INTRODUCTION

Recent instrumental improvements in the diagnosis of small intestine diseases, including video capsule endoscopy (1) and double balloon endoscopy (2), have revealed that non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin induce intestinal mucosal lesions (3, 4). Although various factors are thought to be involved in the pathophysiology of NSAID-induced intestinal lesions (5-11), no clinical therapy for intestinal lesions has been established. Additionally, NSAIDs are known to cause intestinal injury in both humans and experimental animal models including mice (12-14).

BTB and CNC homolog 1 (Bach1) is a known transcriptional repressor of heme oxygenase-1 (HO-1). It plays an important role in the feedback regulation of HO-1 expression, which protects cells from various insults including oxidative stress and inflammatory cytokines. However, the role of Bach1 in intestinal inflammation remains unclear. In this study, the role of Bach1 in intestinal mucosal injury was elucidated using 8-week-old female C57BL/6 (wild-type) and homozygous Bach1-deficient C57BL/6 mice. Intestinal mucosal injuries induced by a single subcutaneous administration of indomethacin were evaluated macroscopically, histologically, and biochemically. Mucosal protein content and chemokine mRNA levels were determined by real-time PCR. Our results showed that the indomethacin-induced intestinal injury was remarkably improved in Bach1-deficient mice. Histological examination showed that the area of injured lesion was decreased in Bach1-deficient mice compared to wild-type mice. Administration of indomethacin induced expression of inflammatory chemokines such as KC, MIP1α and MCP1, which was suppressed in Bach1-deficient mice. Myeloperoxidase activity in the intestinal mucosa was also significantly decreased in Bach1-deficient mice. Additionally, Bach1 deficiency enhanced immunopositivity of HO-1 in the intestinal mucosa after indomethacin administration. Disruption of the Bach1 gene thus caused inhibition of mucosal injury, indicating that inhibition of Bach1 may be a novel therapeutic strategy for treating indomethacin-induced intestinal injury.

Key words: non-steroidal anti-inflammatory drugs, indomethacin, intestinal injury, myeloperoxidase activity, inflammatory chemokine, heme oxygenase-1, Bach1

MATERIALS AND METHODS

Experimental animals

Eight-week-old female C57BL/6 wild-type mice and homozygous Bach1-deficient mice were used in this study. Wild-type mice were obtained from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan), while Bach1-deficient mice were kindly provided by Prof. Igarashi (Tohoku University, Japan) (18). The mice were housed in stainless steel cages with
wire bottoms and maintained on a 12-h light-dark cycle, under temperature and relative humidity conditions of 21–23°C and 55–65%, respectively. The experiments were performed using five to seven non-fasting mice per group under unanesthetized conditions. All experimental procedures were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

**Induction of small intestinal lesions**

After 16 h of fasting, the animals were fed for 3 h and subcutaneously administered indomethacin (1-(4-chlorobenzyloxy)-5-methoxy-2-methyl-1H-indole-3-acetic acid; Wako Pure Chemical Industries, Ltd, Osaka, Japan) at a dose of 10 mg/kg, and killed 24 h later under deep anesthesia. The jejunum and ileum were then removed, opened along the anti-mesenteric attachment, and examined for lesions under a stereomicroscope with square grids. The area (mm²) of visible lesions was macroscopically measured, totalled per 15 cm of small intestine, and expressed as a lesion score. The investigator measuring the lesions was blind to the treatment conditions of the animals. Both wild-type and Bach1-deficient mice were randomized into groups receiving indomethacin or distilled water containing carboxymethyl cellulose sodium salt (Wako Pure Chemical Industries) at the same concentration as the indomethacin diluent (vehicle).

**Measurement of myeloperoxidase (MPO) activity**

The intestinal mucosa was scraped off using two glass slides, then homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) containing 30 mM KCl in a Teflon Potter-Elvehjem homogenizer. The total protein in the tissue homogenates was measured by the method of Lowry (23). As an index of neutrophil accumulation, tissue-associated MPO activity in the intestinal mucosa was determined by a modification of the method of Grisham et al. (24). The mucosal homogenates were centrifuged at 20000 g for 15 min at 4°C to pellet the insoluble cellular debris. The pellet was then re-homogenized in an equivalent volume of 0.05 M potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20000 g for 15 min at 4°C and the supernatants saved. MPO activity was assessed by measuring the H₂O₂-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO required to cause a change in absorbance of 1.0/μmin at 645 nm and 25°C.

**Real-time PCR for KC, MIP1α, and MCP-1**

RNA was isolated by the acid guanidinum phenol chloroform method using an Isogen kit (Nippon Gene, Tokyo, Japan). The RNA concentration was determined by absorbance at 260 nm in relation to absorbance at 280 nm. RNA was stored at -70°C until reverse transcription was performed. An 1 μg aliquot of extracted RNA was reverse transcribed into first-strand complementary DNA (cDNA) at 42°C for 40 min, using 100 U/ml reverse transcriptase (Takara Biochemicals, Shiga, Japan) and 0.1 μM oligo (dT)-adapter primer (Takara Biomedicals) in a 50 μl reaction mixture. Real-time PCR was carried out with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the DNA binding dye SYBR-Green I for the detection of PCR products. The reaction mixture (RT-PCR kit, code RRO43A, Takara Biomedicals) contained 12.5 μl Premix Ex Taq, 2.5 μl SYBR-Green I, custom-synthesized primers, ROX reference dye, and cDNA (equivalent to 20 ng total RNA) to give a final reaction volume of 25 μl. The primers were as follows: for KC, sense 5′-TGTCAAGTGCTGCAGACCCAT-3′ and antisense 5′-TGTCAAGTGCTGCAGACCCAT-3′; for MCP1, sense 5′-CTCCCTCCACCACTGCA-3′ and antisense 5′-CCAGGCCGCAACTGTGA-3′; for MIP1α, sense 5′-ACAAGCAGCGAGGTACC-3′ and antisense 5′-CATGATCGACGGTGACAGA-3′; and for β-actin, sense 5′-TATCCACCTTCACCAGATGTT-3′ and antisense 5′-AGCTACGTAACACGTCCGCTA-3′. The PCR conditions were as follows: initial denaturation for 10 sec at 95°C, followed by 40 cycles of amplification for 5 sec at 95°C and 31 sec at 60°C. The PCR products were quantified using standard DNA for each, purified by PCR products of reverse-transcribed RNA. The relative expression was then calculated as the density of the product of the respective target gene divided by that for β-actin from the same cDNA.

**Immunohistochemical staining**

After twenty four hours of fixation in formalin, the samples were embedded in paraffin, and sections were cut to 5 μm thickness using a microtome cryostat and then mounted on MAS-coated slides. We performed antigen retrieval using proteinase K solution, and the sections were rinsed with distilled water for 5 min, and then incubated with 3% hydrogen peroxide in methanol for 30 min to block the endogenous peroxidase activity. After incubation, the sections were washed in phosphate buffered saline (PBS)-Tween for 5 min each. Non-specific binding was blocked by incubating the slides with Dako Cytomation protein block (Dako, Tokyo, Japan) for 30 min at room temperature. The sections were then incubated with primary antibody (anti-HO-1 (Hsp32) antibody, Assay Designs, Ann Arbor, MI, USA) diluted at 1:200 with antibody dilution (Dako) overnight at 4°C. The sections were then washed three times in PBS-Tween for 5 min each, and incubated with secondary antibody (Histfine Simple Stain mouse MAX PO (rabbit), Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. Unbound antibodies were removed by three washes in PBS for 5 min, and the bound antibodies were visualized using diaminobenzidine as a chromogen substrate reagent. Negative controls for non-specific binding incubated with secondary antibodies were confirmed to produce no signal. All sections were counterstained with haematoxylin. The sections were finally dehydrated, cleared, and coverslipped.

**Statistical analysis**

All values are expressed as means±SEM. The data were compared by one-way analysis of variance (ANOVA) followed by Bonferroni’s Multiple Comparison Test. A probability value less than 5% was considered statistically significant.

**RESULTS**

Effects of Bach1 deficiency on macroscopic findings, ulcer score and histology

A single administration of indomethacin at a dose of 10 mg/kg induced multiple erosions in the small intestine (Fig 1A). The development of intestinal lesions induced by indomethacin was significantly suppressed in Bach1-deficient mice compared with wild-type mice (Fig 1B). Histological examination showed defects of the villi, epithelial stratification, basal lamina degeneration, and infiltration of the epithelium prior to infiltration of the mucosa by inflammatory cells. In Bach1-deficient mice, however, reduced erosions and inflammatory changes were observed in the small intestine (Fig 2).
Effects of Bach1 deficiency on MPO activity in the intestinal mucosa

Neutrophil accumulation was evaluated by measuring tissue-associated MPO activity in the intestinal mucosal homogenates (Fig. 3). In the sham groups, there was no difference in the MPO activity between wild-type mice and Bach1-deficient mice. On the other hand, MPO activity in the intestinal mucosa was remarkably increased in the indomethacin-treated group compared with the sham group. The increase in MPO activity in the intestinal mucosa was significantly suppressed in Bach1-deficient mice compared with that in wild-type mice.

mRNA expression of inflammatory chemokines in the intestinal mucosa

The levels of inflammatory chemokines including KC, MCP-1, and MIP1-α in the small intestine were determined by real-time PCR (Fig. 4). In the sham group, no difference was found in the level of inflammatory chemokines between wild-type mice and Bach1-deficient mice. However, a single administration of indomethacin caused a significant increase in mRNA levels of inflammatory chemokines in the wild-type mice compared with those in the Bach1-deficient mice, suggesting that Bach1 deficiency suppresses the increase of inflammatory chemokines in the intestinal mucosa.

Fig. 1. (A) Macroscopic findings of the small intestine in mice treated with indomethacin. The administration of indomethacin provoked multiple erosions in the small intestine in wild-type mice. On the other hand, in Bach1-deficient mice, the number and the severity of lesions were clearly diminished. (B) Effect of Bach1 deficiency on ulcer index in the intestinal mucosa treated with indomethacin. Data are expressed as means±SEM of five to seven mice. *P < 0.05 compared to wild-type mice.

Fig. 2. Histological findings of the small intestine. The small intestine was removed and fixed in 10% neutral buffered formalin. After fixation, the tissues were stained with haematoxylin and eosin. (A) Wild-type, vehicle. (B) Bach1-deficient, vehicle. (C) Administration of indomethacin resulted in defects in the villi, epithelial stratification, basal lamina degeneration, and infiltration of inflammatory cells. (D) Bach1 deficiency caused smaller erosions with less infiltration of inflammatory cells.
Localization of HO-1 in inflamed intestinal tissue

To investigate HO-1 expression in the small intestinal mucosa, we performed HO-1 immunohistochemical staining of the small intestinal mucosa (Fig. 5). Induced HO-1 expression was mainly observed in mononuclear cells in mucosa and submucosa. After treatment with indomethacin, HO-1 immunopositivity was stronger in Bach1-deficient mice than in wild-type mice. In the Bach1-deficient mice, smaller erosions and much more HO-1-positive mononuclear cells were seen in the epithelium.

DISCUSSION

The present study demonstrates that Bach1 deficiency attenuates indomethacin-induced intestinal mucosal injury and inflammation in mice. We assessed intestinal injury by various methods, including ulcer index and histology. In each assessment, Bach1 deficiency significantly inhibited small intestinal injury. In addition, we showed that MPO activity and mRNA expression of KC, MIP1α and MCP1, which are involved in chemotaxis and cell activation of neutrophils and monocytes, were enhanced in indomethacin-induced intestinal inflammation, and that such increases were suppressed in Bach1-deficient mice.

It has been hypothesized that neutrophil-mediated inflammation is involved in the development of indomethacin-induced intestinal injury (25-30). In this study, we found that MPO activity representing neutrophil infiltration in the intestinal mucosa was markedly elevated after administration of indomethacin, and this increase is considered to be correlated with various conditions such as prostaglandin (PG) deficiency, bacterial flora (31), nitric oxide (NO), and hypermotility of the intestine (30). We also found that MPO activity, an index of tissue-associated neutrophil accumulation, significantly increased in the intestinal mucosa after indomethacin administration and that Bach1 deficiency significantly inhibited this increase. These results indicate that the inhibition of neutrophil accumulation by Bach1 deficiency may be one of the protective factors helping to decrease indomethacin-induced intestinal injury.

KC is a keratinocyte-derived chemokine, which is considered to be a murine homologue of human GRO. This chemokine is involved in cell activation of neutrophils and plays a key role in the pathogenesis of gastrointestinal inflammation. Our previous study showed that the expression of KC in gastric mucosa inflamed with Helicobacter pylori was exacerbated by aspirin administration in gerbils (32). Moreover, various agents have been reported to cause the upregulation of KC in the inflamed intestinal mucosa (33, 34). In this study, the expression of KC in indomethacin-treated intestinal mucosa was significantly suppressed in Bach1-deficient mice compared with that in wild-type mice. We also showed the anti-inflammatory properties of Bach1 deficiency in vivo by demonstrating a reduction in mucosal inflammatory chemokines, confirming previous reports that the expression of macrophage chemokines, including MCP1 and MIP1α, which are important for triggering gastrointestinal inflammatory response (35, 36), were significantly inhibited in Bach1-deficient mice.

Immunohistochemical analysis of the expression of HO-1, a cytoprotective factor, in Bach1-deficient mice showed that HO-1-immunopositive mononuclear cells were induced after indomethacin administration. This induction in the small intestinal mucosa was significantly higher in Bach1-deficient mice. As in our previous study, HO-1 was localized in macrophages in human assessment, Bach1 deficiency significantly inhibited small intestinal injury. In addition, we showed that MPO activity and mRNA expression of KC, MIP1α and MCP1, which are involved in chemotaxis and cell activation of neutrophils and monocytes, were enhanced in indomethacin-induced intestinal inflammation, and that such increases were suppressed in Bach1-deficient mice.

**Fig. 3.** Effect of Bach1 deficiency on neutrophil accumulation in the intestinal mucosa of mice treated with indomethacin. Data represent the means±SEM of five to seven mice. *P<0.05 compared to wild-type mice.

**Fig. 4.** Effect of Bach1 deficiency on mRNA expression of KC (a), MCP-1 (b), and MIP-1α (c) in the intestinal mucosa of indomethacin-treated mice. Each value indicates the means±SEM for five to seven mice. *P<0.05 compared to wild-type mice treated with indomethacin.
inflamed colonic mucosa (37). These results indicate that HO-1 expressed in mononuclear cells have an important role, which leads to the suppression of MPO through inhibiting expression of various chemokines. Our previous study also showed that HO-1 had cytoprotective effects in the inflammatory response of the gastrointestinal tract (37-39), while the function of Bach1 was partly unknown. A previous report using Bach1-deficient mice proved that the extent of myocardial infarction was suppressed by the effect of HO-1 (22). On the other hand, although hyperoxic lung injury was also inhibited in Bach1-deficient mice, it was not due to the effect of HO-1, but to IL-6 upregulation (21). Therefore, it remains unclear whether factors other than HO-1 are involved in the cytoprotective effect of Bach1 deficiency, as HO-1 must be induced in Bach1-deficient mice. Further studies are required to clarify the function of Bach1. In the future, however, the inhibition of Bach1 may be a novel therapeutic strategy to treat indomethacin induced intestinal injury.

Abbreviations: Bach1- BTB and CNC homolog 1; HO-1- heme oxygenase-1; NSAIDs- nonsteroidal anti-inflammatory drugs; MPO- myeloperoxidase; MARE- Maf-recognition element.

Acknowledgements: This research was supported in part by grants (to Y.N.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interests: None declared.

REFERENCES

Fig. 5. Immunohistochemical analysis for HO-1. (A) Wild-type, vehicle (B) Bach1-deficient, vehicle (C) Administration of indomethacin resulted in ulceration with slight HO-1 induction (D) Bach1 deficiency caused smaller erosions showing higher HO-1 immunopositivity.


Received: October 15, 2009
Accepted: December 11, 2009

Author’s address: Prof. Yuji Naito, MD, PhD; Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi-hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan; Phone: +81-75-251-5508; Fax: +81-75-251-0710; E-mail: ynaito@koto.kpu-m.ac.jp