INTRODUCTION
Nonsteroidal anti-inflammatory drugs (NSAIDs) are used as anti-inflammatory agents for several diseases. However, NSAIDs have adverse effects on the stomach and small bowel, including the duodenum. NSAID-induced small bowel injury is generally ignored due to the difficulty in diagnosis. However, NSAIDs have been reported to cause damage to the gastric and small bowel mucosa with a relatively high frequency (1, 2).

Gastrointestinal mucosal injury caused by NSAIDs can manifest as simple inflammatory mucosal changes, ulceration, bleeding, and even perforation (3, 4). Proton pump inhibitors have been used as the standard treatment for such gastric injuries. Moreover, other agents such as misoprostol, probiotics, lactoferrin, rebamipide, NSAID pro-drugs, and selective COX-2 inhibitors have been evaluated in terms of their ability to prevent or treat small bowel injury (5). However, no effective drug is available for the prevention or treatment of small bowel injury. NSAID-induced gastrointestinal injury occurs by various mechanisms: inhibition of COX-1 enzyme and gastro-protective prostaglandin synthesis, increased intestinal permeability, uncoupling of mitochondrial oxidative phosphorylation, and inflammation (6).

Statins are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and play important roles in cholesterol synthesis. For this reason, they are used as lipid-lowering agents. The efficacy of statins against other conditions such as osteoporosis (7), several type of cancer (8-10), and rheumatoid arthritis (11) have been evaluated. Anti-inflammatory and antioxidant effects of statins have also been reported (12-15). Therefore, simvastatin, an HMG-CoA reductase inhibitor, has potential as a prophylactic and therapeutic agent for NSAID-induced small bowel injury.

MATERIALS AND METHODS

Reagents and materials

Simvastatin was purchased from Peprotech (Minneapolis, MN). Simvastatin was obtained from Sigma-Aldrich (St. Louis, MO).
2', 7-dichlorofluorescein diacetate (DCFH-DA) and Alexa Fluor 488 goat anti-mouse antibody were purchased from Molecular Probes (Eugene, OR). Antibodies to phospho NF-κB p65 (Ser 536), NF-κB p65, Akt, phospho Akt (Ser 473), IκBα, and phospho-IκBα were purchased from Cell Signaling Technology (Beverly, MA). 8-Hydroxyguanosine antibody was purchased from Abcam. TNF-α antibody was purchased from Santa Cruz biotechnology.

**Cell culture**

Rat normal small bowel IEC-6 cells were purchased from the Korean Cell Lines Bank (KCLB, Seoul, Korea) and cultured in Dulbecco’s modified Eagle’s medium (GIBCO, NY) supplemented with 10% fetal bovine serum (GIBCO) and 1% antibiotic-antimycotic in a humidified 5% CO₂ atmosphere. The cells were treated with TNF-α (50 ng/ml) or simvastatin (0.5 μM).

**Real-time quantitative PCR**

Total RNA was purified using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). For relative quantitation, the reactions were tested using TaKaRa Premix Ex taq II (TaKaRa, Japan), and PCR processing was carried out in an iCycler (Bio-Rad, Hercules, CA). The sequences of primers for rat IL-6 were 5'-AAAGGCAATGCTCAGACG-3' (sense) and 5'-GAGCATTGGAAGTTGGAATGA-3' (antisense), and those for rat IL-8 were 5'-CATTTATTTGCGATGATGCTTACGTC-3' (sense) and 5'-GCCCATTACCTTAACTGCAACTATGTCG-3' (antisense). The sequences of primers for rat COX-2 were 5'-TGATCGGAAGACTCGCTGCAACA-3' (sense) and 5'-AAAAGCAGCTCTGGGTCGAA-3' (antisense). The copy number of the target gene was normalized to an endogenous reference, GAPDH. The fold change from normal samples was set at 1-fold, and the normalized fold change ratio was calculated.

**Immunoblotting**

Cells were lysed in cold RIPA lysis buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) containing protease and phosphatase inhibitors (GenDEPOT, USA). Cell lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% skim milk or 5% bovine serum albumin, the membrane was probed with anti-phospho Akt, anti-phospho IκBα, and anti-phospho NF-κB p65 antibodies, followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The membranes were then stripped and reprobed with anti-Akt, anti-IκBα, anti-NF-κB p65, and anti-β-actin antibodies.

**Reactive oxygen species assay**

Intracellular ROS was assayed by measuring the fluorescence of DCF, which is the oxidized product of DCF-DA in stained cells. IEC-6 cells were stimulated with TNF-α or simvastatin for 15 min. Cells were then washed in PBS and incubated with 25 μM DCF-DA in the dark for 40 min. DCF fluorescence was detected by confocal microscopy.

**Animal model**

Animals were handled in an accredited animal facility in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) guidelines under the facility named CACU (The Center of Animal Care and Use) of Gachon University Lee Gil Ya Cancer and Diabetes Institute after IRB approval (LCDI-2014-0051). Seven weeks old SPF male C57BL/6 mice ( Orient Bio, Sungnam, Korea) were used for the experiments.

A total of 32 mice were divided into 4 groups (n = 8):
- Group 1, a non-treated control group;
- Group 2, indomethacin (12 mg/kg) induced damage group;
- Group 3, indomethacin (12 mg/kg) with PPI (pantoprazole, 5 mg/kg);
- Group 4, indomethacin (12 mg/kg) plus simvastatin (15 mg/kg).

All groups were starved for 4 hours before treatments, then animals were treated for 24 hours and sacrificed. All stomachs and small intestines of mice were removed and opened along the greater curvature and then washed with ice cold PBS solutions. Isolated proximal small intestines were subjected to H&E, immunohistochemistry, and immunofluorescencestaining.

**Histological scoring**

Histologic score for specimen was analyzed according to the previous report (16). Previous histologic score system for colitis evaluation was modified for analyzing the small bowel inflammation. Degree of lamina propria and submucosal mononuclear cellular infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion were used for histologic score.

**Immunohistochemistry**

Proximal small intestinal in paraffin-embedded blocks were sectioned and mounted on frost-free slides. Sections (3 – 10 μm) were deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen retrieval was achieved by immersion of the sections in 10 mM sodium citrate buffer (pH 6.0), heating to 95°C for 10 min, and cooling in cold water for 30 min. Slides were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase, rinsed in phosphate-buffered saline (PBS) for 5 min, and then incubated with a mouse monoclonal antibody to 8-hydroxyguanosine (1:200, ab85808; Abcam, Cambridge, MA) at 4°C for 2 hours. After three PBS washes (5 min), sections were incubated with a secondary horseradish peroxidase-conjugated anti-rabbit/mouse antibody (Dako, Carpinteria, CA, USA) at room temperature for 20 min, followed by DAB + chromogen solution (Dako) for 5 min. Nuclei were counterstained with hematoxylin. Relatively normal mucosa in 4 slides of each IHC sample were observed at 20 x using microscope and images captured by an Olympus DPT2 microscope (Olympus, Tokyo, Japan). Pictures were quantified by DP2-BSW (Olympus soft imaging solution GmbH, Germany).

**Immunofluorescence**

Immunofluorescence was used a TNF-α antibody (1:100, sc-52746, Santa Cruz biotechnology, Santa Cruz, CA) and secondary Alexa Fluor 488 antibody (1:500, A11001, molecular probes Inc., Eugene OR, USA). Nuclei were stained using Vectashield mounting medium for fluorescence with DAPI (H-1200, Vector Laboratories, Burlingame, CA). Images were performed using Zeiss LSM confocal microscope.
Statistical analysis

Histologic scores were analyzed with Mann Whitney test (SAS; Cary, NC). The paired Student’s T-test was used to compare all other data. Statistical significance was defined as P < 0.05 for comparisons indicated. Data are reported as mean values ± standard error of the mean (S.E.), unless otherwise indicated.

RESULTS

Effects of simvastatin on TNF-α-induced inflammatory gene expression

Interleukin-6 (IL-6) and IL-8, inflammatory cytokines, contribute to inflammation caused by TNF-α. The effect of simvastatin on TNF-α-induced inflammation was investigated in IEC-6 cells, nontransformed small intestinal cells. IEC-6 cells were incubated in the presence of TNF-α for 24 hours. TNF-α treatment induced the expression of IL-6 and IL-8, but the effect was diminished by treatment with simvastatin (Fig. 1).

Simvastatin inhibits TNF-α-induced phosphorylation of Akt

Activation of TNF-α can trigger the activation of Akt. We examined whether simvastatin modulates TNF-α-induced Akt phosphorylation and found that simvastatin treatment inhibited TNF-α-mediated Akt phosphorylation (Fig. 2a). TNF-α induced Ser-473 phosphorylation of Akt in control cells within 15 min, and the peak effect occurred at 45 min. However, TNF-α did not accelerate Akt phosphorylation in cells pretreated with 0.5 µM simvastatin. These results indicate that simvastatin inhibits TNF-α-induced Akt phosphorylation.

Simvastatin promotes TNF-α-mediated degradation of IκBα

Translocation of NF-κB to the nucleus is preceded by proteolytic degradation of IκBα (17). To determine whether the NF-κB-inhibitory activity of simvastatin is the result of IκBα degradation, we exposed IEC-6 cells pretreated with simvastatin to 50 ng/ml TNF-α for various times and examined the IκBα status in the cytoplasm by Western blotting. TNF-α induced IκBα degradation in control cells within 15 min, and the effect had disappeared at 45 min. Simvastatin maintained TNF-α-induced IκBα degradation (Fig. 2b). Under these conditions, simvastatin inhibited phosphorylation of IκBα by treatments of TNF-α. These results indicate that simvastatin directly activated TNF-α-induced IκBα degradation.

Fig. 1. Inhibition of IL-6 or IL-8 expression in 50 ng/ml TNF-α induced inflammation with 0.5 uM simvastatin in IEC-6 cell line. IEC-6 cells were treated with 50 ng/ml TNF-α and/or 0.5 uM simvastatin for 24 hours. mRNA expressions of IL-6 and IL-8 were determined by qRT-PCR. Asterisks indicate statistical significance (P < 0.05).

Fig. 2. Simvastatin prevents TNF-α induced down-regulation of Akt phosphorylation in rat normal intestinal cells. (a) Total and phosphorylation of Akt were detected in IEC-6 cells after treatments of 50 ng/ml TNF-α and/or 0.5 uM simvastatin for time points. (b) Immunoblots represent the time dependent effect of simvastatin on the total and phosphorylation of IκBα in IEC-6 cells. (c) Effect of simvastatin on total and phosphorylation of NF-κB using immunoblotting in IEC-6 cells.
Simvastatin inhibits TNF-α-induced activation of p65

TNF-α induces the phosphorylation of NF-κB p65, which is required for its transcriptional activity (18). We next determined whether simvastatin affects TNF-α-induced NF-κB activation. IEC-6 cells were pretreated with 0.5 µM simvastatin and then stimulated with 50 ng/ml TNF-α. We examined whether simvastatin modulates TNF-α-induced Ser-536 phosphorylation of p65 through the Akt pathway. Western blot analysis showed that TNF-α induced the phosphorylation of p65, and the maximum effect occurred at 45 min (Fig. 2c). These results suggest that simvastatin inhibits TNF-α-mediated phosphorylation of NF-κB p65 via reducing of Akt phosphorylation.

Simvastatin inhibits TNF-α-induced cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) expression

To investigate the influence of simvastatin on TNF-α-induced COX-1 and COX-2 expression, we assessed their mRNA levels by qRT-PCR. Treatment with 50 ng/ml TNF-α induced overexpression of both COX-1 and COX-2 mRNA, while pretreatment with simvastatin reduced TNF-α-induced expression of these genes in IEC-6 cells (Fig. 3a). However,

![Fig. 3. Simvastatin affects mRNA expression of COX-1 and COX-2. (A) mRNA expression of COX-1 or COX-2 was determined by qRT-PCR in IEC-6 cells after treatment with 50 ng/ml TNF-α and/or 0.5 µM simvastatin for 24 hours. (B) Western blot of COX-1 or COX-2 in IEC-6 cells after treatment with 50 ng/ml TNF-α and/or 0.5 µM simvastatin. Experiments were performed in triplicate. Asterisks indicate statistical significance (P < 0.05).](image)

![Fig. 4. Suppression of TNF-α induced generation of ROS in simvastatin pretreated IEC-6 cells. IEC-6 cell were treated with 0.5 uM simvastatin for 20 min and/or 50 ng/ml TNF-α for 20 min. The cells were stained with 25 uM DCF-DA for 30 min.](image)
western blotting showed that the effect on COX-1 and COX-2 protein levels of treatment with the combination of TNF-α and simvastatin was similar to that of TNF-α alone (Fig. 3b). These results suggest that simvastatin inhibits the TNF-α-induced transcription of the COX-1 and COX-2 genes but does not affect COX-1 and COX-2 protein levels.

**Simvastatin inhibits intracellular ROS generation in TNF-α-stimulated IEC-6 cells**

TNF-α has been shown to bind to receptors and generate ROS in various cell lines (19). To investigate whether ROS production was induced during TNF-α-mediated inflammation, intracellular ROS levels were analyzed using the cell-permeable, oxidation-sensitive dye DCF-DA. ROS-mediated oxidation of this dye was detected by confocal laser-scanning microscopy. TNF-α-treated cells exhibited greater DCF-DA fluorescence intensity than untreated cells (Fig. 4). However, DCF-DA fluorescence intensity was decreased by treatment with simvastatin. These findings suggest that simvastatin reduces TNF-α-induced ROS production.

**Effect of simvastatin in vivo**

To investigate the anti-inflammatory effect of simvastatin (10 mg/kg), an indomethacin (12 mg/kg)-induced mouse model of inflammation was established (Fig. 5a).

Histopathological examination showed that indomethacin disrupted epithelial stratification and induced basal lamina degeneration and infiltration of inflammatory cells (Fig. 5b). The number of villi was significantly lower in mice after oral administration of indomethacin than control mice. In contrast, simvastatin pretreatment reduced the structural damage to the small intestine and increased the mean villus height compared with indomethacin treatment alone. The histological score for inflammation was evaluated for each group. Comparing to control group, indomethacin groups showed the statistically higher histological score. However, histological score for inflammation of duodenum was decreased after treatment with simvastatin (Fig. 5c).

Indomethacin-induced damage induced expression of TNF-α (20, 21). Immunofluorescence image of TNF-α show that simvastatin reduced indomethacin induced expression of TNF-α in small intestine (Fig. 6a).

One of the key molecular mechanisms of NSAID-induced damage is the robust induction of ROS in the small intestine. 8-Hydroxydeoxyguanosine (8-OHdG) is a useful marker for assessing DNA damage due to ROS. Representative images of 8-OHdG staining of the small intestine are show in Fig. 6b. Treatment with indomethacin resulted in a higher level of 8-OHdG than in the control mucosal area. The numbers of 8-OHdG-positive cells were statistically smaller after treatment with the combination of indomethacin plus PPI or simvastatin than treatment with indomethacin alone.
NSAIDs can reduce inflammation and pain. However, in the presence of \textit{Helicobacter pylori} infection, NSAIDs are one of two major causes of peptic ulcers (22, 23). Inhibition of COX1 and prostaglandin (24) increased membrane permeability (25), and generation of pro-inflammatory mediators are the three main mechanisms of NSAID-induced gastrointestinal injury (26). Many inflammatory mediators can be involved in response to gastrointestinal injury (27-29). Pro-inflammatory mediators, such as TNF-\(\alpha\), can release oxygen-derived free radicals in damaged microvessels, which can damage or destroy the mucosa by lipid peroxidation (30).

This study investigated the effect of simvastatin on gastrointestinal inflammation by assessing TNF-\(\alpha\)-induced expression of NF-\(\kappa\)B-mediated genes, COX expression, ROS production, and NSAID-induced inflammation in the mouse small intestine.

We found that simvastatin inhibited the expression of inflammatory genes induced by TNF-\(\alpha\) (Fig. 1). This effect of simvastatin correlated with down-regulation of NF-\(\kappa\)B-mediated gene expression, which was regulated by TNF-\(\alpha\). Simvastatin suppressed constitutive, but not inducible, NF-\(\kappa\)B expression. Suppression of NF-\(\kappa\)B activation by simvastatin was due to inhibition of TNF-\(\alpha\)-induced NF-\(\kappa\)B phosphorylation.

We report here for the first time that the anti-inflammatory effect of simvastatin was due to inhibition of TNF-\(\alpha\)-induced inflammation in the small intestine. Simvastatin specifically reduced TNF-\(\alpha\)-induced Akt phosphorylation at Ser373. However, it had no effect on Akt phosphorylation at Thr308 (data not shown). Therefore, simvastatin affects a specific site of

\[ \text{Fig. 6. Simvastatin reduced indomethacin-induced expression of TNF-\(\alpha\) and 8-hydroxydeoxyguanosine in mouse proximal intestine.} \]

\(a\) Immunofluorescence staining with TNF-\(\alpha\) in proximal small intestinal. TNF-\(\alpha\) was visualized with Alexa Fluor 488 antibody (Green). Nuclei stained with DAPI. Original magnification \(\times 10\). \(b\) Immunohistochemical staining with 8-hydroxyguanosine. Staining of Nuclei were used with hematoxylin. Calculation of 8-OHdG positive cell of mucosal area was counted by DP2-BSW (Olympus soft imaging solution). Original magnification \(\times 20\) (\(P < 0.03\)).
Akt or upstream protein kinases that phosphorylates Akt at the Ser373 residue. Moreover, TNF-α-induced IkBα degradation was increased by simvastatin. Simvastatin did not affect phosphorylation of IkBα at Ser 32. However, simvastatin treatment increased the degradation of IkBα, which in turn inhibited phosphorylation of NF-κB p65.

Simvastatin inhibited TNF-α-induced transcription of the COX-1 and COX-2 genes. However, simvastatin did not affect total COX-1 and COX-2 protein levels. This result suggests that simvastatin does not influence gastrointestinal mucosal protection regulated by prostaglandins.

We also found that simvastatin reduced TNF-α-induced generation of ROS. Akt activation can be increased by ROS. Simvastatin reduced TNF-α-mediated activation of Akt and NF-κB via reducing ROS production. Thus, simvastatin inhibited TNF-α-induced inflammation in the small intestine. Increased level of ROS by indomethacin enhanced intestinal damage (31). IHC staining for 8-OHdG in mice small intestine exposure to indomethacin suggested that simvastatin also functions as an ROS scavenger. Detection of a high level of 8-OHdG is suggestive of ROS-induced DNA damage. 8-OHdG is also an indicator of recovery of DNA damage (32). The reduced 8-OHdG level following treatment of simvastatin suggested amelioration of indomethacin-induced ROS production.

The structure of the mouse small intestine was disrupted by indomethacin-induced inflammation, as determined by histology (Fig. 5). However, simvastatin treatment reduced this indomethacin-induced loss of small intestinal structural integrity. Therefore, simvastatin protects the small intestine by reducing ROS production in indomethacin-mediated inflammation. Indeed, simvastatin has been reported to protect against damage to the small intestine in a murine model of hyper-acute Th1-type ileitis with Toxoplasma gondii by inactivating Th1-type immune responses (13).

No definitive effective drug is available for the treatment of small bowel injury. Satoh et al. reported that a diet without or with small amounts of dietary fiber could decrease gastrointestinal inflammation associated with the use of NSAIDs (33). Statin has therapeutic effects on gastrointestinal damage. Anti-inflammatory effect of HMG-CoA reductase inhibitors, flavastatin, pravastatin and atorvastatin on ulcer formation in 5-bromo-2-((4-fluorophenyl)-3-((4-methylsulfonylphenyl) thiophene (BFMeT)-treated rats (34). Simvastatin reduced the risk of progression from inflammatory bowel disease (35) to colorectal cancer (36, 37). Simvastatin reduced lesion score of the colitis, GSH level, and myeloperoxidase (38) activity on trinitrobenzene sulfonic acid (TNBS)-induced colonic inflammation in rats (39). Antiulcer effect of simvastatin decreased the formation of gastric lesions. Simvastatin altered expression of antioxidant proteins and enzymatic activity of antioxidant proteins on indomethacin- and ethanol-induced gastric ulcer in rats (40, 41). Simvastatin decreased gastric TNF-α and gastric ulcer area in diabetic rats (42).

Anti-inflammatory effect of statin was dependent on their antioxidant role and unrelated to its cholesterol-lowering activity. Previous studies suggested that simvastatin has an antioxidant and anti-inflammatory effect regardless of cholesterol-lowering action. Mason et al. reported that atorvastatin could reduce blood pressure, nitrooxidative stress and rantes level in hypertensive rats with diabetes by enhancing nitric oxide (NO) release (43). In normocholesterolemic rats, simvastatin reduced leukocyte-endothelial cell interaction and expression of P-selectin, as an endothelial cell adhesion molecule, on the mesenteric venular endothelium after superfusion with either N(G)-nitro-L-arginine methyl ester (L-NAME) or thornbin (44). A study of Huang et al. showed that simvastatin suppressed H2O2 mediated oxidative stress through decreased protein level of Nox4 in murine osteoblastic cells (45).

In summary, we investigated simvastatin-mediated inhibition of TNF-α-induced inflammation and found that it acts as an ROS scavenger. Our results also showed that simvastatin suppresses TNF-α-mediated inflammation through inhibition of ROS-induced Akt signaling pathways. Thus, the simvastatin suppressed the inflammatory related production of intracellular ROS levels and reduced inflammatory reaction in indomethacin-induced small bowel injury of mice. This phenomenon may have contributed to protection against NSAID-mediated injury in gastrointestinal tract.

Abbreviations: 8OHdG, 8-hydroxy-2-deoxyguanosine; COX, cyclooxygenase; DCFH-DA, 2′, 7′-dichlorofluorescein diacetate; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IL, interleukin; L-NAME, N(G)-nitro-L- arginine methyl ester; NF-κB, nuclear factor-κB; NSAIDs, nonsteroidal anti-inflammatory drugs; ROS, reactive oxygen species; SFE, specific-pathogen-free; TNBS, trinitrobenzene sulfonic acid, TNF-α, tumor necrosis factor alpha.

Eun-Kyung Kim and Jae Hee Cho equally performed the majority of the work including collecting, analyzing, and interpreting the data and writing the manuscript. A Reum Jeong, Eui Joo Kim, and Dong Kyun Park contribute to designing experiments of mouse model. Kwang An Kwon, Jun-Won Chung, and Kyoung Oh Kim edited the report. Yun Soo Kim, Ju Hyun Kim, and Yoon Jae Kim designed and coordinated the study.

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