Aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via claudin-7

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Oshima T, Miwa H, Joh T. Aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via claudin-7. Am J Physiol Cell Physiol 295: C800–C806, 2008. First published July 30, 2008; doi:10.1152/ajpcell.00157.2008.—Tight junctions create a paracellular permeability barrier that is breached when nonsteroidal anti-inflammatory drugs cause gastrointestinal injury, including increased gastrointestinal permeability. However, the mechanism by which aspirin affects the function of gastric epithelial tight junctions is unknown. Thus, we examined the effect of aspirin on gastric mucosal barrier properties and tight junction organization using MKN28, a human gastric epithelial cell line that expresses claudin-3, claudin-4, claudin-7, zona occludens (ZO)-1, and occludin, but not claudin-2 or claudin-5, as determined by immunoblot analysis and immunofluorescent staining. Aspirin (5 mM) treatment of MKN28 gastric epithelial monolayers significantly decreased transepithelial electrical resistance and increased dextran permeability. Both aspirin-mediated permeability and phosphorylation of p38 MAPK were significantly attenuated by SB-203580 (a p38 MAPK inhibitor) but not by U-0126 (a MEK1 inhibitor) or SP-600125 (a JNK inhibitor). Aspirin significantly decreased the quantity of claudin-7 protein produced by MKN28 cells but not the quantity of claudin-3, claudin-4, ZO-1, or occludin. The aspirin-induced decrease in claudin-7 protein was completely abolished by SB-203580 pretreatment. These results demonstrate, for the first time, that claudin-7 protein is important in aspirin-induced gastric barrier loss and that p38 MAPK activity mediates this epithelial barrier dysfunction.

Tight junction; p38 mitogen-activated protein kinase; permeability

Nonsteroidal anti-inflammatory drugs (NSAIDs) are well known to induce gastrointestinal injury (1, 2, 10), which is our main concern with this group of drugs. Multiple factors, including deficiency of prostaglandins, neutrophil activation, microcirculatory disturbances, oxygen free radicals, and luminal acid, contribute to the development of NSAID-induced gastric injury (39, 44, 45). We have previously reported that a representative NSAID, indomethacin, inhibited cyclooxygenase (COX)-1-dependent monolayer integrity, and an indomethacin-induced increase in intercellular permeability was attenuated by PGE2 administration (13). PGE2 reportedly stimulates increases in intracellular Ca2+ and cAMP and also promotes the sealing of tight junctions (TJs) (4). However, PGE2 did not prevent aspirin-induced damage to TJs of the gastric epithelium (23), suggesting that COX inhibition is not the underlying mechanism whereby aspirin induces gastric epithelial permeability. Aspirin causes damage in the stomach when administered orally through esophageal intubation but not subcutaneously, despite suppressing the production of PGE2 in the gastric mucosa similarly via both routes of administration (17). Thus, to assess the direct effect of aspirin on TJs may provide useful information about the mechanism of aspirin-induced gastropathy.

Aspirin activates p38 MAPK and JNK; however, indomethacin and ibuprofen fail to induce significant activation of JNK or p38 MAPK, suggesting that signal transduction differs among NSAIDs. The role of MAPK signaling in TJ regulation has been of interest (22, 36). Constitutive MAPK activation inhibits the formation of TJs in Madin-Darby canine kidney cells. Moreover, pharmacological inhibition of MEK1 signaling in these cells permits TJ formation (3). The mitogenic effect of MAPK activity is logically opposed to TJ formation. We have previously found that MAPK and p38 MAPK pathways play an important role in hydrogen peroxide-mediated disruption of endothelial and epithelial TJs (15, 31). However, the detailed mechanisms, including the involvement of claudins, in the regulation of epithelial TJs in aspirin-induced gastrointestinal damage are as yet unclear.

TJs are the most apical component of intercellular junctional complexes (6) and are composed of occludin, the claudin family, and junctional adhesion molecule (9, 42). The claudin family is a family of 24 distinct transmembrane proteins. Multiple claudin isoforms are expressed in a homophilic and heterophilic manner and in varied patterns of expression that are tissue specific (11) to regulate junctional permeability, and the selectivity and strength of the TJs are conferred by these proteins in most cell types (25). There has been no report explaining how aspirin modulates TJ proteins, including claudins, or how the activation of MAPK is involved in this system. In the present study, we investigated the mechanisms of aspirin-induced effects on gastric mucosal barrier function and TJ organization and clarified which claudins are involved in aspirin-mediated gastric epithelial barrier dysfunction. We also investigated the role of MAPKs using specific inhibitors.

Materials and Methods

Cell lines and culture conditions. MKN28, a human (70-yr-old Japanese woman) gastric adenocarcinoma cell line, was obtained from the American Type Culture Collection (Rockville, MD). RPMI-1640 (Sigma, St. Louis, MO) was supplemented with 10% FBS, 2.5 mg/ml amphotericin B, 50 U/ml penicillin, and 50 mg/ml streptomycin. The culture medium was changed every 48–72 h.

Reagents and antibodies. Rabbit anti-claudin-2, anti-claudin-3, anti-claudin-4, anti-claudin-7, and anti-occludin antibodies as well as...
Adding 500 per minute as previously described (30). Cell lysates were prepared by nase (LDH; LDH assay, Sigma) into the media. All data represent the /H9262 U-0126 (1 μM) pretreatment did not have any effect on aspirin-induced decreases in TEER. *P < 0.05 vs. aspirin.

Mouse anti-zonula occludens (ZO)-1 antibodies were purchased from Zymed Laboratories (San Francisco, CA). Alexa 488-conjugated anti-mouse IgG antibody was obtained from Invitrogen (Carlsbad, CA). Cy3-conjugated goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-phospho-p38 MAPK, p42/44 MAPK, and JNK antibodies, MEK inhibitor U-0126, and p38 MAPK inhibitor SB-203580 were obtained from Promega (Madison, WI). All other reagents were purchased from Sigma unless stated otherwise.

Transepithelial electrical resistance. MKN28 cells were plated on 9-mm-diameter, 0.4-μm pore size tissue culture inserts (Becton Dickinson, Bedford, MA) with “chopstick” electrodes. The value obtained from the monolayer was measured using MILLICELL-ERS (Millipore, Bedford, MA) and corrected units (Ω·cm²). The transmonolayer electrical resistance was measured at 100% confluence. The change in electrical resistance was represented by the percent baseline resistance, which was calculated as follows: percent baseline resistance = [(resistance from each time point) − (resistance from a blank insert)]/[baseline resistance] × 100, where baseline resistance was the resistance at the 0-min time point (29).

When resistance was stable (at >280 Ω·cm²), the culture medium from the upper (apical) compartment of the monolayer was removed and replaced with medium containing aspirin (5 mM) or control medium. In some experiments, monolayers were pretreated with U-0126 (1 μM for 30 min), SB-203580 (1 μM for 30 min), or SP-600125 (1 μM for 30 min) before the addition of aspirin. Cell viability was assessed by measuring the release of lactate dehydrogenase (LDH; LDH assay, Sigma) into the media. All data represent the average of four independently treated monolayers.

Western blot analysis of cell lysates. Western blot analysis was performed as previously described (30). Cell lysates were prepared by adding 500 μl of boiling sample buffer [125 mM Tris·HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, protease inhibitor cocktail, and 0.025% bromphenol blue] directly to a P60 culture dish. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions.

Equal quantities of protein from each sample were electrophoretically separated on 7.5% or 12% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL) and blocked with 5% milk powder in PBS at 4°C (overnight). The membrane was washed three times at 5 min/wash with wash buffer (0.1% milk powder in PBS) and then incubated with the appropriate primary antibody for 1 h at room temperature. The membrane was washed again in 0.1% milk-PBS for three times at 5 min/wash with wash buffer and then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit or antimouse IgG antibody for 1 h at room temperature. Finally, the membrane was washed three times and developed using the enhanced chemiluminescence detection system (Amersham, La Jolla, CA). For the small interfering (si)RNA assay, MKN28 cells were transiently transfected with negative control siRNA or claudin-7-specific siRNA, a cocktail of three siRNA designed by B-Bridge (Mountain View, CA), by LipofectAMINE 2000 (Invitrogen). Seventy-two hours after transfection, cells were harvested, and immunoblot analysis was performed. All experiments were reproduced three times, and the results of a typical experiment are shown.

Epithelial solute permeability. We also measured epithelial permeability in response to aspirin exposure using FITC-labeled dextran (FITC-dextran, molecular mass: 4,000) as a permeable tracer that passes across the epithelial monolayer using the method described by Maruo et al. (21) with minor modifications (28). MKN28 cells were grown on the surface of 0.4-μm-pore size tissue culture inserts.
These cell monolayers were washed twice with HBSS and then placed on 24-well plates with 1 ml HBSS in the lower chamber. A 500-μl aliquot of HBSS containing 5 mg/ml FITC-dextran was added to the luminal chamber, and the apparatus was then placed in a CO2 incubator at 37°C. After an incubation for 30 min, a 100-μl sample was taken from the lower chamber, and the absorbance of FITC-dextran was determined at 492 nm using a spectrophotometer (Fluoroskan Ascent, Thermo Fisher Scientific, Waltham, MA). Data were expressed as follows: FITC-dextran permeability index (%) = [(experimental clearance) - (spontaneous clearance)]/(clearance of filter alone) - (spontaneous clearance)) × 100.

Permeability measurements were made under control conditions, after an exposure to aspirin (5 mM), and after pretreatment with U-0126 (1 μM for 30 min), SB-203580 (1 μM for 30 min), or SP-600125 (1 μM for 30 min). After the pretreatment, aspirin was added to the culture, and permeability was measured at 24 h after stimulation. For the siRNA assay, MKN28 cells were transiently transfected with negative control siRNA or claudin-7-specific siRNA.

Immunofluorescent staining of junctional proteins. MKN28 cells were seeded onto 1.2-cm-diameter coverslips and allowed to reach confluency. Monolayers were exposed to aspirin or the appropriate buffer controls. Samples were fixed with ethanol (30 min) and acetone (1 min) (27) and stained for claudins. Primary rabbit anti-claudin-7 polyclonal antibody and/or mouse anti-ZO-1 monoclonal antibody were used at a concentration of 4 μg/ml. Cy3-conjugated goat anti-rabbit IgG and Alexa488 anti-mouse IgG secondary antibodies were used.

Fig. 3. Effect of the p38 MAPK inhibitor on aspirin-induced change in tight junction proteins. A: representative data of zonula occludens (ZO)-1, occludin, claudin-2, claudin-3, claudin-4, and claudin-7 proteins after stimulation with aspirin (5 mM for 24 h) and SB-203580 pretreatment. B: aspirin did not change the quantities of ZO-1, occludin, claudin-3, or claudin-4. However, the quantity of claudin-7 protein was reduced by aspirin stimulation, and this decrease was blocked by SB-203580 (1 μM) pretreatment. Claudin-2 was not detected in MKN28 cells. SB-203580 pretreatment did not have any effect on the quantities of ZO-1, occludin, claudin-2, claudin-3, or claudin-4 proteins. C: SB-203580 (1 μM) treatment did not change the quantity of claudin-7.

*P < 0.05 vs. untreated control; #P < 0.05 vs. aspirin.

Fig. 4. Aspirin activates MAPK. A: aspirin (5 mM) induced p42/44 MAPK, p38 MAPK, and JNK phosphorylation (P). SB-203580 (1 μM) blocked aspirin-induced p38 MAPK phosphorylation but not aspirin-induced p42/44 MAPK and JNK phosphorylation. B: U-0126 (1 μM) and SP-600125 (1 μM) blocked aspirin-induced p42/44 MAPK and JNK phosphorylation, respectively.
used at a dilution of 1:200. The specificity of the reaction was tested by an incubation with HBSS, nonimmune rabbit serum, or mouse IgG1. Slides were viewed using a fluorescence microscope. Images were recorded on a Windows computer using a ×40 objective.

Statistical analysis. All values are expressed as means ± SD. Data were analyzed using one-way ANOVA with Bonferroni’s correction for multiple comparisons. Significance was accepted at \( P < 0.05 \).

RESULTS

Effect of aspirin on the transepithelial electrical resistance of a MKN28 monolayer. Aspirin (5 mM) treatment of gastric epithelial MKN28 cell monolayers caused a significant decrease in transepithelial electrical resistance (TEER) at 4, 12, and 24 h after stimulation (Fig. 1). There was no evidence of any cytotoxic effects of aspirin (5–10 mM) at any time point, as determined by the LDH release assay (9.1 ± 1.5% LDH release for control cells and 7.9 ± 2.2% for cells treated with 10 mM aspirin for 24 h). Although pretreatment with the MEK inhibitor U-0126 (1 μM) or the JNK inhibitor SP-600125 (1 μM) did not have any effect on the aspirin-induced decrease in TEER, pretreatment with the p38 MAPK inhibitor SB-203580 (1 μM) significantly inhibited the aspirin-induced decrease in TEER. Pretreatment of monolayers with U-0126, SP-600125, and SB-203580 did not influence baseline TEER.

Effects of MAPK inhibitors on aspirin-induced permeability. The paracellular permeability of MKN28 cells was also measured using FITC-dextran (molecular mass: 4,000). The amount of FITC-dextran that passed across an epithelial monolayer was measured with a plate spectrophotometer. Aspirin (5 and 10 mM for 24 h) significantly increased the FITC-dextran permeability (Fig. 2A) of MKN28 cells, suggesting that aspirin induced a break in the mucosal barrier of these cells. Pretreatment with the p38 MAPK inhibitor SB-203580 (1 μM) significantly attenuated the aspirin-induced increase in FITC-dextran permeability (Fig. 2B). Pretreatment with the MEK inhibitor U-0126 (1 μM) or the JNK inhibitor SP-600125 (1 μM) did not have any effect on the aspirin-induced increase in permeability. These data are consistent with the TEER data. U-0126 (1 μM), SB-203508 (1 μM), or SB-600125 (1 μM) treatment (24.5 h) did not have any effect on gastric epithelial permeability.

Aspirin reduces the quantity of claudin-7. The quantities of TJ proteins claudin-2, claudin-3, claudin-4, claudin-7, ZO-1, and occludin were determined by Western blot analysis. There were no changes in the quantities of claudin-3, claudin-4, ZO-1, or occludin in total cell extracts after stimulation with aspirin (5 mM for 24 h; Fig. 3A). Claudin-2 was not detected

Fig. 5. Immunofluorescent staining of claudin-7 and ZO-1. Claudin-7 and ZO-1 were stained at junctions under control conditions. Both claudin-7 and ZO-1 were colocalized at the junctions. Aspirin stimulation decreased the staining of claudin-7. Pretreatment with SB-203580 blocked the aspirin-induced decrease in claudin-7 staining. Bar = 30 μm.
in MKN28 cells. The quantity of claudin-7 protein was significantly lower after stimulation with aspirin (Fig. 3B). The quantities of claudin-3, claudin-4, ZO-1, and occludin were not affected by SB-203580 pretreatment. On the other hand, the aspirin-induced decrease in the quantity of claudin-7 was attenuated by SB-203580 pretreatment. SB-203580 (1 μM) treatment (24.5 h) did not change the quantity of claudin-7 (Fig. 3C).

Aspirin induced p42/44 MAPK, p38 MAPK, and JNK phosphorylation. SB-203580 pretreatment blocked aspirin-induced p38 MAPK phosphorylation (Fig. 4A). Aspirin-induced p42/44 MAPK and JNK phosphorylation were blocked by U-0126 and SP-600125 pretreatment, respectively (Fig. 4B).

Claudin-7 and ZO-1 were stained at junctions under control conditions, as determined by immunofluorescence staining (Fig. 5). Both claudin-7 and ZO-1 were colocalized at the junctions. Aspirin stimulation decreased claudin-7 staining, and this decrease was abolished by SB-203580 pretreatment (Fig. 5). ZO-1 staining was not affected by aspirin stimulation. siRNA against claudin-7, siRNA against claudin-7 treatment successfully and specifically decreased claudin-7 protein, as shown by immunoblot analysis (Fig. 6A). Claudin-3 protein was not affected by siRNA against claudin-7. Gastric epithelial permeability was significantly increased with the treatment of siRNA against claudin-7 but not by control siRNA 72 h after transfection (Fig. 6B). These findings suggest that the quantity of claudin-7 protein is related to impaired barrier function in aspirin-treated MKN28 cells.

**DISCUSSION**

Aspirin, as with all NSAIDs, induces gastric epithelial damage. However, although PGE2 administration recovers an indomethacin-induced increase in gastric epithelial permeability (13), aspirin-induced damage to TJs of gastric epithelial cells is not prevented by PGE2 (23). Thus, aspirin induces gastric epithelial damage via a different mechanism from other NSAIDs. To understand the mechanisms that are involved in aspirin-induced gastric damage might provide a new strategy for the prevention of aspirin-induced gastropathy.

In the present study, we examined how paracellular junctional permeability changed when gastric epithelial cells were exposed to aspirin in vitro. Aspirin reduced the TEER of gastric epithelial cells and increased solute permeability (Figs. 1 and 2). One group of cells that may be involved in these aspirin-induced effects are the members of the p38 MAPK and JNK subfamilies of MAPKs, which can be activated in many types of cells by stress stimuli such as ultraviolet light, hyperosmolarity, and inhibition of protein synthesis (18, 24). Recent studies have suggested that TJs are regulated in some tissues through the p38 MAPK signaling pathway (15, 19, 31, 46). We have previously reported that p38 MAPK activation is involved in the hydrogen peroxide-induced increase in endothelial and epithelial permeability (15, 31). Moreover, TGF-β3 regulates the blood-testis barrier via the p38 MAPK pathway (19). Schwenger et al. (37) have reported that sodium salicylate produces a coordinate activation of JNK and p38 MAPK in human colonic epithelial cells. Although a study (14) has indicated that MEK1 signaling increases paracellular permeability, some disparity exists in observed cellular responses. In one study (20), inhibition of MEK1 signaling did not influence the expression of occludin or claudin-1 or affect TJ function in several breast cancer cells. Another study (34) using Escherichia coli showed that ERK1/2 was activated in colonic epithelial cells but did not induce TJ barrier disruption, as measured by TEER. Thus, the exact role of MAPKs in epithelial barrier function and TJs appears to vary, possible due to cell type or stimulant specificity. The present study showed that aspirin phosphorylates MAPKs, including p42/44 MAPK, p38 MAPK, and JNK. However, only the p38 MAPK inhibitor SB-203580 attenuated the aspirin-induced decrease in TEER and aspirin-induced increase in permeability (Figs. 1 and 2). These data suggest that p38 MAPK activation is involved in aspirin-induced gastric epithelial permeability and is important for signal transduction in gastric epithelial permeability. Additional studies are needed to evaluate how aspirin modulates p38 MAPK activity.

The concentration of aspirin required for gastric mucosal cell death in vitro is 10 times higher than that of indomethacin (40). This is in agreement with the corresponding concentrations of these NSAIDs required for gastrointestinal effects in vivo (7). Higher aspirin concentrations were not included in this study because of signs of toxicity in cells treated 24–48 h with aspirin at levels >10 mM. Studies on the oral administration of indomethacin and aspirin in rats have shown that the concentrations in the stomach were 1–8 and 10–100 mM, respectively, when these drugs caused gastric injury (7). Thus, the in vitro concentrations of aspirin used in this study are physiologically significant in vivo. Aspirin at millimolar concentration also has COX-independent effects and is able to interfere with several intracellular molecular pathways (5).

Gastrointestinal epithelial barrier function is maintained by intracellular junctional complexes, including TJs, adherens junction, and desmosomes (12, 16, 26, 35). This selective barrier function is critical for maintaining mucosal homeostasis. TJs separate the apical from basolateral cell surface domains to establish cell polarity and also provide barrier func-

**Fig. 6. Effect of small interfering (si)RNA against claudin-7.** A: claudin-7 siRNA specifically reduced the quantity of claudin-7 protein, as shown by immunoblot analysis. B: claudin-3 protein was not affected by claudin-7 siRNA. Claudin-7 siRNA significantly increased gastric epithelial permeability. A control siRNA, however, did not have any effect on gastric epithelial permeability (n = 4). *P < 0.01 vs. untreated control or control siRNA.
tion, inhibiting solute and water flow through the paracellular space (41). Among TJ proteins, claudins are important for TJ formation and have specific tissue distribution patterns. We found that at least claudin-3, claudin-4, and claudin-7 are expressed by MKN28 cells, but not claudin-2. We have also detected claudin-3, claudin-4, and claudin-7 in human gastric biopsy specimens (unpublished observations). Loss of claudin-4 has been reported to induce epithelial barrier disruption (8, 38), and overexpression of claudin-4 increases TEER and the number of junctional strands (43). In the present study, aspirin decreased the quantity of claudin-7 but not that of claudin-3, claudin-4, occludin, or ZO-1 (Fig. 3).

Previously, claudin-7 expression has been reported to be increased in gastric cancer (32). In the present study, we demonstrated claudin-7 knockdown by siRNA actually decreased the quantity of claudin-7 and increased gastric epithelial permeability (Fig. 6). These data indicate that claudin-7 is indeed involved in the TJ formation of gastric epithelial cells and is involved in aspirin-induced gastric epithelial barrier dysfunction. Occludin was originally found as the first surface-expressed TJ protein and exists at TJs. However, a previous knockout study (33) has indicated that the function of occludin is still unclear. To precisely define other TJ proteins that modulate epithelial permeability after aspirin exposure, more research is required. Moreover, additional studies are needed to evaluate the effect of aspirin on other cell lines or to examine whether this mechanism also occurs in patients taking aspirin.

This is the first report showing that aspirin induces gastric barrier loss and decreases the quantity of claudin-7, both effects of which are abolished by a p38 MAPK-specific inhibitor.

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