Free Radical Research, June 2011; 45(6): 717–727

informa
healthcare

Suppression of indomethacin-induced apoptosis in the small intestine due to Bach1 deficiency

AKIHITO HARUSATO[1], YUJI NAITO[1], TOMOHISA TAKAGI[1], KAZUHIKO UCHIYAMA[1], KATSURA MIZUSHIMA[1], YASUKO HIRAI[1], SHINYA YAMADA[1], TOSHIFUMI TUJI[1], HIROYUKI YORIKI[1], RYUSUKE HORIE[1], KEN INOUE[1], KOHEI FUKUMOTO[1], OSAMU HANDA[1], TAKESHI ISHIKAWA[1], SATOSHI KOKURA[1], YUKIKO MINAMIYAMA[1], HIROSHI ICHIKAWA[2], AKIHIKO MUTO[3], KAZUHIKO IGARASHI[3] & TOSHIKAZU YOSHIKAWA[1]

[1]*Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi-hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan,* [2]*Department of Medical Life Systems, Faculty of Life and Medical Sciences, Doshisha University, Tatara Miyakodani 1-3, 610-0394, Kyotanabe-City, Japan, and* [3]*Department of Biochemistry, Tohoku University School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai, 980-8575, Japan*

(Received date: 22 November 2010; Accepted date: 18 March 2011)

Abstract
BTB and CNC homologue 1 (Bach1) is a transcriptional repressor of heme oxygenase-1 (HO-1). This study hypothesized that Bach1 plays an important role in the indomethacin-induced apoptosis in the case of small-intestinal mucosal injury. Eight-week-old male C57BL/6 (wild-type) and homozygous Bach1-deficient C57BL/6 mice were included in this study. Mucosal injuries induced by subcutaneously administering indomethacin were evaluated macroscopically, histologically and biochemically. Indomethacin-induced injuries were improved in Bach1-deficient mice. Immunohistochemistry showed an increase in the number of HO-1-positive cells, which were mainly F4/80 positive macrophages, in Bach1-deficient mice. Indomethacin administration increased the expression of HO-1 mRNA and protein in the small intestine in Bach1-deficient mice. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) staining showed that the extent of apoptosis was suppressed in Bach1-deficent mice. In conclusion, deficiency of the Bach1 gene inhibited apoptosis and thus suppressed mucosal injury, indicating that Bach1 is a novel therapeutic target for indomethacin-induced intestinal injury.

Keywords: *Non-steroidal anti-inflammatory drugs, apoptosis, small intestine, heme oxygenase-1, Bach1*

Introduction

Development of endoscopic diagnostic techniques for small intestine diseases, such as video capsule endoscopy [1] and balloon enteroscopy [2], has led to the revelation that non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin can induce intestinal mucosal injuries [3,4]. Although the pathophysiology of NSAID-induced intestinal injuries has been elucidated [5–10], practical therapies for such injuries are unavailable. Therefore, it is important to investigate preventive and therapeutic strategies for NSAID-induced intestinal injuries.

Expression of BTB and CNC homologue 1 (Bach1), a transcriptional repressor of heme oxygenase-1 (HO-1) and a cytoprotective enzyme, is induced by various injuries, including oxidative stress [11,12]. Bach1 can bind Maf proteins and then act as a repressor of HO-1 via the Maf-recognition element (MARE) of the HO-1 promoter. Bach1 binds heme with high affinity, thus preventing it from binding to MARE, and regulates the nuclear export of Bach1. Thus, the repressor function of Bach1 is inhibited by heme [13–15]. To investigate the function of Bach1, homozygous deficient mice resistant to various injuries involving

Correspondence: 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. Tel: +81-75-251-5508. Fax: +81-75-251-0710. Email: ynaito@koto.kpu-m.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.574287

many organs, including the heart, lung, spinal cord and liver, were created [16–19]. Although the function of Bach1 in the gastrointestinal tract had been unknown, we first reported that Bach1 deficiency ameliorated indomethacin-induced intestinal mucosal injury in mice [20]. However, the precise underlying mechanisms remain unclear.

The administration of NSAIDs is known to induce apoptosis of small intestinal epithelial cells and we have also focused on the relationship between small intestinal injury and NSAIDs-induced apoptosis. This study aimed to elucidate the mechanisms of Bach1 deficiency mediated amelioration of indomethacin-induced mucosal injury with a particular focus on apoptosis in the small intestine.

Methods

Experimental animals

We used 8-week-old male C57BL/6 wild-type mice and homozygous Bach1-deficient mice. Wild-type mice were obtained from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan), whereas Bach1-deficient mice were kindly provided by Professor Igarashi (Tohoku University, Japan) [14]. The mice were housed in stainless steel cages with wire bottoms and maintained under conditions of 12-h light-dark cycle at 21–23°C and 55–65% humidity. All experimental procedures were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

Induction of small intestinal injury

After 15 h of fasting, the animals were fed for 2 h and subcutaneously administered indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a dose of 10 mg/kg body weight; they were euthanized 24 h later under deep anaesthesia. We removed 15 cm of the small intestine from the terminal ileum, opened along the anti-mesenteric attachment and used for each experiment. The area (mm^2) of visible lesions was macroscopically measured and expressed as an ulcer index, as we reported previously [20].

RNA analysis

RNA was isolated by the acid-guanidinium-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. A 1-μg aliquot of extracted RNA was reverse transcribed into first-strand complementary DNA (cDNA) at 42°C for 40 min by using 100 U/ml of reverse transcriptase (Takara Biochemicals, Shiga, Japan) and 0.1 μM of oligo (dT)-adapter primer (Takara Biochemicals) in a 50-μl reaction mixture. Real-time polymerase chain reaction (PCR) was performed as described previously [20]. The following primers were used: for HO-1, sense 5′-CCTCACTGGCAGGAAATCATC-3′ and anti-sense 5′-CCTCGTGGAGACGCTTTACATA-3′; for TNF-α sense 5′- ATCCGCGACGTGGAACTG-3′ and anti-sense 5′-ACCGCCTGGAGTTCTGGAA-3′; and for β-actin, sense 5′-TATCCACCTTCCAGCAGATGT-3′ and anti-sense 5′-AGCTCAGTAACAGTCCGCCTA-3′. The relative expression was then calculated as the density of the product of the target gene divided by that for β-actin from the same cDNA.

Immunosorbent assay of TNF-α

We determined the concentration of TNF-α in the supernatant of mucosal homogenates using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Inc., MN) specific for mouse TNF-α The assay was performed according to the manufacturer's instructions. After colour development, optimal densities were measured at 450 nm with a microplate reader (MPRA4i, Tosoh, Tokyo, Japan).

Protein extraction and Western blotting

Tissue specimens of the small intestine were homogenized in ice-cold lysis buffer (CelLyticTM MT Cell Lysis Reagent; Sigma-Aldrich, St. Louis, MO) with a protease inhibitor cocktail (Sigma-Aldrich). Total protein extract and cytosolic fractions separated using a Mitochondria Isolation Kit for Tissue (PIERCE, Rockford, IL) were used for Western blotting of HO-1 and caspase-3, respectively. Specimens were then purified by microcentrifugation at 10 000 *g* for 10 min at 4°C. Protein concentrations of the supernatants were adjusted to 1 mg/mL by dilution in 4 × sodium dodecyl sulphate (SDS) gel loading buffer (Invitrogen Japan K.K., Tokyo, Japan) and boiled for 10 min before loading. Next, 20 mg of total protein were loaded onto each lane of 10% acrylamide gel and separated using SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto a Hybond-P polyvinylidene fluoride (PVDF) transfer membrane (0.45 mm; GE Healthcare UK, Buckinghamshire, UK). Thereafter, the membranes were incubated for 1 h at room temperature (RT) in blocking buffer (Tris-buffered saline (TBS) containing 4% low-fat milk powder). The membrane was incubated overnight at 4°C in blocking buffer with primary antibodies against caspase-3 and HO-1. After the excess antibodies were washed, bound antibody

was detected using horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (1/3000; GE Healthcare UK) in blocking buffer for 1 h and enhanced chemiluminescence (ECL) (GE Healthcare UK). Densitometric analyses were performed using the software, Multigauge, ver3.11 (Fuji film, Tokyo, Japan). The primary antibodies were obtained from the following sources: anti-caspase-3 antibody (sc-7148) ,anti-actin antibody (sc-10731) and anti-tubulin antibody (sc-5286) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-HO-1 (Hsp32) antibody (SPA-895) from Assay Designs Inc. (Ann Arbor, MI); and all secondary antibodies from GE Healthcare Bio-Sciences KK (Tokyo, Japan).

Immunohistochemical and immunofluorescence staining

After 24-h fixation in formalin, the samples were embedded in paraffin; 5-μm thick sections were cut using a microtome cryostat and mounted on MAS-coated slides. We performed antigen retrieval using proteinase K solution; 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. Non-specific binding was blocked by incubating the slides with Dako Cytomation protein block (Dako, Tokyo, Japan) for 30 min at RT. The sections were then incubated with primary antibody against HO-1 and F4/80 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 and incubated with secondary antibody at RT according to the manufacturer's recommendations. Unbound antibodies were removed by three washes in PBS for 5 min. For HO-1 immunohistochemical staining, the bound antibodies were visualized as previously reported [20]. For immunofluorescence staining of HO-1 and F4/80, bound antibodies were visualized using secondary antibody anti-rabbit Alexa 594 and anti-rat Alexa 488, respectively. Negative controls used for non-specific binding and incubated with secondary antibodies were confirmed to produce no signal. All sections were counterstained with hematoxylin. Fluorescence staining was observed under an inverted fluorescence microscope (IX70-23FL/DIC-SP; Olympus, Tokyo, Japan). The primary antibodies were obtained from the following sources: anti-HO-1 (Hsp32) antibody (SPA-895) from Assay Designs (Ann Arbor, MI); anti-F4/80 antibody (NB600-404) from Novus Biologicals, Inc. (Littleton, CO). The secondary antibodies were obtained from the following sources: Histofine Simple Stain mouse MAX PO (414341, rabbit) from Nichirei Biosciences Inc. (Tokyo, Japan) for HO-1 immunohistochemistry; Labelled Chicken Anti-Rabbit IgG Antibodies (A-21442) for HO-1 immunofluorescence and Labelled Donkey Anti-Rat IgG Antibodies (A-21208) for F4/80 immunofluorescence from Molecular Probes, Inc. (Eugene, OR).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was also performed using the *In Situ* Apoptosis Detection Kit (Takara Biochemicals) according to the manufacturer's instructions. This assay measures DNA strand breaks and therefore identifies cells undergoing apoptosis. We sampled 10 microscopic fields (at 60 × magnification) of small intestinal mucosa without overlap. The numbers of TUNEL-positive cells were counted in each field and expressed as the apoptosis score.

HO activity assay

Mucosal homogenates of small intestine were prepared by mixing them with a buffer containing 100 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100 and protease inhibitors; the mixture was then lysed by sonication (Sonifier250; Branson, Danbury, CT) on ice. The total protein in the tissue homogenates was measured by the method of Lowry et al. [21]. We determined HO activity with fluorescence by modifying the method previously reported [22]. The fluorescence was detected in a fluorescence reader (ARVOMX; Perkin Elmer, Waltham, MA) at 37°C with excitation at 441 nm, and emission was detected at the wavelength of 528 nm.

Statistical analysis

All values are expressed as means ± SEM. The data were compared by Student's *t*-test for unpaired variance and one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. $p < 0.05$ was considered statistically significant.

Results

Bach1 deficiency ameliorated indomethacin-induced intestinal injury

Indomethacin administration induced multiple erosions in the small intestine. The extent of the injury induced by indomethacin was significantly inhibited in Bach1-deficient mice compared to wild-type mice (Figure 1A). Hematoxylin-eosin staining of intestinal mucosa showed defects in the villi, ulceration with destruction and necrosis of the epithelium and infiltration of inflammatory cells in the wild-type mice. In Bach1-deficient mice, however, the number

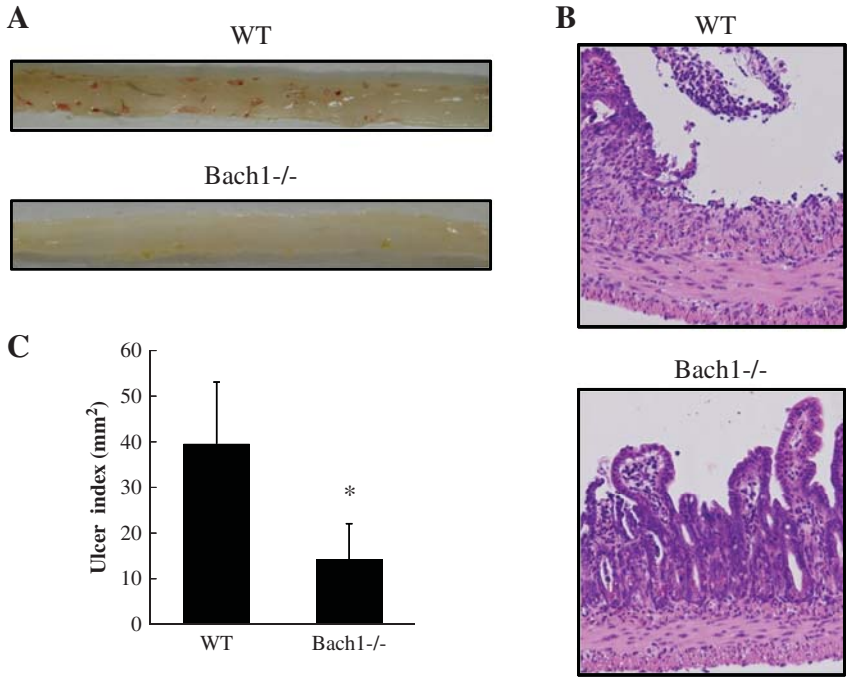

Figure 1. (A) Macroscopic findings of the small intestine in mice treated with indomethacin. Indomethacin administration induced multiple ulcers and erosions in the small intestine in the wild-type mice. On the other hand, in the Bach1-deficient mice, the number and severity of the lesions were clearly ameliorated. (B) Findings of histological examination of the small intestine of the indomethacin-treated mice (100×). (C) Ulcer index of Bach1-deficient and wild-type mice treated with indomethacin. Data were compared by Student's *t*-test for unpaired variance and expressed as mean ± SEM of seven mice. $^{*}p < 0.05$ compared to wild-type mice.

of erosions and inflammatory changes were clearly diminished (Figure 1B). Ulcer index was also significantly lower in Bach1-deficient mice as compared with wild-type mice (Figure 1C).

Expression of HO-1 mRNA and protein in small intestinal mucosa

From the real-time PCR results, indomethacin significantly increased HO-1 mRNA expression in the small intestinal mucosa of Bach1-deficient mice compared to the wild-type mice (Figure 2A). Because the expression of HO-1 mRNA in intestinal mucosa of indomethacin-treated mice increased, we also evaluated HO-1 protein expression by using western blotting. Similar to the observed HO-1 mRNA expression, HO-1 expression was faint in the wild-type mice. On the other hand, in Bach1-deficient mice, HO-1 expression was remarkably increased, especially after indomethacin administration (Figure 2B).

Localization of HO-1-immunopositive cells in intestinal mucosa

To investigate HO-1 expression in the small intestine, we performed HO-1 immunohistochemical staining of the small intestinal mucosa (Figure 3). After indomethacin administration, HO-1-immunopositivity was stronger in Bach1-deficient mice than in wild-type mice. In the Bach1-deficient mice, many more HO-1-positive mononuclear cells with smaller erosions were observed in the intestinal mucosa. To precisely investigate the HO-1-immunopositive cells, we also performed immunofluorescence staining of the intestinal mucosa. HO-1 was immunostained with an anti-monoclonal antibody, followed by Alexa Fluor 594-conjugated anti-mouse antibody. Macrophages were stained with anti-F4/80, followed by Alexa Fluor 488-conjugated secondary antibodies. As shown in Figure 4, the merged images show that the HO-1-immunopositive cells accumulated in the intestinal tissue were confirmed to be mainly F4/80-positive macrophages.

Expression of TNF-α in small intestinal mucosa

To investigate the mechanism of indomethacin-induced small intestinal injury in Bach1 deficient mice, we evaluated the expression of TNF-α in small intestinal mucosa using immunosorbent assay as well as real time PCR (Figure 5). Administration of indomethacin induced the intestinal production of TNF-α in wild type mice, but in Bach1-deficient mice the increased production of TNF-α was significantly inhibited.

HO activity assay using HO inhibitor

We also performed HO activity assay using the HO inhibitor tin protoporphyrin (SnPP) to confirm the

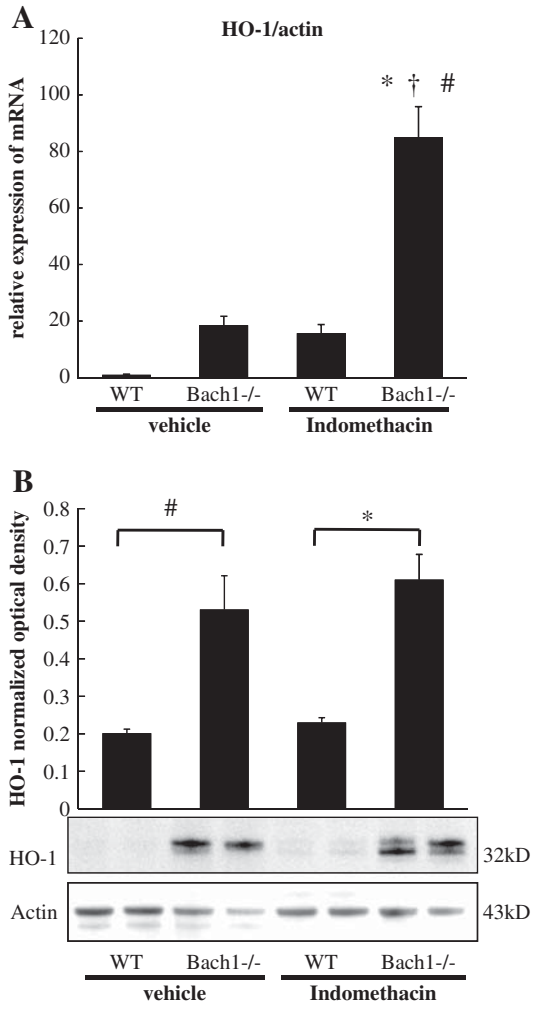

Figure 2. (A) Expression of HO-1 mRNA in small intestinal mucosal tissue determined by real-time polymerase chain reaction (PCR). The data were compared by ANOVA followed by Bonferroni's Multiple Comparison Test. Each value indicates the mean ± SEM for six mice. (B) The levels of HO-1 protein in the small intestinal mucosa were determined using western blotting. The densitometric data are shown as mean ± SEM for six mice. Results are normalized to β-actin. The data were compared by ANOVA followed by Bonferroni's Multiple Comparison Test. A representative western blotting is shown in the lower panel. Each treatment condition is represented by two lanes. $^{*}p < 0.05$ compared to the wild type mice treated with indomethacin. †$p < 0.05$ compared to the Bach1-deficient mice, vehicle. #$p < 0.05$ compared to the wild-type mice, vehicle.

protective actions of HO-1 in Bach1-deficient mice with intestinal mucosal injury. As shown in Figure 6A, the inhibitory effect on the ulcer index in the case of the Bach1-deficient mice against indomethacin-induced injury was significantly reversed by the administration of SnPP. HO activity in the tissue after the indomethacin treatment, determined by monitoring the levels of bilirubin, was significantly increased in the Bach1-deficient mice compared to the wild-type mice and this increase was significantly inhibited by SnPP (Figure 6B).

TUNEL staining of intestinal mucosa

TUNEL staining was performed to determine the location and number of the apoptotic cells in tissue subjected to indomethacin-induced intestinal injury (Figure 7). Few TUNEL-positive cells were detected in the intestinal epithelium of the control mice. Indomethacin markedly increased the number of TUNEL-positive cells on the top of the villi in the case of the wild-type mice, with destruction of the normal intestinal structure. However, the number of apoptotic cells and mucosal damage were significantly attenuated in Bach1-deficient mice (Figure 8). Furthermore the administration of Snpp to Bach1-deficient mice increased the number of apoptotic cells in Bach1 deficient mice (Figure 9A).

Expression of caspase-3 in small intestinal mucosa

We used an active caspase-3-specific antibody and analysed the expression of caspase-3 in the purified cytosolic fractions of mucosal homogenates. The results of western blotting showed that caspase-3 activation induced by indomethacin in wild-type mice was suppressed in the Bach1-deficient mice (Figure 10) and administration of Snpp counteracted this suppressive effect (Figure 9B). These results suggest that the suppression of caspase-3 activation in Bach1-deficient mice is involved in the HO-1-mediated mechanism in the case of indomethacin-induced small intestinal injury.

Discussion

This study provides concrete evidence for the involvement of HO-1 in the protection of the small intestine from indomethacin-induced injury and epithelial cell apoptosis. Firstly, Bach1 deficiency significantly attenuated indomethacin-induced injury in mice, and this was confirmed by the ulcer index and histological examination. Second, we showed the up-regulation of HO-1 mRNA and proteins in the small intestine and HO-1 localization in tissue macrophages and showed that HO-1 expression in Bach1-deficient mice contributed, at least in part, to the suppression of intestinal mucosal injury. Finally, our results suggest the involvement of apoptosis in the pathogenesis of indomethacin-induced small intestinal injury and the correlation between the suppression of apoptosis and the cytoprotective properties in Bach1-decificient mice.

We previously reported that the number of HO-1-immunopositive mononuclear cells increased after indomethacin administration and that the extent of this induction in the small intestinal mucosa was significantly higher in Bach1-deficient mice [20]. In another study that we previously performed, HO-1 was localized in the macrophages in inflamed colonic mucosa in patients with active ulcerative colitis [23]

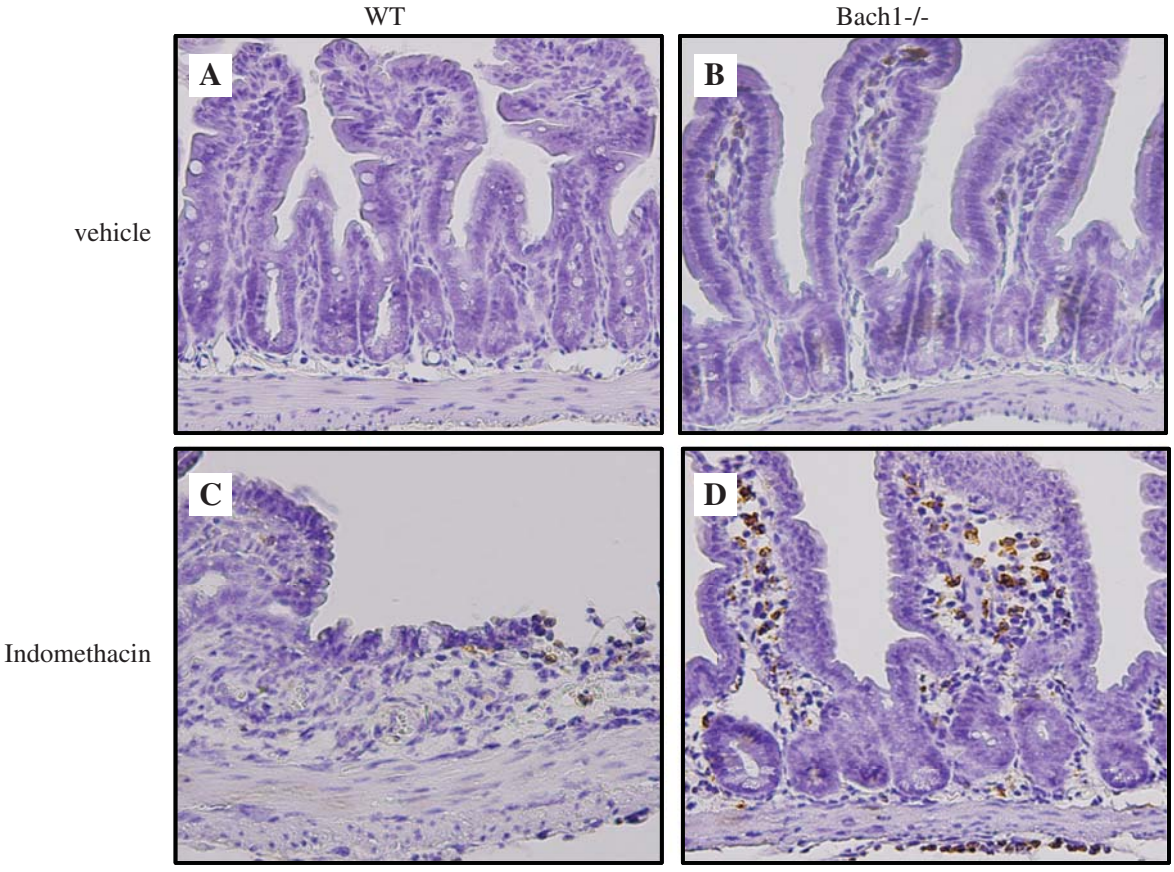

Figure 3. Results of immunohistochemical analysis of the small intestine for HO-1 (200×). (A) Wild-type, vehicle. (B) Bach1-deficient, vehicle. (C) Indomethacin-induced ulceration with slight induction of HO-1 expression. (D) Deficiency of Bach1 reduced mucosal erosions showing higher HO-1-immunopositivity.

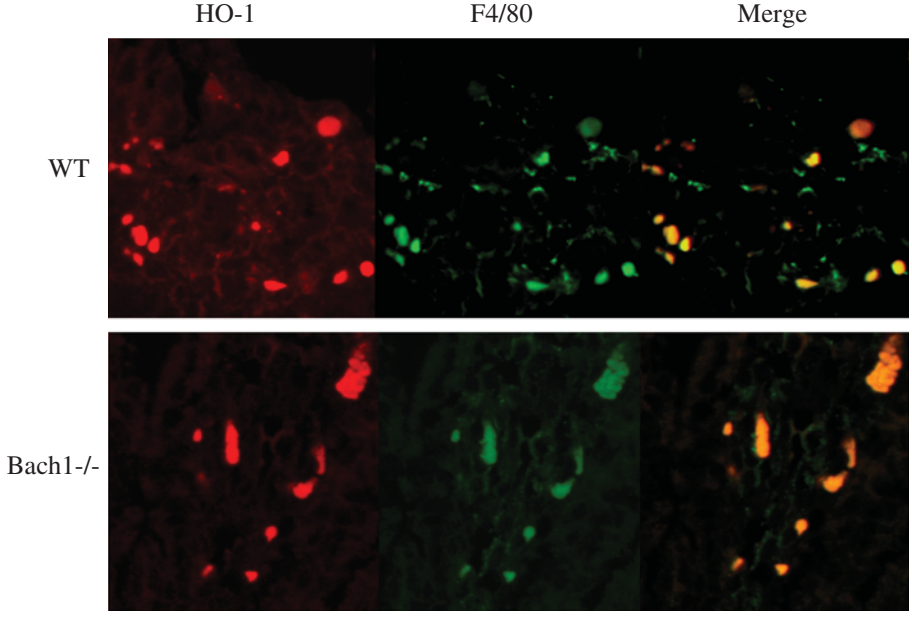

Figure 4. Results of immunofluorescence staining of the intestinal mucosa treated with indomethacin. HO-1 and F4/80 were visualized under a laser scanning confocal microscope using secondary antibody anti-rabbit Alexa 594 and anti-rat Alexa 488, respectively (200×). The left row shows the HO-1-stained images; the middle row shows the F4/80-stained images; and the right row shows the merged left and middle images. A representative image of the three independent experiments is shown.

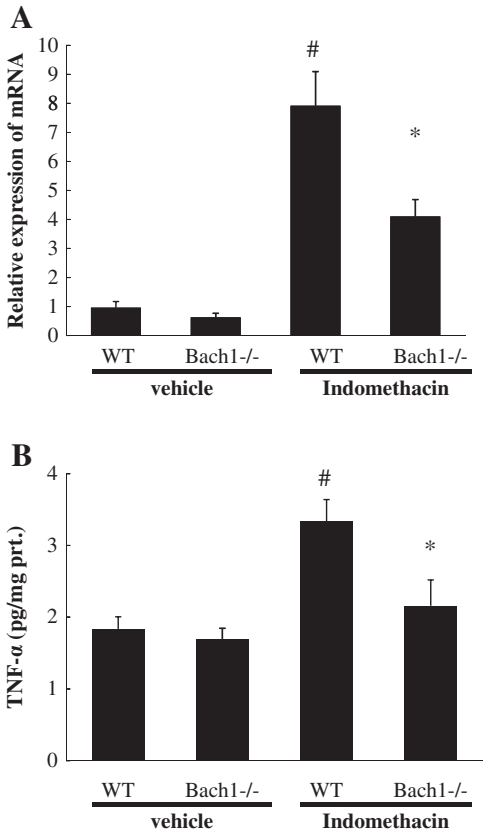

Figure 5. (A) Expression of TNF-α mRNA in small intestinal mucosal tissue determined by real-time PCR. (B) The result of immunosorbant assay for mouse TNF-α in the supernatant of small intestinal mucosal homogenates. These data were compared by ANOVA followed by Bonferroni's Multiple Comparison Test. Each value indicates the mean ± SEM for six mice. $^{*}p < 0.05$ compared to the wild-type mice treated with indomethacin. #$p < 0.05$ compared to the wild-type mice, vehicle.

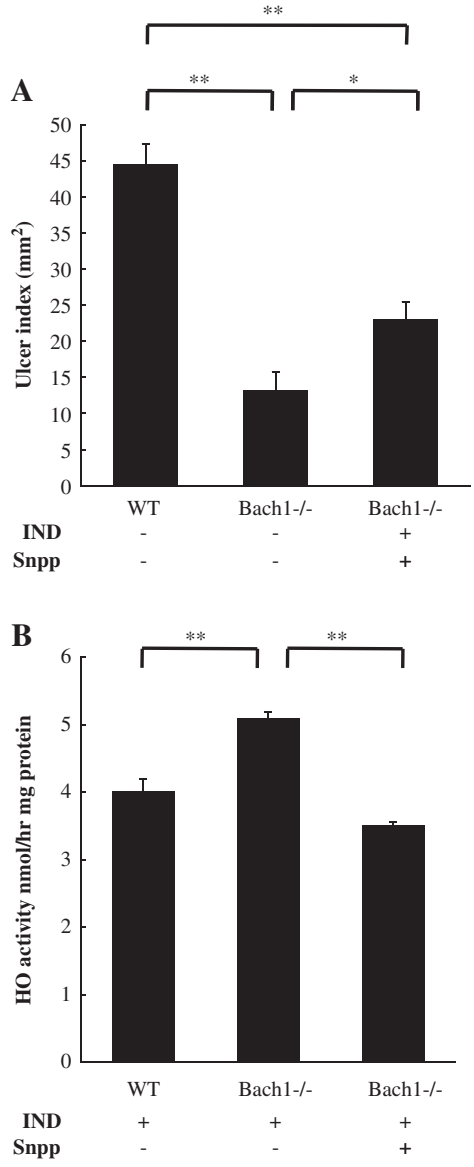

Figure 6. Comparison between the ulcer index (A) and the result of the HO activity assay (B) of the small intestine using an HO inhibitor, tin protoporphyrin. Data were compared by ANOVA followed by Bonferroni's Multiple Comparison Test. Each value indicates the mean ± SEM for six mice ($^{**}p < 0.001$, $^{*}p < 0.05$).

and this finding is similar to the findings of the immunohistochemical staining of intestinal mucosa in the present study. As shown in Figure 4, HO-1-immunopositive cells were found to be mainly F4/80-positive macrophages in the mucosa and submucosa. These data suggest that HO-1-positive macrophages may play a role in the suppression of indomethacin-induced intestinal injury.

Findings of recent studies support that HO-1 has important functions in the inflammatory response of the gastrointestinal tract and these results suggest the involvement of HO-1 in the intestinal mucosal defence system [23–26]; however, the function of Bach1 in gastrointestinal diseases has not been completely elucidated. Concerning the studies using Bach1-deficient mice, one study proved that the extent of myocardial infarction was suppressed by the HO-1 [17]. On the other hand, Bach1 deficiency ameliorated hyperoxic lung injury; however, this was not attributable to the effect of HO-1 but due to IL-6 up-regulation [16]. To investigate the function of HO-1 in the suppression of indomethacin-induced intestinal injury, we examined the effects of SnPP, an HO inhibitor, on ulcer index and HO activity in indomethacin-treated mice. The tissue activity of HO after the indomethacin treatment significantly increased in the Bach1-deficient mice and this increase was significantly inhibited by SnPP to the levels observed in the case of wild-type mice. However, the inhibitory effect on the ulcer index observed in the case of Bach1-deficient mice against indomethacin-induced injury was in part reversed by the administration of SnPP.

Although it is certain that HO-1 plays an essential role in the suppression of indomethacin-induced small intestinal injury, these results indicate that additional factors are involved in the cascade of the Bach1-deficiency-mediated anti-inflammatory response in the small intestine. Bach1 has also recently been

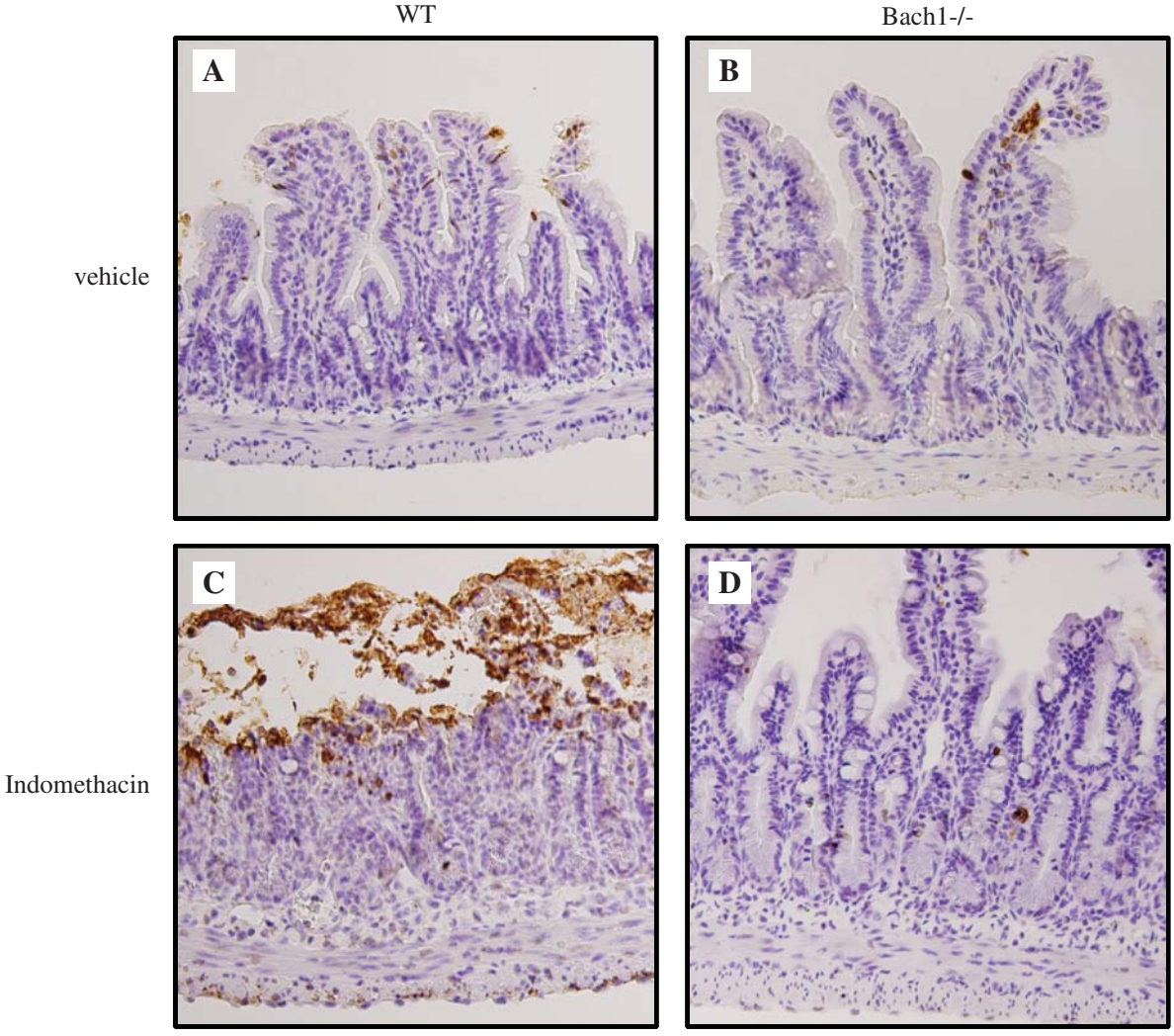

Figure 7. Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) assay. Representative fields are shown (200×). (A) Wild-type, vehicle. (B) Bach1-deficient, vehicle. (C) Wild-type, treated with indomethacin. (D) Bach1-deficient, treated with indomethacin.

reported to inhibit oxidative stress-induced cellular senescence by impeding the function of p53 [27], but it is difficult to reasonably explain that Bach1 deficiency could ameliorate acute intestinal inflammation without the effect of HO-1.

The administration of NSAIDs has been found to induce apoptosis of intestinal epithelial cells. In general, several mechanisms have been proposed for NSAIDs-induced apoptosis, which include COX-dependent and COX-independent mechanisms. Regarding COX-2-inhibitor-induced apoptosis, some researchers have suggested that decreased cellular PGE2 and increased arachidonic acid might be involved in the induction of apoptosis [28] and that the increased cellular concentration of arachidonic acid alters mitochondrial membrane permeability and releases cytochrome c, leading to apoptosis [29,30]. On the other hand, several COX-independent mechanisms have been reported, including the down-regulation of nuclear factor-kappa B (NF-kB) activity,

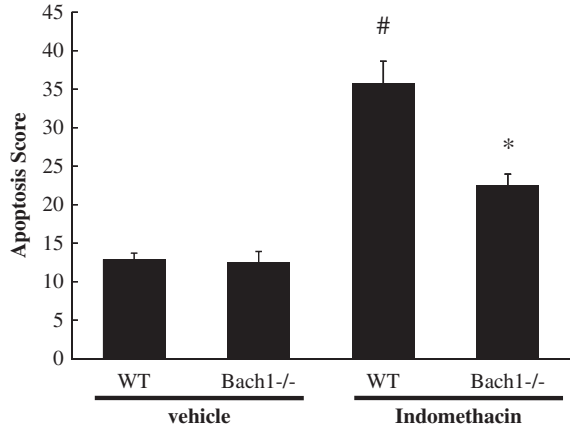

Figure 8. The apoptotic score was calculated by counting 10 randomly selected villi and crypts in the sections after the TUNEL staining. The score represents the means of TUNEL-positive cells. Data were compared by ANOVA followed by Bonferroni's Multiple Comparison Test. Each value indicates the mean ± SEM for three mice. $^{*}p < 0.05$ compared to the wild-type mice treated with indomethacin. $\#p < 0.05$ compared to the wild-type mice, vehicle.

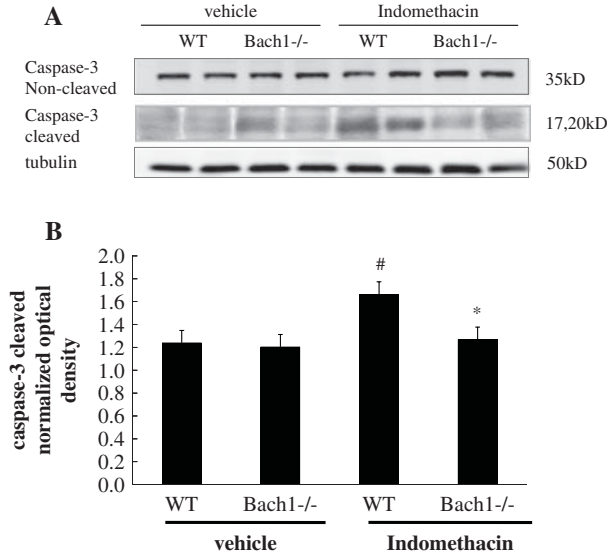

Figure 9. (A) The apoptotic score was calculated by counting 10 randomly selected villi and crypts in the sections after the TUNEL staining. The score represents the means of TUNEL-positive cells. Data were compared by Student's *t*-test for unpaired variance and each value indicates the mean ± SEM for three mice. $^{*}p < 0.05$ compared to Bach1-deficient mice treated with indomethacin only. (B) Activation of caspase-3 in Bach1-deficient mice treated with indomethacin was analysed using an anti-active caspase-3 antibody in the cytosolic fractions of mucosal homogenates by western blotting. Each treatment condition is represented by two lanes.

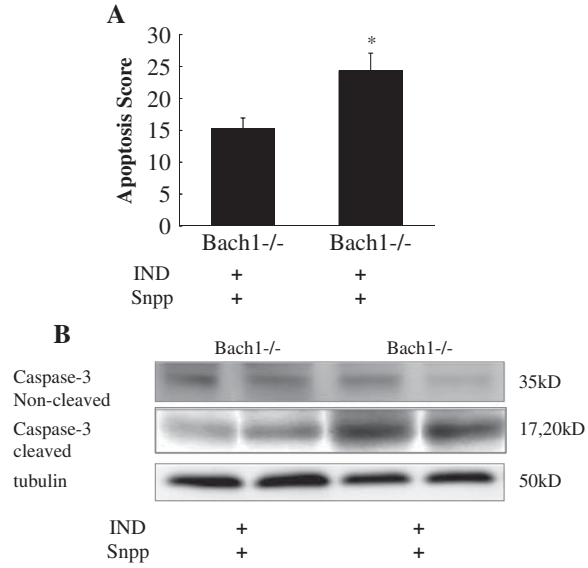

Figure 10. (A) Activation of caspase-3 was analysed using an anti-active caspase-3 antibody in the cytosolic fractions of mucosal homogenates by western blotting. Each treatment condition is represented by two lanes. (B) The densitometric data of western blotting for cleaved caspase-3. Data were compared by ANOVA followed by Bonferroni's Multiple Comparison Test and each value indicates the mean ± SEM of six mice. $^{*}p < 0.05$ compared to the wild type mice treated with indomethacin. $^{\#}p < 0.05$ compared to the wild-type mice, vehicle.

alteration in the levels of pro- and anti-apoptotic proteins and activation of extrinsic and intrinsic pathways of apoptosis [31–33]. We have also focused on the relationship between gastrointestinal mucosal injury and NSAIDs-induced apoptosis [34,35].

From the results of this study, we concluded that regulation of HO-1 by Bach1 was involved in the resistance to indomethacin-induced apoptosis in the Bach1-deficient mice with indomethacin-induced small intestinal injury. The results of the TUNEL assay proved that Bach1 deficiency reduced the number of apoptotic cells in the intestinal epithelium. The results of western blot analysis showed that cleaved caspase-3 expression in indomethacin-treated intestinal mucosa was significantly suppressed in Bach1-deficient mice compared to the wild-type mice. We also showed that the production of TNF-α which plays an important role in indomethacin-induced apoptosis in the small intestine [36], was markedly suppressed in Bach1-deficient mice after indomethacin administration. Furthermore, the administration of Snpp significantly reduced not only the number of apoptotic cells, but also inhibited activation of caspase-3. Several studies also support the anti-apoptotic property of HO-1 [37,38]. These results suggest that over-expressed HO-1 enhances protection against apoptosis. However, it is still unknown whether Bach1 has several important functions in addition to repressing HO-1 activity, because HO-1 must be induced in Bach1-deficient mice.

In conclusion, our data indicate that Bach1 deficiency ameliorated small intestinal injuries through, at least partly, increased HO-1 expression. Although further studies are warranted to elucidate the underlying mechanisms, inhibition of Bach1 can be a novel therapeutic strategy to treat patients with indomethacin-induced small intestinal injury.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (B) to T.Y. (no. 21390184) and (C) to Y.N. (no. 22590705) from the Japan Society for the Promotion of Science; by a City Area Program to T.Y. and Y.N. from the Ministry of Education, Culture, Sports, Science and Technology, Japan; and by an Adaptable and Seamless Technology Transfer Program through target-driven R&D to Y.N. from the Japan Science and Technology Agency.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Iddan G, Meron G, Glukhovsky A, Swain P. Wireless capsule endoscopy. Nature 2000;405:417–418.

[2] Yamamoto H, Sekine Y, Sato Y, Higashizawa T, Miyata T, Iino S, Ido K, Sugano K. Total enteroscopy with a nonsurgical steerable double-balloon method. Gastrointest Endoscopy 2001;53:216–220.
[3] Goldstein JL, Eisen GM, Lewis B, Gralnek IM, Zlotnick S, Fort JG. Video capsule endoscopy to prospectively assess small bowel injury with celecoxib, naproxen plus omeprazole, and placebo. Clin Gastroenterol Hepatol 2005;3: 133–141.
[4] Maiden L, Thjodleifsson B, Theodors A, Gonzalez J, Bjarnason I. A quantitative analysis of NSAID-induced small bowel pathology by capsule enteroscopy. Gastroenterology 2005; 128:1172–1178.
[5] Whittle BJR. Temporal relationship between cyclooxygenase inhibition, as measured by prostacyclin biosynthesis, and the gastrointestinal damage induced by indomethacin in the rat. Gastroenterology 1981;80:94–98.
[6] Whittle BJR, Laszlo F, Evans SM, Moncada S. Induction of nitric oxide synthase and microvascular injury in the rat jejunum provoked by indomethacin. Br J Pharmacol 1995; 116:2286–2290.
[7] Weissenborn U, Maedge S, Buettner D, Sewing KF. Indometacin-induced gastrointestinal lesions in relation to tissue concentration, food intake and bacterial invasion of the rat. Pharmacology 1985;30:32–39.
[8] Redfern JS, Blair Iii AJ, Lee E, Feldman M. Gastrointestinal ulcer formation in rabbits immunized with prostaglandin E2. Gastroenterology 1987;93:744–752.
[9] Yamada T, Deitch E, Specian RD, Perry MA, Balfour Sartor R, Grisham MB. Mechanisms of acute and chronic intestinal inflammation induced by indomethacin. Inflammation 1993; 17:641–662.
[10] Basivireddy J, Jacob M, Ramamoorthy P, Pulimood AB, Balasubramanian KA. Indomethacin-induced free radical-mediated changes in the intestinal brush border membranes. Biochem Pharmacol 2003;65:683–695.
[11] Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA 1997; 94:10925–10930.
[12] Lin Q, Weis S, Yang G, Weng YH, Helston R, Rish K, Smith A, Bordner J, Polte T, Gaunitz F, Dennery PA. Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. J Biol Chem 2007; 282:20621–20633.
[13] Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, Hayashi N, Yamamoto M, Shibahara S, Fujita H, Igarashi K. Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. EMBO J 2001;20:2835–2843.
[14] Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, Tashiro S, Takahashi S, Shibahara S, Alam J, et al. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. EMBO J 2002;21:5216–5224.
[15] Suzuki H, Tashiro S, Hira S, Sun J, Yamazaki C, Zenke Y, Ikeda-Saito M, Yoshida M, Igarashi K. Heme regulates gene expression by triggering Crm1-dependent nuclear export of Bach1. EMBO J 2004;23:2544–2553.
[16] Tanimoto T, Hattori N, Senoo T, Furonaka M, Ishikawa N, Fujitaka K, Haruta Y, Yokoyama A, Igarashi K, Kohno N. Genetic ablation of the Bach1 gene reduces hyperoxic lung injury in mice: role of IL-6. Free Radic Biol Med 2009; 46:1119–1126.
[17] Yano Y, Ozono R, Oishi Y, Kambe M, Yoshizumi M, Ishida T, Omura S, Oshima T, Igarashi K. Genetic ablation of the transcription repressor Bach1 leads to myocardial protection against ischemia/reperfusion in mice. Genes Cells 2006;11: 791–803.
[18] Kanno H, Ozawa H, Dohi Y, Sekiguchi A, Igarashi K, Itoi E. Genetic ablation of transcription repressor Bach1 reduces neural tissue damage and improves locomotor function after spinal cord injury in mice. J Neurotrauma 2009;26:31–39.
[19] Iida A, Inagaki K, Miyazaki A, Yonemori F, Ito E, Igarashi K. Bach1 deficiency ameliorates hepatic injury in a mouse model. Tohoku J Exp Med 2009;217:223–229.
[20] Harusato A, Naito Y, Takagi T, Yamada S, Mizushima K, Hirai Y, Horie R, Inoue K, Fukumoto K, Hirata I, et al. Inhibition of Bach1 ameliorates indomethacin-induced intestinal injury in mice. J Physiol Pharmacol 2009;60(Suppl 7):149–154.
[21] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–275.
[22] Klemz R, Mashreghi MF, Spies C, Volk HD, Kotsch K. Amplifying the fluorescence of bilirubin enables the real-time detection of heme oxygenase activity. Free Radic Biol Med 2009;46:305–311.
[23] Takagi T, Naito Y, Mizushima K, Nukigi Y, Okada H, Suzuki T, Hirata I, Omatsu T, Okayama T, Handa O, Kokura S, Ichikawa H, Yoshikawa T. Increased intestinal expression of heme oxygenase-1 and its localization in patients with ulcerative colitis. J Gastroenterol Hepatol 2008;23(Supppl 2):S229–S233.
[24] Yoda Y, Amagase K, Kato S, Tokioka S, Murano M, Kakimoto K, Nishio H, Umegaki E, Takeuchi K, Higuchi K. Prevention by lansoprazole, a proton pump inhibitor, of indomethacin -induced small intestinal ulceration in rats through induction of heme oxygenase-1. J Physiol Pharmacol 2010;61:287–294.
[25] Sakamoto N, Kokura S, Okuda T, Hattori T, Katada K, Isozaki Y, Nakabe N, Handa O, Takagi T, Ishikawa T, Naito Y, Yoshida N, Yoshikawa T. Heme oxygenase-1 (Hsp32) is involved in the protection of small intestine by whole body mild hyperthermia from ischemia/reperfusion injury in rat. Int J Hyperther 2005;21:603–614.
[26] Naito Y, Takagi T, Yoshikawa T. Heme oxygenase-1: a new therapeutic target for inflammatory bowel disease. Aliment Pharmacol Ther Suppl 2004;20:177–184.
[27] Dohi Y, Ikura T, Hoshikawa Y, Katoh Y, Ota K, Nakanome A, Muto A, Omura S, Ohta T, Ito A, Yoshida M, Noda T, Igarashi K. Bach1 inhibits oxidative stress-induced cellular senescence by impeding p53 function on chromatin. Nat Struct Mol Biol 2008;15:1246–1254.
[28] Chan TA, Morin PJ, Vogelstein B, Kinzler KW. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. Proc Natl Acad Sci USA 1998;95:681–686.
[29] Cao Y, Pearman AT, Zimmerman GA, McIntyre TM, Prescott SM. Intracellular unesterified arachidonic acid signals apoptosis. Proc Natl Acad Sci USA 2000;97: 11280–11285.
[30] Scorrano L, Penzo D, Petronilli V, Pagano F, Bernardi P. Arachidonic acid causes cell death through the mitochondrial permeability transition. Implications for tumor necrosis factor-alpha aopototic signaling. J Biol Chem 2001;276: 12035–12040.
[31] Wong BC, Jiang X, Fan XM, Lin MC, Jiang SH, Lam SK, Kung HF. Suppression of RelA/p65 nuclear translocation independent of IkappaB-alpha degradation by cyclooxygenase-2 inhibitor in gastric cancer. Oncogene 2003;22:1189–1197.
[32] Redlak MJ, Power JJ, Miller TA. Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 2005;289:731–738.
[33] Gu Q, Wang JD, Xia HH, Lin MC, He H, Zou B, Tu SP, Yang Y, Liu XG, Lam SK, Wong WM, Chan AO, Yuen MF, Kung HF, Wong BC. Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer. Carcinogenesis 2005;26:541–546.
[34] Omatsu T, Naito Y, Handa O, Mizushima K, Hayashi N, Qin Y, Harusato A, Hirata I, Kishimoto E, Okada H, Uchiyama K, Ishikawa T, Takagi T, Yagi N, Kokura S, Ichikawa H,

Yoshikawa T. Reactive oxygen species-quenching and anti-apoptotic effect of polaprezinc on indomethacin-induced small intestinal epithelial cell injury. J Gastroenterol 2010; 45:692–702.

[35] Hirata I, Naito Y, Handa O, Hayashi N, Mizushima K, Adachi S, Omatsu T, Okayama T, Kishimoto E, Ichikawa H, Takagi T, Kokura S, Otaka M, Yoshikawa T. Heat-shock protein 70-overexpressing gastric epithelial cells are resistant to indomethacin-induced apoptosis. Digestion 2009;79:243–250.

[36] Fukumoto K, Naito Y, Takagi T, Yamada S, Horie R, Inoue K, Harusato A, Hirata I, Omatsu T, Mizushima K, Hirai Y, Yoshida N, Uchiyama K, Ichikawa T, Handa O, Konishi H, Wakabayashi N, Yagi N, Kokura S, Ichikawa H, Kita M, Yoshikawa T. Role of tumor necrosis factor-alpha in the pathogenesis of indomethacin-induced small intestinal injury in mice. Int J Mol Med 2011;27:353–359.

[37] Aburaya M, Tanaka K, Hoshino T, Tsutsumi S, Suzuki K, Makise M, Akagi R, Mizushima T. Heme oxygenase-1 protects gastric mucosal cells against non-steroidal anti-inflammatory drugs. J Biol Chem 2006;281:33422–33432.

[38] Yeh CH, Chen TP, Wang YC, Lin YM, Lin PJ. HO-1 activation can attenuate cardiomyocytic apoptosis via inhibition of NF-kappaB and AP-1 translocation following cardiac global ischemia and reperfusion. J Surg Res 2009;155:147–156.

This paper was first published online on Early Online on 18 April 2011.