously (45). Five mice in each group were randomly assigned after they were weighted. Control mice received only filtered water. DSS (4%) was dissolved in drinking water and was administered for 5 days. Fluoxetine (i.e., 10 mg/kg per day and 20 mg/kg per day) was suspended in PBS and administered once daily by oral gavage, beginning 2 days before DSS administration. Vehicle-treated mice were administered with 100 µl of PBS once daily by oral gavage. The mice were assessed daily for water/chow consumption, body weight, stool consistency, and the presence of gross blood in the stool or at the anus. They were then euthanized on day 8. To evaluate whether fluoxetine can show the therapeutic effect on DSS-induced colitis, a therapeutic murine model was used as described previously (38). Briefly, colitis was induced by 4% DSS for 4 days. After the induction of colitis, mice were administrated daily fluoxetine (20 mg/kg per day) by gavage for the next 5 days.

Induction of CAC in mice. The induction of CAC in mice was established in a previous study by the authors (11). Briefly, five C57BL/6 mice in each group were randomly assigned after they were weighted. The mice were given a single intraperitoneal injection of azoxymethane (AOM, 12 mg/kg initial body wt, Sigma) on day 0. After 7 days, 2% DSS was administered via the drinking water for 5 days, and this was followed by 16 days of consumption of free water. This cycle was repeated three times. Fluoxetine (i.e., 10 mg/kg per day and 20 mg/kg per day) suspended in PBS or the same volume (100 µl) of vehicle was administered once daily by oral gavage, beginning on day 8. Mice were observed daily for water intake, body weight, stool consistency, and for the presence of hematochezia. They were then euthanized 10 days after the final 2% DSS administration.

Macroscopic assessment and histological analysis. The disease activity index (DAI) was assessed according to the weight loss, stool consistency, and gross rectal bleeding, as previously described (14). Mice were anesthetized with isoflurane. Postmortem, the entire colon was removed from the cecum to the anus, and the colon was then opened longitudinally. Subsequently, the removed colon tissues were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin. Histological quantification to evaluate the severity of inflammation was performed using a scoring system, as described previously (10, 16), by a pathologist who was blinded to the treatment. For each mouse, the histological evaluation was performed on at least three samples.

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Fig. 1. Effects of fluoxetine (FLX) on interleukin (IL)-8 expression in COLO 205 cells stimulated with tumor necrosis factor (TNF-α). A: COLO 205 cells were transfected with pIL8-luciferase transcriptional reporter, as indicated. After 24 h, transfected cells were pretreated with the indicated concentration of fluoxetine for 24 h and were then combined with TNF-α (10 ng/ml) for 4 h. Data are expressed as mean fold induction ± SE of luciferase activity relative to unstimulated controls (n = 5). The mean fold induction of β-actin reporter gene activity relative to the unstimulated controls remained relatively constant throughout experiments. B: COLO 205 cells were pretreated with the indicated concentration of fluoxetine for 24 h and then stimulated with TNF-α (10 ng/ml) for 4 h. IL-8 mRNA expression was measured by real-time RT-PCR. Levels are normalized to β-actin. Data are expressed as fold change in mRNA transcript levels relative to the unstimulated control (means ± SE, n = 3). C: COLO 205 cells were pretreated with fluoxetine for 24 h and then stimulated with TNF-α (10 ng/ml) for 24 h. Secretion of IL-8 was measured by ELISA (means ± SE, n = 5). *P < 0.05 compared with TNF-α alone.

Immunohistochemical analysis and in situ TUNEL assay. Immunohistochemistry was performed as previously described (10, 51). For antigen retrieval, slides were immersed in a Tris/EDTA buffer (pH 9.0), heated in a decloaking chamber at 125°C for 3 min, and then cooled for 10 to 20 min. After an addition 3% hydrogen peroxide, sections were incubated for 10 min. After washing with TBS Tween-20 (pH 7.6), the slides were stained with rabbit polyclonal anti-phospho-IKK-α/β antibody (Cell Signaling Technology) or 5-hydroxytryptamine (HT) rabbit antibody (Sigma) in an autoimmunostainer (Autostainer 2D; Lab Vision, Fremont, CA) for 1 h at room temperature according to the manufacturer’s instructions. The slides were washed three times with TBS Tween-20 and were incubated with secondary antibody for 30 min. After the slides were reacted with streptavidin for 20 min, the reaction was visualized by 3,3′-diaminobenzidine tetrahydrochloride for 5 min, and the slides were counterstained with Meyer’s hematoxylin. Immunoreactivity was analyzed with a microscope. The number of 5-HT-reactive cells in each of four nonadjacent fields (magnification: ×100) of view was counted per colon sample, as well as the number of 10 crypts, and then the average number of enterochromaffin (EC) cells per 10 crypts was calculated. Each slide for phospho-IKK-α/β immunohistochemistry was assessed for intensity of immunoreactivity on a 0 to +4 scale as previously described (51).

Apoptosis was examined by in situ TUNEL assay in mouse colon samples. TUNEL assay was performed using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon, Temecula, CA) following by a manufacturer’s instruction. The apoptosis index was determined by the ratio of TUNEL-positive cells to the total number of cells per each of four nonadjacent field (magnification: ×100), which was randomly selected as previously described (51).

Primary IEC isolation. To study the effect of fluoxetine on primary IECs, primary epithelial cells were isolated as previously described (42). The colons were cut longitudinally and washed three times in PBS. The colons were cut into pieces 0.5 cm long and then incubated for 90 min at room temperature in solution containing 3 mM EDTA and 0.5 mM DTT with shaking. The resulting supernatant was filtered through nylon mesh (BD Bioscience, San Jose, CA). The cellular suspension was centrifuged, washed, and resuspended in RPMI-1640 with 10% FBS and antibiotics.

Statistical analysis. Data were expressed as the means ± SE. Differences between groups were compared using an analysis of variance with Bonferroni correction. P values <0.05 were considered statistically significant.

Fig. 2. Fluoxetine inhibits NF-κB DNA binding activity in COLO 205 cells stimulated with TNF-α. A: COLO 205 cells were pretreated with the indicated concentration of fluoxetine for 24 h and then stimulated with TNF-α (10 ng/ml) for 1 h. NF-κB DNA binding activity in the nuclear extracts was assessed by EMSA. The data are representative of more than 3 independent experiments. B: COLO 205 cells were transfected with 2x pNF-κB-luciferase transcriptional reporter. After 24 h, the transfected cells were pretreated with the indicated concentration of fluoxetine for 24 h and were then combined with TNF-α (10 ng/ml) for 1 h. Data are expressed as mean fold induction ± SE of luciferase activity relative to unstimulated controls (n = 5). The mean fold induction of the β-actin reporter gene activity relative to the unstimulated controls remained relatively constant throughout each experiment. *P < 0.05 compared with TNF-α alone.
Fluoxetine inhibits TNF-α-induced IL-8 expression in COLO 205 cells. To determine whether fluoxetine could reduce inflammatory responses, IL-8 mRNA expression was evaluated in COLO 205 cells stimulated with TNF-α. As shown in Fig. 1A, pretreatment with fluoxetine showed that inhibition of IL-8 expression was dependent on the concentration of fluoxetine. Consistent with this, fluoxetine significantly downregulated mRNA levels of IL-8 in COLO 205 cells stimulated with TNF-α, as assessed by real-time RT-PCR (Fig. 1B). In addition, significant inhibition of IL-8 release by fluoxetine was observed in TNF-α-stimulated COLO 205 cells (Fig. 1C).

Fluoxetine suppresses NF-κB activity in TNF-α-stimulated COLO 205 cells. Because IL-8 expression is regulated primarily by the transcription factor NF-κB in TNF-α-stimulated IEC, we asked whether fluoxetine could prevent TNF-α-induced NF-κB activity. For this experiment, the NF-κB DNA binding activity was determined by EMSA. Pretreatment with fluoxetine reduced the NF-κB DNA binding activity in a dose-dependent manner (Fig. 2A). To confirm this result, a reporter gene assay was performed. As shown in Fig. 2B, pretreatment of cells with fluoxetine decreased the TNF-α-induced NF-κB activity significantly.

Fluoxetine suppresses NF-κB signaling in IEC by blocking IKK. TNF-α strongly induced IκBα phosphorylation/degradation in COLO 205 cells, whereas IκBα phosphorylation/degradation was suppressed in fluoxetine-pretreated cells (Fig. 3A). To determine the effect of fluoxetine on IKK activity, an HTScan IKK-β kinase assay was performed. As shown in Fig. 3B, stimulation of COLO 205 cells with TNF-α resulted in an increase of IKK activity, whereas pretreatment of cells with fluoxetine significantly reduced the TNF-α-induced IKK activity. To confirm the results in normal IEC, we isolated primary epithelial cells from mouse. As shown in Fig. 3C, TNF-α strongly induced IκBα phosphorylation and IKK activation in normal IEC. However, pretreatment of fluoxetine inhibited IκBα phosphorylation and IKK activation. These results indicate that fluoxetine can inhibit NF-κB signaling pathway by blocking IKK activity.

Fluoxetine attenuates the severity of DSS-induced acute murine colitis. To test the physiological relevance of the fluoxetine-mediated suppression of inflammatory reactions in vivo, we conducted an in vivo study using a murine model of DSS-induced acute colitis. In the preventive model, all mice treated with 4% DSS showed symptoms compatible with acute colitis. Oral administration of fluoxetine significantly reduced the severity of DSS-induced acute murine colitis, as evaluated according to the weight loss, clinical DAI, and reduction of the colon length (Table 1).

We next evaluated the severity of acute murine colitis by blinded histological injury scoring in the proximal and distal colon. The histological examination of proximal colon from DSS-induced colitis mice showed inflammatory lesions that included total impairment of the glandular structure, mucosal ulceration, crypt damage, and the infiltration of inflammatory cells composed of macrophages, lymphocytes, and neutrophils.

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<th>Table 1. Clinical indices of DSS-induced acute murine colitis treated with or without fluoxetine</th>
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<td>Body Weight Change, % of day 0</td>
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<td>DSS + PBS</td>
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Values are means ± SD of 5 mice. Percent indicates body weight change at day 7 compared with day 0. DSS, dextran sulfate sodium; PBS, phosphate-buffered saline; FLX (10 qd), fluoxetine 10 mg/kg per day; FLX (20 qd), fluoxetine 20 mg/kg per day. *P < 0.05 compared with DSS + PBS group; †P < 0.05 compared with control group.
In contrast, an administration of fluoxetine markedly reduced the impairment of the glandular architecture and the infiltration of inflammatory cells. Histological grading showed that the treatment with fluoxetine significantly attenuated the overall score compared with the vehicle-treated control group (Fig. 4A). Similar results were observed in samples of the distal colon (Figures 4B). To evaluate the unexpected effect of fluoxetine in the murine colon, we administered fluoxetine (10 mg/kg) without DSS exposure. The result showed that an administration with fluoxetine did not change the normal colon histological findings (data not shown). We next evaluated the therapeutic effect of fluoxetine on DSS-induced murine colitis. Administration of fluoxetine (20 mg/kg per day) attenuated clinical indices such as body-weight change and DAI. In addition, fluoxetine resulted in a significant attenuation of acute colitis histologically (Table 2).

In addition to the clinical and histological severity, the level of MPO activity, as a parameter of neutrophil accumulation, was increased in colons of mice with DSS-induced colitis (Fig. 5A). In this experimental model, inoculation with fluoxetine signifi-

| Table 2. Effects of fluoxetine on clinical indices and histological injury score in the therapeutic model of colitis |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | Body Weight Change, % of day 0 | Disease Activity Index | Histological Injury Score |
| Control                        | 101.4 ± 6.4      | —                | —                |
| DSS + PBS                      | 81.1 ± 2.6†      | 3.1 ± 0.6†       | 22.0 ± 8.3†      |
| DSS + FLX (20 qd)              | 88.4 ± 1.6*      | 2.0 ± 0.7*       | 9.3 ± 3.6*       |

Values are means ± SD of 5 mice. Percent indicates body weight change at day 9 compared with day 0. *P < 0.05 compared with DSS + PBS group; †P < 0.05 compared with control group.
cantly reduced upregulated MPO activity. Furthermore, the DSS-induced secretion of MIP-2, a mouse homolog of IL-8, was significantly decreased in fluoxetine-pretreated mice (Fig. 5B).

Given that the data from the in vitro study here showed that fluoxetine could exert its anti-inflammatory effects by blocking the IKK activity in IEC, we investigated this signaling in a DSS-colitis model to reconfirm it in vivo. To determine the IKK activity in the in vivo model of colitis, immunohistochemistry was performed on colon sections of the mice. As shown in Fig. 6, A and B, DSS colitis was accompanied by an increased expression of phosphorylated-IKK in the colonic mucosa and submucosa. In contrast, IKK phosphorylation was markedly reduced with an inoculation of fluoxetine. The score for the phospho-IKK-α/β significantly decreased in fluoxetine-treated mice compared with vehicle-treated mice. To confirm the results, immunoblot analysis was performed. Primary IECs were isolated from mice with DSS-induced colitis. Exposure to DSS strongly induced IkBα phosphorylation and IKK activation. However, administration of fluoxetine reduced IkBα phosphorylation and IKK activation (Fig. 6C).

Fluoxetine inhibits colitis-associated colon carcinogenesis in a murine model. Because tumor development is closely associated with an inflammatory process (35), it is possible that fluoxetine inhibits tumorigenesis in the mice treated with AOM/DSS. To evaluate this hypothesis, we investigated the effects of fluoxetine on colitis-associated colon cancer in mice. Weight change was evaluated daily as a clinical parameter in the DSS-induced acute murine colitis model during the experimental period. Although weight reduction was observed 2 or 3 days after 2% DSS administration, there was no significant difference in terms of the weight change among treatment groups. We next evaluated the severity of murine colitis using a blinded histological scoring system. The severity of experimental colitis in the fluoxetine-treated group was significantly reduced compared with vehicle-treated control mice (Fig. 7A).
Fluoxetine inhibits intestinal inflammation regardless of 5-HT signaling. Because 5-HT is reported to be important in the pathogenesis of mucosal inflammation in experimental colitis (23), we evaluated NF-κB activation and IL-8 expression in IEC stimulated with TNF-α in the presence or absence of 5-HT. As shown in Fig. 9, A and B, 5-HT did not significantly affect the increased reporter gene of NF-κB and IL-8 in COLO 205 cells stimulated with TNF-α. In contrast, fluoxetine significantly reduced the NF-κB and IL-8 reporter gene expression. These results suggest that fluoxetine seems to directly inhibit NF-κB signaling in TNF-α stimulated COLO 205 cells.

We next evaluated the number of 5-HT-expressing cells in fluoxetine-treated mice. The results showed that a significant increase in the number of 5-HT immunoreactive EC cells was observed in the mice treated with DSS (Fig. 10, A and B). However, there was no significant difference in the number of EC cells between vehicle-treated group and fluoxetine-treated group.

**DISCUSSION**

The present study was performed to investigate the potential anti-inflammatory effect of fluoxetine and to elucidate the molecular mechanism involved. Previous studies showed anti-inflammatory and immunomodulatory effects of antidepressants both in vitro and in vivo (15, 43). In particular, a recent study reported that both fluoxetine and desipramine ameliorated the extent and severity of colitis induced by acetic acid in rats, which was associated with the decreased level of proinflammatory cytokines (27). However, no report has explained the mechanism of the anti-inflammatory effect of fluoxetine on IEC and murine colitis. Therefore, we hypothesized that fluoxetine modulates NF-κB signaling through which it could ameliorate murine colitis. In the present study, fluoxetine significantly inhibited NF-κB pathway in IEC. The inhibition of both NF-κB activation and IkB phosphorylation/degradation results in the suppression of chemokine expression and secretion in COLO 205 cells stimulated by TNF-α. Fluoxetine also attenuated acute murine colitis and tumorigenesis induced by DSS administration. Finally, our results demonstrate that fluoxetine suppressed intestinal inflammation by inhibiting NF-κB signaling by way of an IKK blockade in IEC both in vitro and in vivo. To the best of our knowledge, this is the first mechanistic approach for the determination of an anti-inflammatory result of fluoxetine on IEC and an acute murine colitis model.

To determine whether fluoxetine can ameliorate intestinal inflammation in experimental models, we used preventive and therapeutic models of DSS-induced murine colitis. We demonstrated that fluoxetine could prevent DSS-induced intestinal inflammation. We next confirmed that fluoxetine ameliorated the established colitis in DSS-treated mice. Furthermore, fluoxetine resulted in a significant improvement in chronic intestinal inflammation induced by three cycles of DSS. These results suggest that fluoxetine has preventive and therapeutic effects in intestinal inflammation, indicating that it has potential clinical usefulness in treating patients with IBD.

Although it is controversial, there is increasing evidence that psychological factors such as chronic stress, adverse life events, and depression may account for the development and relapse of inflammatory bowel disease (25). It has been demonstrated that...
patients with IBD have higher anxiety and depression levels (25). Previous studies reported that stress was associated with the disease activity of ulcerative colitis (17, 46). Moreover, a high response rate in patients treated with a placebo appears to be associated with a feeling of well-being in many clinical trials (30, 49). In addition, recent studies have demonstrated that antidepressants attenuated the severity of colitis in experimental mouse models (22, 57). Some antidepressants also showed a specific benefit during the clinical course of IBD (33, 36). Nevertheless, there is substantial skepticism regarding the effectiveness of antidepressant agents or the role of psychological factors in the outcome of patients with IBD (50). Therefore, these results provide evidence that fluoxetine may be beneficial in treating patients with IBD with psychological problems.

Serotonin (5-HT) is a well-known monoamine neurotransmitter in the central nervous system and regulates mood, sleep, body temperature, and appetite (21). Enteric 5-HT, which is produced in EC cells, plays a critical role in gut homeostasis and intestinal inflammation. Previous studies have found that the numbers of EC cells and mucosal 5-HT change in patients with ulcerative colitis or Crohn’s disease (6, 12). In addition, a recent study found that 5-HT is involved in the pathogenesis of inflammation in experimental colitis (23). Although SSRIs may affect 5-HT metabolism through the serotonin reuptake transporter, it has been demonstrated that fluoxetine had no significant effect on 5-HT release in murine colitis induced by DSS, despite the increased number of EC cells (5). Our study demonstrated that fluoxetine downregulated the increased levels of NF-κB and IL-8 reporter genes in TNF-α-stimulated

Fig. 8. Immunostaining for in vitro IKK and in situ TUNEL assay in AOM/DSS murine model. A: in the group treated with vehicle, phospho-IKK-α/β was strongly stained in colonic epithelial and submucosal inflammatory cells. Treatment of fluoxetine significantly attenuated phospho-IKK activity in colonic sections. B: in the TUNEL assay, tumors induced by AOM/DSS showed numerous apoptotic cells (brown staining of nuclei) compared with normal control. However, there was no significant difference in apoptosis index between vehicle-treated and fluoxetine-treated mice. Results are representative of at least 3 separate examined sites (magnification: ×100).
COLO 205 cells regardless of the presence of 5-HT. Moreover, there was no significant difference in the number of EC cells between fluoxetine-treated and vehicle-treated mice. These results suggest that fluoxetine may have an anti-inflammatory effect on DSS-induced colitis regardless of 5-HT signaling from EC cells.

In the process of tumorigenesis, chronic inflammation plays a crucial role in tumor promotion and progression (35). A variety of proinflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6, and IL-8 are regulated by NF-κB pathway, and these can promote tumor growth and progression (3). On the basis of these reports, suppression of NF-κB pathway may be associated with the inhibition of tumorigenic process. In the present study, fluoxetine reduced intestinal inflammation as assessed by the DAI and histological severity through the inhibition of NF-κB pathway. Furthermore, fluoxetine significantly reduced upregulated MPO activity and MIP-2 secretion. Thus fluoxetine can inhibit the production of proinflammatory cytokines and chemokines that contribute to reduce tumor proliferation. Therefore, we have deduced that a possible mechanism of inhibition of tumor development by fluoxetine is NF-κB suppression although the treatment with fluoxetine did not induce apoptosis.

We demonstrated that fluoxetine directly inhibits NF-κB signaling in IEC through the IKK blockade and ameliorates experimental colitis and colitic cancer in mice. These results seem to be inconsistent with a study that genetic ablation of IKK-β does not prevent DSS-induced intestinal inflammation (26). However, several reports have demonstrated that suppression of IKK-β is associated with attenuation of colitis. Thus the IKK-β-deleted intestinal epithelium is more susceptible to ischemia-reperfusion-induced apoptosis (9). In addition, IKK-β-dependent NF-κB signaling is known to be necessary for mucosal regeneration after DSS-induced acute murine colitis (19). The present study showed that fluoxetine can suppress IKK signals in vitro and in vivo models. Therefore, the hypothesis that fluoxetine may promote the process of mucosal regeneration in intestinal epithelium is supported by those reports and our results. However, further research is needed to elucidate the effects of fluoxetine in modulating intestinal inflammation.

A limitation of our study is that fluoxetine concentrations were higher than the dose usually prescribed for patients. The serum concentration in this study may be slightly higher than the serum concentration in clinically relevant doses, which range from 20 to 40 mg daily (47). In addition, it is unclear whether the anti-inflammatory effect is a class of SSRIs or...
whether it is specific for fluoxetine. Further studies are required to understand whether other SSRIs have a similar effect. This will help to clarify the anti-inflammatory mechanism of fluoxetine.

In conclusion, this study reveals that fluoxetine suppresses the TNF-α-induced NF-κB signal pathway by way of an IKK blockade in IEC and that it ameliorates acute murine colitis. Therefore, the novel protective effects of fluoxetine on colitis support its potential usefulness for the treatment in patients with IBD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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