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Protective Effects of Acetaminophen on Ibuprofen-Induced Gastric Mucosal Damage in Rats with Associated Suppression of Matrix Metalloproteinase

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Acetaminophen reduces ibuprofen-induced gastric damage

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APAP, acetaminophen, N-(4-hydroxyphenyl) acetamide; CL-82198, N-[4-(4-Morpholinyl)

butyl]-2-benzofurancarboxamide; COX, cyclooxygenase; IBP, ibuprofen, 2-[4-(2-methylpropyl) phenyl] propanoic acid; IPA, Ingenuity Pathway Analysis; MMP, matrix metalloproteinase; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are known to cause gastric mucosal damage as a side effect. Acetaminophen, widely used as an analgesic and anti-pyretic drug, has gastroprotective effects against gastric lesions induced by absolute ethanol and certain NSAIDs. However, the mechanisms that underlie the gastroprotective effects of acetaminophen have not yet been clarified. In the present study, we examined the role and protective mechanism of acetaminophen on ibuprofen-induced gastric damage in rats. Ibuprofen and acetaminophen were administered orally, and the gastric mucosa was macroscopically examined 4 h later. Acetaminophen decreased ibuprofen-induced gastric damage in a dose-dependent manner. To investigate the mechanisms involved, transcriptome analyses of the ibuprofen-damaged gastric mucosa were performed in the presence and absence of acetaminophen. Ingenuity Pathway Analysis (IPA) revealed that acetaminophen suppressed the pathways related to cellular assembly and inflammation, whereas they were highly activated by ibuprofen. Based on gene classifications from the IPA Knowledge Base, we identified the following 5 genes that were related to gastric damage and showed significant changes in gene expression, i.e., IL-1β, CCL2, MMP-10, MMP-13, and FOS. Expression of these salient genes was confirmed using real-time PCR. The expression of MMP-13 was the most reactive to the treatments, showing strong induction by ibuprofen and suppression by acetaminophen. Moreover, MMP-13 inhibitors decreased ibuprofen-induced gastric damage. In conclusion, these results suggest that acetaminophen decreases ibuprofen-induced gastric mucosal damage and that the suppression of MMP-13 may play an

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important role in the gastroprotective effects of acetaminophen.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen (IBP), loxoprofen, and aspirin have been widely used for treatment of acute and chronic pain. The analgesic mechanism of NSAIDs involves inhibition of cyclooxygenase (COX) and suppression of prostaglandin production. However, it has been documented that NSAIDs cause gastrointestinal damages as adverse effects; the annual incidence of upper gastrointestinal bleeding has been reported to range from 43 to 140 per 100,000 people (Hernandez-Diaz and Rodriguez, 2002).

One of the important factors in gastric damage induced by NSAIDs is endogenous prostaglandin (PG) deficiency caused by inhibition of COX-1 and COX-2 (Takeuchi, 2012; Okada et al., 1989). PGs protect the gastric mucosa against necrosis induced by NSAIDs or ethanol (Takeuchi, 2012; Okada et al., 1989). COX-1 is constitutively expressed in various tissues, including the stomach, as a housekeeping protein, whereas COX-2 is expressed in response to cytokines in most tissues under pathological conditions such as inflammation (O'Neill and Ford-Hutchinson, 1993). Studies using selective COX-1 or COX-2 inhibitors indicated that the gastric ulcerogenic properties of NSAIDs are caused by inhibition of both COX-1 and COX-2 (Wallace et al., 2000; Tanaka et al., 2001). However, several reports suggest that other elements such as free radicals, disturbances of microcirculation, and hypermotility may be involved in the pathogenic mechanisms (Takeuchi, 2012; Naito and Yoshikawa, 2006; Funatsu et al., 2007). Further, it has been reported that other risk factors, including diabetes and concomitant use of other agents, aggravate gastric lesions induced by

NSAIDs (Singh et al., 2011; Takeuchi et al., 2011; Kwiecien et al., 2012). Taken together, although PG deficiencies due to inhibition of COX-1 and COX-2 play important roles in gastric side effects, the mechanisms by which NSAIDs induce gastric damage remain elusive.

Acetaminophen (APAP) is another analgesic and anti-pyretic drug. However, unlike NSAIDs, it does not cause gastric mucosal damage (Lanza et al., 1998; Yoon et al., 2012). Although the analgesic mechanism of APAP remains unclear and requires further elucidation, it is suggested that APAP may act centrally and be a weak inhibitor of PG synthesis (Anderson, 2008).

APAP reportedly reduces gastric lesions induced by absolute ethanol, acidic aspirin, IBP, and indomethacin in rats (Stern et al., 1984; Konturek et al., 1982; Seegers et al., 1980; Van Kolfschoten et al., 1983). However, the effects on IBP-induced gastric damage were limited because the suppression by APAP was examined in a fixed dose (Van Kolfschoten et al., 1983). Various theories have been proposed to explain the gastroprotective effects of APAP, e.g., activation of PG synthesis and scavenging of free radicals (van Kolfschoten et al., 1981). However, the precise mode of action has not yet been completely elucidated.

In the present study, we aimed to examine the protective effects of APAP on IBP-induced gastric mucosal damage using various APAP doses. In addition, transcriptome analyses were performed on the IBP-damaged gastric mucosa in the presence and absence of APAP. The gene expression profiles were analyzed using Ingenuity Pathway Analysis (IPA) software to assess the involvement of gene expression changes in gastric protection by APAP. To the best of our knowledge, this is the first study to show gene expression profiling following oral

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administration of NSAIDs and APAP in rats.

Materials and Method

Reagents

IBP (Shiratori pharmaceutical, Chiba, Japan), APAP (Iwaki pharmaceutical, Tokyo, Japan), and the matrix metalloproteinase-13 (MMP-13) inhibitor N-[4-(4-Morpholinyl)butyl]-2-benzofurancarboxamide (CL-82198; Sigma, USA) were purchased from each manufacture.

Animals

Male Sprague-Dawley rats (6 weeks old) were purchased from Charles River Japan、Kanagawa, Japan. The animals were fed standard rat chow and tap water *ad libitum*. They were housed in stainless steel cages with wire bottoms and maintained on a 12-h light/12-h dark cycle with the temperature and relative humidity of the animal room controlled at 21°C–23°C and 40%–60%, respectively. All experimental procedures were carried out in accordance with the Declaration of Helsinki and approved by the Animal Care and Use Committee of LION Corporation.

Induction of gastric mucosal damage by IBP

Gastric mucosal damage was induced by oral administration of 200 mg/kg IBP suspended in a 5% gum arabic solution. Rats were divided into the following treatment groups (n = 9-10 individuals per group): 1) 200 mg/kg IBP; 2) 200 mg/kg IBP and 100 mg/kg APAP; 3) 200 mg/kg IBP and 125 mg/kg APAP; and 4) 200 mg/kg IBP and 150 mg/kg APAP; 5) 200 mg/kg

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IBP and 175 mg/kg APAP; 6) 200 mg/kg IBP and 200 mg/kg APAP; and 7) 200 mg/kg IBP and 400 mg/kg APAP. IBP and APA were administered at the same time in the groups 2)-7). Prior to the experiments, the animals were deprived of food overnight. They were euthanized 4 h post-drug administration under isoflurane anesthesia, and the gastric tissues were collected.

Measurement of gastric damage

The stomachs were removed, inflated by injecting 10 ml of saline, immersed in 1% formalin for 1 h to fix the gastric tissue, and opened along the greater curvature. Subsequently, the lengths of hemorrhagic lesions were measured using a dissecting microscope with a ruler. The sum of the lesion lengths per stomach was used as a score of damage (Wallace et al., 1993).

Histology

The gastric mucosa was examined with a microscope after the administration of IBP 200 mg/kg with or without APAP 200 mg/kg. The animals were euthanized 4h after the drug administration under isoflurane anesthesia, and the stomachs were excised, immersed in 10% neutralized formalin and embedded in paraffin. The sections (8 μm) were cut using microtome and stained with hematoxylin and eosin.

RNA extraction and microarray analysis

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Rats were divided into the following three treatment groups: 1) 200 mg/kg IBP (n = 5); 2) 200 mg/kg IBP and 200 mg/kg APAP (n = 5); and 3) 5% gum arabic solution (control; n = 5). Prior to the experiments, the animals were deprived of food overnight. They were euthanized 2 h or 4 h post-drug administration under isoflurane anesthesia, and the stomachs were removed and incised along the greater curvature. The corpus mucosa was scraped using two glass slides and the samples were stored in RNAlater (Ambion, Austin, TX, USA) until RNA extraction. Total RNA was extracted from each sample using a QIAGEN RNeasy kit (Valencia, CA) according to the manufacturer's protocol. Subsequently, total RNA from 5 samples was pooled for each group. Microarray analysis was performed using a SurePrint G3 Rat GE 8x60K Microarray (Agilent Inc., Santa Clara, CA) at the TAKARA Bio Co., Ltd. A total of 100 ng of RNA was labeled using the fluorescent probe Cy3 and 0.6 μg of the labeled RNA was used for hybridization. The cDNA microarray was scanned using an Agilent DNA Microarray Scanner (G2565CA) and images were analyzed using Agilent Feature Extraction Software v10.7 for background subtraction, normalization, and assessment of quality control. Signal evaluation was also performed with the Agilent Feature Extraction Software v10.7 to assess the reliability of the transcripts of each spot using the following three levels: 0, not detected; 1, detected but insufficient for analysis; 2, detected sufficiently for analysis. Subsequently, fold changes in mRNA expression were calculated relative to control samples.

Ingenuity pathway analysis

Probes were excluded from analysis of gene expression using IPA software (Ingenuity

Systems, Redwood City, CA) under any of the following conditions: 1) if signal evaluation values were 0 or 1 in every group and 2) if fold changes between two groups were greater than 0.5 or less than 2. Genes that met these criteria were uploaded into the IPA website (http://www.ingenuity.com/) to annotate biofunctions and construct molecular interaction networks. In the present study, two major methods of pathway analysis were used, network analysis and canonical pathway analysis.

In network analysis, the significance and specificity of IPA-generated networks were based on the scores of each network. High scores indicated that gene networks are highly specific to each cluster. For example, a molecular network score of 3 indicates a 1 in 10^3 chance of getting a network containing the same number of network eligible molecules, with the same number of molecules randomly picked from the IPA Knowledge Base. That is, a score of 3 indicates 99.9% confidence of not being generated by random chance alone.

In canonical pathway analysis, the significance of the association between the data set and a canonical pathway was measured as follows: 1) The ratio of the number of identified genes in a particular pathway to the total number of genes that constitute the pathway was calculated. 2) Fischer's exact test was used to calculate the probability (*P*) of the association between genes in the dataset and the canonical pathway occurring due to chance alone.

Quantitative real-Time PCR confirmation

The expression of salient genes was confirmed by real-time PCR. Total RNA was extracted as described above. Aliquots (1 μg) of extracted RNA were reverse transcribed into cDNA at

37°C for 15 min using reverse transcriptase (Takara Biochemicals, Japan). Real-Time PCR was performed using a CFX96 Real-Time System and C1000 Thermal Cycler (Bio-Rad, USA) and the products were detected using the DNA-binding dye SYBR Green II. Primer sequences used in this study are summarized in **Table 1**. The PCR settings were as follows: initial denaturation at 95°C for 30 s followed by 40 cycles of amplification at 95°C for 5 s and 60°C for 30 s, and subsequent melting curve analysis increasing the temperature from 60°C to 95°C. The values obtained were normalized to those of GAPDH mRNA.

MMP-13 inhibitor assay on IBP-induced gastric damage

CL-82198 is a selective inhibitor of MMP-13 (IC_{50} = 10 μM), which does not inhibit MMP-1, MMP-9, or TACE (Chen et al., 2000). Rats were divided into the following five treatment groups: 1) 5% gum arabic solution (n=4); 2) 200 mg/kg IBP (n=8); 3) 200 mg/kg IBP and 200 mg/kg APAP (n=8); 4) 200 mg/kg IBP and 0.2 mg/kg CL-82198 (n=8); and 5) 200 mg/kg IBP and 1.0 mg/kg CL-82198 (n=8). APAP or CL-82198 was administered to rats with IBP at the same time in the groups 3)-5). They were euthanized 4 h post-drug administration under isoflurane anesthesia, the gastric tissue was collected, and gastric lesions were measured as mentioned above.

Statistical Analysis

Gastric damage data and real-time PCR results are presented as mean ± S.E. with 4–10 rats per group. Statistical analyses were performed using a two-tailed unpaired *t* test or Dunnett's

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multiple comparison test. Values of $P < 0.05$ were considered to be significant.

Results

Effects of APAP on IBP-induced gastric damage

To investigate the effects of APAP on IBP-induced gastric damage, the lengths of hemorrhagic lesions were measured. Co-treatment with APAP at 100 mg/kg, 125 mg/kg, 150 mg/kg, 175 mg/kg, 200 mg/kg, and 400 mg/kg led to 35.0%, 81.0%, 98.7%, 98.6%, 97.0%, and 98.0% decrease in gastric lesions, respectively, compared with of IBP treatment (200 mg/kg) alone (**Fig 1A**). APAP significantly suppressed IBP-induced gastric damage in a dose-dependent manner. Histological analysis revealed that IBP administration caused epithelial cell damage, whereas the combination of IBP and APAP prevented the mucosal tissue damage (**Fig 2A**).

Microarray analysis for gene expression induced by IBP and APAP

Expression profiles of differentially regulated transcripts were generated at two time points: 2 h and 4 h post-drug administration. Of the 30,003 probes studied, 222 were upregulated at least 2.0-fold by IBP compared with the control group and downregulated at least 2.0-fold by IBP and APAP co-treatment compared with IBP alone at 2 h post-drug administration. In contrast, 160 probes were downregulated by IBP compared with the control group and upregulated by IBP and APAP co-treatment compared with IBP alone by at least 2.0-fold. In total, 382 probes were selected at 2 h post-drug administration, and 370 of these were mapped to IPA-defined genetic networks. Similarly, 525 probes were upregulated by IBP and downregulated by IBP and APAP co-treatment, and 55 probes were

downregulated by IBP and upregulated by IBP and APAP co-treatment by at least 2.0-fold at 4 h post-drug administration. In total, 580 probes were selected at this time point, and 566 of these were mapped to IPA-defined genetic networks.

Selected genes were analyzed using IPA to identify major functional molecular networks (**Table 2**). By network analysis, the following 5 top molecular networks were significantly activated with IBP and APAP co-treatment compared with IBP alone at 2 h post-drug administration: (1) Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry; (2) Post-Translational Modification, Embryonic Development, Organ Development; (3) Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry; (4) Embryonic Development, Organismal Development, Tissue Development; and (5) Molecular Transport, Cell Cycle, DNA Replication, Recombination and Repair. At 4 h post-drug administration, the following 5 top molecular networks were significantly activated: (1) Cellular Assembly and Organization, Cellular Movement, Hematological System Development and Function; (2) Inflammatory Disease, Inflammatory Response, Ophthalmic Disease; (3) Organismal Development, Dermatological Diseases and Conditions, Cellular Assembly and Organization; (4) Carbohydrate Metabolism, Small Molecule Biochemistry, Cellular Growth and Proliferation; and (5) Drug Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry. As shown in **Table 2**, the top scores at 4 h were greater than those at 2 h.

Further, canonical pathway analysis identified other major pathways related to inflammatory disease (**Table 3**). Among these, the LXR/RXR activation pathway was highly activated at 2 h post-drug administration and pathways involving CYP genes were highly activated at 4 h

post-drug administration. Genes involved in the LXR/RXR activation pathway included CD14, interleukin-6 (IL-6), and prostaglandin endoperoxide synthase 2 (PTGS2). Although the expression of the PTGS2 gene was upregulated by IBP and downregulated by IBP and APAP co-treatment, the expression pattern of prostaglandin endoperoxide synthase 1 (PTGS1) was not altered by either drug administration compared with the control group at any time point (**Table 4**).

Moreover, using the gene classification from the IPA Knowledge Base, 47 genes were selected as gastric damage-related genes, having functions in apoptosis (in epithelial cells), vasoconstriction, ulcer formation, gastrointestinal disease (inflammatory, immune), loss of gastric acid secretion, imbalance of extracellular matrix, disorder of cell proliferation (in gastric), bleeding, disorder of cell adhesion (in epithelial cells), formation of vascular lesion, disorder of cell migration (in epithelial cells) and imbalance of bicarbonate ion secretion. Comparison of this gastric damage profile with the expression patterns of 47 genes revealed 21 genes with good correspondence. Of these genes, matrix metalloproteinase 10 (MMP-10), matrix metalloproteinase 13 (MMP-13), and caspase 12 (CASP12) may participate directly in the induction of gastric damage because they encode enzymes involved in tissue destruction. In particular, MMP-13 was upregulated by IBP and downregulated by IBP and APAP co-treatment.

Furthermore, the IPA Knowledge Base revealed that the change in the expression of MMP-13 may be associated with the genes interleukin-1β (IL-1β), chemokine (C-C motif) ligand 2 (CCL2), MMP-10, and FBJ osteosarcoma oncogene (FOS) (**Fig 2**). IL-1β, CCL2,

and MMP-10 were significantly upregulated by IBP treatment and downregulated by IBP and APAP co-treatment. FOS was not upregulated by IBP but was downregulated by IBP and APAP co-treatment (**Table 4**).

To confirm the expression patterns identified by cDNA microarray at 2 h and 4 h post-drug administration, expression of the genes IL-1β, CCL2, MMP-10, MMP-13, and FOS was confirmed using real-time PCR (**Fig 3**). Although the microarray results overestimated the fold-changes observed during real-time PCR for some genes, the methods agreed on the direction of regulation in all cases. Of these 5 genes, the expression of MMP-13 was most upregulated by IBP and downregulated by IBP and APAP co-treatment at 4 h post-drug administration.

Effects of a MMP-13 inhibitor on IBP-induced gastric damage

Gene expression analysis indicated that MMP-13 may be a direct pathogenic factor during the induction of gastric damage. To investigate the contribution of MMP-13 downregulation during gastroprotection by APAP, we evaluated the effects of the MMP-13 inhibitor CL-82198 on IBP-induced gastric damage. CL-82198 decreased gastric lesions in a dose-dependent manner in the presence of IBP (**Fig 4**). CL-82198 administered at 0.2mg/kg and 1.0mg/kg led to 40.3% and 72.1% decrease in gastric lesion, respectively, compared with IBP administration alone.

Discussion

With the exception of selective COX-2 inhibitors, traditional NSAIDs are classified into three types: those carrying an acetic acid moiety, those with a propionic acid moiety, and those carrying other acidic moieties such as salicylate. In the present study, we examined the gastroprotective effects of APAP on gastric damage induced by IBP, which carries a propionic acid moiety. Oral doses of APAP at 200 mg/kg blocked 97.0% of gastric damage caused by same amounts of IBP. In a previous report, APAP suppressed gastric lesions induced by other NSAIDs aspirin and indomethacin (Stern et al., 1984; Konturek et al., 1982; Seegers et al., 1980), suggesting that gastric damage caused by various types of NSAIDs may be suppressed by combined administration with APAP.

In the present study, we investigated the protective mechanisms of APAP using gene expression profiling in the gastric mucosa. Using IPA, we found that two major pathways, the cellular assembly pathway and inflammation pathway, were highly activated by IBP and suppressed by APAP. This result agreed with the previous observation of Naito et al. (2007) which showed that administration of indomethacin induced the inflammation-signaling pathway in the gastric mucosa of rats.

In addition, our results demonstrated that IBP upregulated genes related to gastric damage, which encode IL-1β, CCL2, MMP-10, and MMP-13. In contrast, treatment with APAP downregulated the expression of these genes and that encoding FOS. IL-1β and CCL2 are cytokines that are involved in immunoregulatory and inflammatory processes (Deshmane et al., 2009; Ren and Torres, 2009). MMP-10 and MMP-13 are proteins of the matrix

metalloproteinase (MMP) family that are involved in the breakdown of the extracellular matrix (ECM) (Hofmann et al., 2000). FOS is a transcriptional regulator related to inflammation and pain perception (Shiozawa and Tsumiyama, 2009). Among these 5 genes, we focused on MMP-13 because it could be a direct pathogenic factor during induction of gastric damage by IBP, and it was suppressed during gastroprotection by APAP.

MMPs are a class of Zn-dependent endopeptidases that regulate cell-matrix composition. Under normal conditions, MMPs are present in tissues at low levels in the latent form and are responsible for normal physiological tissue turnover (Sengupta and MacDonald, 2007). However, MMPs play a critical role in ECM degradation and remodeling during inflammation and wound healing processes (Egeblad and Werb, 2002). Several studies describe the involvement of MMPs in degradation and remodeling of ECM during pathogenesis and healing of gastric ulcers (Sengupta and MacDonald, 2007; Lempinen et al., 2000; Shahin et al., 2001; Swarnakar et al., 2005). In particular, MMP-1, -8, -13, and -14 are responsible for cleavage of both collagen I and III, the major types of collagen in the stomach wall (Chakraborti et al., 2003; Yan and Boyd, 2007; Fanjul-Fernandez et al., 2010). In the present study, MMP-13 was upregulated in the gastric mucosa damaged by IBP, whereas it was suppressed to near normal levels by APAP during prevention of gastric damage. The MMP-13 inhibitor CL-82198 decreased IBP-induced gastric damage, indicating that MMP-13 may be a pathogenic and not a healing factor in gastric lesions. In addition, it was considered that the protective action of APAP was possibly mediated by suppression of MMP-13.

The catalytic activities of MMPs are highly regulated at multiple levels, including gene expression, spatial localization, zymogen activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs). Most MMPs are secreted as inactive proproteins that are activated when cleaved by extracellular proteinases (Chakraborti et al., 2003; Yan and Boyd, 2007; Fanjul-Fernandez et al., 2010). Further, it is suggested that MMP-10 is responsible for activation of proMMP-13 (Barksby et al., 2006). In the present study, the expression of MMP-10 and MMP-13 in the gastric mucosa was downregulated by APAP during gastroprotection. Thus, it is possible that the suppression of MMP-10 may contribute to the decrease activity of MMP-13 in the gastric mucosa during gastroprotection by APAP.

Furthermore, the promoter of MMP-13 has several *cis*-elements such as TATA boxes and activator protein-1 (AP-1)-binding sites that regulate its expression. External stimuli activate the nuclear AP-1 transcription factor complex and AP-1 dimers consisting of members of *jun* and *fos* gene families. AP-1 dimers bind to cognate *cis*-elements, resulting in the activation of gene transcription (Chakraborti et al., 2003; Yan and Boyd, 2007; Fanjul-Fernandez et al., 2010). Chakraborti et al. (2003) reported that over-expression of c-*fos* in transgenic mice induced the expression of mouse MMP-13 predominantly in bone and also in the thymus and spleen. In the present study, downregulation of FOS and MMP-13 mRNA expression was observed after administration of APAP. These findings suggest that MMP-10 and FOS may regulate the transcription or activity of MMP-13 during prevention of gastric damage by APAP.

In addition, it is possible that other elements, such as PGs, may decrease gastric damage

independent of MMP-13 inhibition by APAP, because the gastric lesion was not fully suppressed by the MMP-13 inhibitor. In the present study, the expression of PTGS1 was not changed at any time point, whereas the expression of PTGS2 was upregulated at 2 h post-drug administration. In contrast, Naito et al. (2007) reported that the expression of PTGS1 in the gastric mucosa was upregulated at 2 h post-indomethacin administration. Although the relationship between COX and gastric damage has not been fully elucidated, other elements such as MMPs may be involved in the pathogenic mechanism.

In summary, this study first demonstrated that APAP suppressed IBP-induced gastric damage in a dose-dependent manner. Administration of APAP downregulated the expression of genes related to inflammation and ECM degradation that were upregulated by IBP during gastric damage. Downregulation of IL-1β, CCL2, MMP-10, MMP-13, and FOS genes were also related to gastroprotection by APAP. In particular, there was a significant reduction in the expression of MMP-13 by APAP, which decreased IBP-induced gastric damage. Collectively, these findings suggested that suppression of MMPs formed an essential part in the gastroprotective effects of APAP. Further studies of the key molecules that regulate catabolic–anabolic balance in the gastric mucosa are essential for understanding the gastrotoxic mechanisms of NSAIDs and gastroprotection by APAP.

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Authorship Contributions

Participated in research design: Fukushima, Monoi, Serizawa, Adachi, Koide, Ohdera, Murakoshi, and Kato

Conducted experiments: Fukushima, Monoi, Mikoshiba, and Hirayama

Performed data analysis: Fukushima, Monoi, and Kato

Wrote or contributed to the writing of the manuscript: Fukushima, Serizawa, Murakoshi, and Kato

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a) This work was not received any grant.

b) This work was not previously published

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Figure Legends

Fig. 1. Effects of acetaminophen on ibuprofen-induced gastric damage.

Animals were given followed drugs and euthanized under isoflurane anesthesia 4hr later: 1) 200 mg/kg ibuprofen (IBP), 2) 200 mg/kg IBP and 100 mg/kg acetaminophen (APAP), 3) 200 mg/kg IBP and 125 mg/kg APAP, 4) 200 mg/kg IBP and 150 mg/kg APAP, 5) 200 mg/kg IBP and 175 mg/kg APAP, 6) 200 mg/kg IBP and 200 mg/kg APAP and 7) 200 mg/kg IBP and 400 mg/kg APAP.

A) Length of gastric lesion. Data are presented as the mean±S.E. (n=9-10, individuals per group). * $P < 0.05$, ** $P < 0.01$ compared with IBP 200 mg/kg (Dunnett's multiplecomparison test). B) Macroscopic and microscopic observation of 200 mg/kg IBP, 200 mg/kg IBP and 200mg/kg APAP. Arrows indicate the location of gastric lesions.

Fig. 2. Molecular networks of affected genes associated with MMP-13.

Biological relationships between genes are described as edges. Edges are displayed with labels that describe the characteristic of the relationships between genes. All edges are supported by at least one reference from the literature or from the canonical information from the Ingenuity Pathway Analysis (IPA) Knowledge Base.

Fig. 3. Analysis of mRNA expression using real-time PCR

Rats were divided into the following treatment groups: 1) 200 mg/kg ibuprofen (IBP, black bar); 2) 200 mg/kg ibuprofen and 200 mg/kg acetaminophen (IBP + APAP, grey bar); and 3)

5% gum arabic solution (control, white bar). Total RNA was extracted from the gastric mucosa of each animal at **A)** 2 h or **B)** 4 h post-drug administration and real-time PCR was performed using the primer set listed in Table 1, as described in the Materials and Methods section. Data are presented as mean ± S.E. (n = 5, individuals per group), and GAPDH was used to normalize the relative mRNA levels. * $P < 0.05$, ** $P < 0.01$ compared with IBP (Dunnett's multiple comparison test).

Fig. 4. Effect of CL-82198 on IBP-induced gastric damage

After overnight fasting, the animals were administered the following drugs and euthanized under isoflurane anesthesia 4 h later: 1) 5% gum arabic solution (n=4); 2) 200 mg/kg ibuprofen (IBP) (n=8); 3) 200 mg/kg IBP and 200 mg/kg acetaminophen (APAP) (n=8); 4) 200 mg/kg IBP and 0.2 mg/kg MMP-13 inhibitor (CL-82198) (n=8); and 5) 200 mg/kg IBP and 1.0 mg/kg CL-82198 (n=8). Data are presented as mean ± S.E. * $P < 0.05$, **$P < 0.01$ compared with IBP 200 mg/kg (Dunnett's multiple comparison test).

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Tables

Table 1. Oligonucleotide primer sequence

Fw, forward; Re, reverse.

Target gene	direction	Sequence(5'-3')
MMP-10	Fw	CCCTGGATTTTATGGAGATGCTC
	Re	TTGGCTCGTGGAGAACCTGTAGAC
MMP-13	Fw	CCCTGGAATTGGCGACAAAG
	Re	GCATGACTCTCACAATGCGATTAC
IL-1β	Fw	GCTGTGGCAGCTACCTATGTCTTG
	Re	AGGTCGTCATCATCCCACGAG
CCL2	Fw	CTATGCAGGTCTCTGTCACGCTTC
	Re	CAGCCGACTCATTGGGATCA
FOS	Fw	GGAGCCGGTCAAGAACATTAGC
	Re	GCATAGAAGGAACCAGACAGG
GAPDH	Fw	GAACGGGAAGCTCACTGGCATGGC
	Re	TGAGGTCCACCACCCTGTTGCTG

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Table 2. Genes in network induced by ibuprofen and acetaminophen treatment.

Networks of the focus molecules are generated based on their association, and ranked by the score of each network. The score is based on a p-value, which calculates the possibility that the focus molecules included in a network are found by random chance alone.

A) 2hr post-drug administration

Network ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	AMBP, amylase, APOC1, ARHGAP4, B3GAT1, C/ebp, C1QTNF2, CBS, CLIP2, cyclooxygenase, ERK1/2, Growth hormone, GSTA1, GZMK, HDL, IGFALS, IRF2, Iti, ITIH4, LCAT, LDL, LRP3, LRP, Nos, NOS1AP, NR1H4, Nr1h, OSBP, Pro-inflammatory Cytokine, Rxr, SAA, SAA2-SAA4/SAA4, STARD4, TNN, VEGFC	34	21	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
2	ADAMTS1, Akt, BCR (complex), CDC42EP4, CDKN3, Cg, CSF1R, Fcer1, FOXC1, FSH, GCNT1, HCRT, IRS, Lh, NFAT5, Nfat (family), NMB, NMDA Receptor, NTRK1, PARD6G, PAX8 (includes EG:18510), PI3K (family), PIGA, PLC gamma, PTK6, Ptk, PTPase, PTPN5, PTPRC, PTPRE, PTPRG, PTPRU, SLC10A1, SYK/ZAP, TP53I11	30	21	Post-Translational Modification, Embryonic Development, Organ Development
3	14-3-3, AMPK, ANP32E, caspase, Collagen Alpha1, CPOX, Cytochrome c, Eif2, EIF2AK3, EPHA4, GFAP, GZMA,	29	20	Carbohydrate Metabolism, Lipid Metabolism, Small

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	HIST1H1T, Histone H1, IgG2b, Ins1, Jnk, KCNK2, LPIN1, MAP2K1/2, MAPT, NARS, PARG, PARP, PEPCK, PFKFB1, PFKFB2, PP2A, RAPGEF4, RGCC, SYNPO2, SYT7, TIMP3, Tnf receptor, trypsin			Molecule Biochemistry
4	CACNA1H, Creb, CSF1 (includes EG:12977), Dynamin, EHD3, ERK, Fgf, FGFR2, Gm-csf, Hsp27, IQUB, JINK1/2, LEFTY2, LOX, MAPK8IP3, Mek, MITF, NNAT, Nuclear factor 1, PACSIN1, PDE10A, Pdgf (complex), PDGF BB, PLA2, POLE, PP1 protein complex group, PRKD2, PRRX2, Rock, RPS6KA2, Rsk, SLC29A1, SOX17, sphingomyelinase, STAT5a/b	28	18	Embryonic Development, Organismal Development, Tissue Development
5	26s Proteasome, Actin, Alpha tubulin, ASF1B, BTNL2, C1QTNF5, Cbp/p300, CD3, CENPF, Cofilin, GAS8, GOT, HELLS, hemoglobin, HISTONE, Histone h3, Histone h4, HLA-C, Hsp90, IgG, IgG2a, IL6, IREB2, KIF15, KLF1, MMP25, Na+,K+ -ATPase, NFATC2IP, NUP107, ORC1 (includes EG:18392), PIAS1, SF3B1, SMC2, Ubiquitin, Vegf	26	18	Molecular Transport, Cell Cycle, DNA Replication, Recombination, and Repair

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B) 4hr post-drug administration

Network ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	ACVRL1, Adaptor protein 1, BCL2A1, CD8, CD244, CELA3B, CIRH1A, DLEU7, elastase, Hspa1l, Ige, Ikb, IL-1R, IL17RB, IL1RAPL2, IL1RL1, Integrin alpha 3 beta 1, ITM2A, LAMC2, MAP3K2, MMP13, NFkB (complex), NKIRAS1, PNPT1, SELP, SELPLG, Smad1/5/8, SPN, STAT1/3/5 dimer, TIMP3, TMEM119, Tnf receptor, UNC5CL, VSNL1, ZFYVE16	40	24	Cellular Assembly and Organization, Cellular Movement, Hematological System Development and Function
2	AMY1A (includes others), AMY2A, amylase, AQP1, AQP5, CCK, chymotrypsin, Collagen type II, Collagen type III, Collagen Type VI, Collagen(s), DGKI, ERK1/2, FGF7, FLT4, GFRA2, GREM1, GZMK, Iti, Kallikrein, LIG4, Pdgf Ab, PDGF-AA, PFKFB1, PLA2, PLA2G2F, RAB11FIP1, Rsk, Secretase gamma, SIT1 (includes EG:27240), SLC17A6, STAM, STX1B, TBX1, TIE1	32	21	Inflammatory Disease, Inflammatory Response, Ophthalmic Disease
3	Angiotensin II receptor type 1, CFD, CSTA, DAB2, ELF5, ELOVL3, ERK, FBN1, Fcgr3, FOXS1, FRZB, Gm-csf, GTF2H1, JAK, JINK1/2, Laminin1, LAMP3, LOXL1,	31	20	Organismal Development, Dermatological Diseases and Conditions, Cellular

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	MAPK15, MEF2C, MYOC, NF1 (includes EG:18015), NFAT (complex), p70 S6k, Pdgf (complex), PDGF BB, PRLR, PTPRC, Rock, S100A3, SOCS2, Sos, STAT5a/b, USHBP1, VAV			Assembly and Organization
4	AASS, Alp, CCL13, CD14, COL9A1, collagen, Collagen type I, Collagen type IV, CPXM1, CRMP1, DPYSL3, EID2, Fibrinogen, Integrin, ITGAL, Laminin, Metalloprotease, MMP10, Mmp, MN1, NKX2-1, OVOL1, PRSS22, ROS1, SLC7A5, Smad, Smad2/3, Tgf beta, TGFB1, THBS1, Tlr, TMEM17, Tnf, trypsin, VNN1	31	20	Carbohydrate Metabolism, Small Molecule Biochemistry, Cellular Growth and Proliferation
5	ADCY2, Ahr-aryl hydrocarbon-Arnt, ATE1 (includes EG:100037311), CACNA1B, CACNB4, CAMKV, CYP1A1 (includes EG:13076), CYP1B1, Cyp2a2, CYP2A13/CYP2A6, CYP2E1, CYP3A43, Cyp4a14, G protein, G protein alphai, G protein beta gamma, GABBR1, Girk, Gpcr, GPR158, GRIA2, KCNJ5, N-type Calcium Channel, NADPH oxidase, NAPB, NPY, Pkc(s), PLC, S1PR5, SLC46A3, TSH, TSHR, unspecific monooxygenase,voltage-gated calcium channel, WDR7	30	22	Drug Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry

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Table 3. Genes in top canonical pathways induced by ibuprofen and acetaminophen treatment.

The ratio of the number of identified genes in a particular pathway to the total number of genes that constitute the pathway was calculated. P-value was calculated using Fischer's exact test.

A) 2hr post-drug administration

Canonical Pathway	Ratio	p-value	Genes in pathway
LXR/RXR activation	10/136	1.49E-04	AMBP, APOC1, CD14, IL6, IL1RAPL1, ITHIH4, LCAT, NR1H4, PTGS2, SAA2-SAA4/SAA4
IL-10 signaling	6/78	2.94E-03	CD14, FCGR2B, IL6, IL1RAPL1, MAPK8, MAPK12
Dendritic cell maturation	9/207	4.06E-03	CD83, FCGR2B, HLA-C, IL6, IL15, IRF8, MAPK8, MAPK12, PLCB1
LPS/IL-1 mediated inhibition of RXR function	11/239	5.78E-03	ACOX2, ACSL4, ACSL6, APOC1, CD14, GSTA1, HS3ST3A1, IL1RAPL1, MAPK8, NR1H4, SLC10A1
Hepatic cholestasis	8/174	7.50E-03	CD14, IL6, IL1RAPL1, Ins1, MAPK8, MAPK12, NR1H4, SLC10A1

B) 4hr post-drug administration

Canonical Pathway	Ratio	p-value	Genes in pathway
Bupropion degradation	6/52	9.24E-04	CYP1A1, CYP1B1, CYP2A2, CYP2A13/ CYP2A6, CYP2E1, CYP3A43
Acetone degradation I (to methylglyoxal)	6/55	1.05E-03	CYP1A1, CYP1B1, CYP2A2, CYP2A13/ CYP2A6, CYP2E1, CYP3A43
Hepatic fibrosis/ Hapatic stellate cell activation	11/146	1.31E-03	AGTR1, CD14, CYP2E1, FLT4, HGF, IL1B, IL1RAPL2, IL1RL1, MMP13, TGFB1, VEGFC
Estrogen biosynthesis	6/68	3.56E-03	CYP1A1, CYP1B1, CYP2A2, CYP2A13/ CYP2A6, CYP2E1, CYP3A43
Nicotine degradation III	6/91	5.58E-03	CYP1A1, CYP1B1, CYP2A2, CYP2A13/ CYP2A6, CYP2E1, CYP3A43

Table 4. The levels of mRNA expression for gastric damage-related genes.

-, Not Detected.

Function	Probe ID	Description	Gene Symbol	Fold Change			
				2hr		4hr	
				IBP /Control	IBP+APAP /Control	IBP /Control	IBP+APAP /Control
Cyclooxygenase	A_44_P137448	prostaglandin-endoperoxide synthase 1	PTGS1	0.96	0.82	1.10	1.13
	A_64_P129316	prostaglandin-endoperoxide synthase 2	PTGS2	8.78	1.61	-	-
Degradation of Extracellular matrix	A_44_P404861	matrix metallopeptidase 10	MMP10	1.72	0.60	2.29	0.84
	A_42_P606126	matrix metallopeptidase 13	MMP13	19.08	1.15	2.09	0.72
Inflammation	A_43_P14911	interleukin 1 beta	IL1B	-	-	3.64	1.19
	A_42_P695401	chemokine (C-C motif) ligand 2	CCL2	5.95	1.42	3.07	1.06
Transcriptional regulator	A_64_P131051	FBJ osteosarcoma oncogene	FOS	1.29	0.66	1.01	0.52

Fig. 1.

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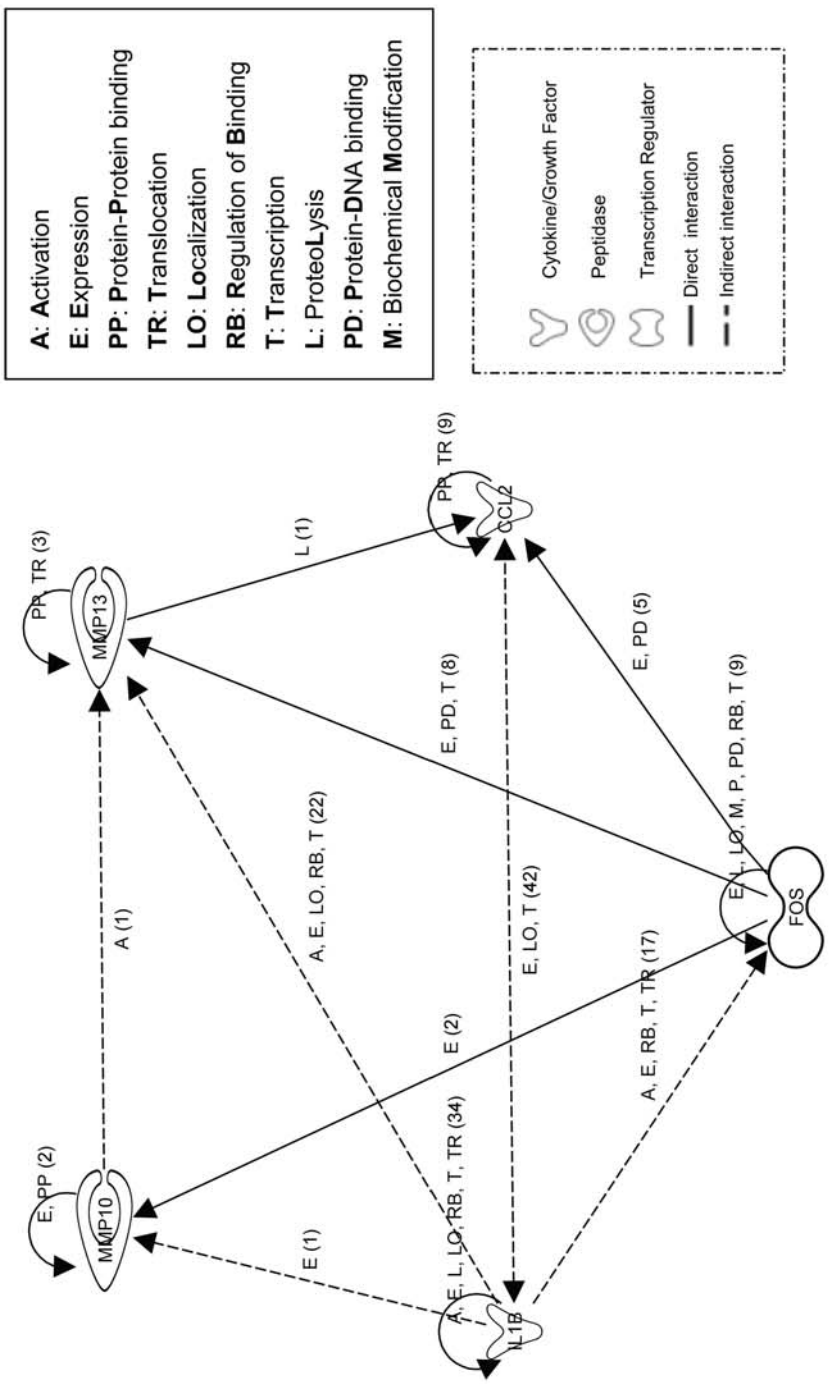

Fig. 2.

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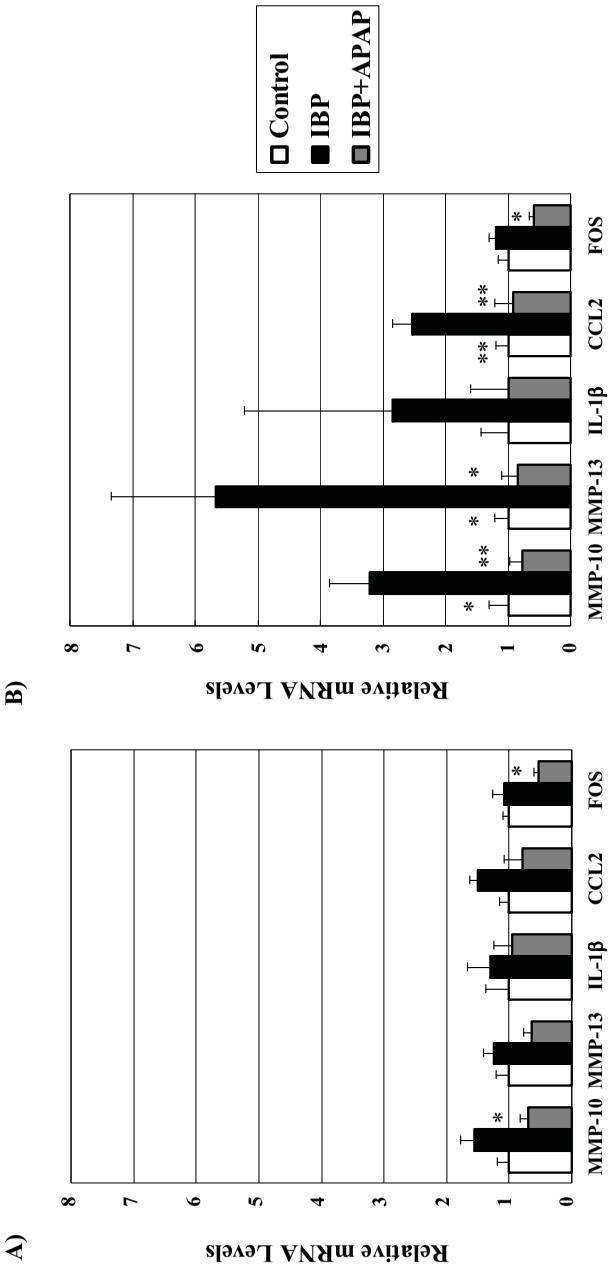

Fig. 3.

Fig. 4.