Acetyl salicylic acid induces damage to intestinal epithelial cells by oxidation-related modifications of ZO-1

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Acetyl salicylic acid (ASA) is one of the most frequently prescribed medications for the secondary prevention of cardiovascular and cerebrovascular events. It has been thought to be affected by gastric acid (6, 35), platelet effects, ASA is known to induce frequent and severe gastrointestinal bleeding. However, recent studies using novel endoscopy techniques, such as capsule endoscopy or balloon-assisted endoscopy, revealed that ASA causes small intestinal mucosal injury at a considerably higher rate than previously believed (19, 21). Until now, it is not known how to treat ASA-induced injury to small intestinal mucosa because the molecular mechanisms behind this phenomenon are not well understood.

The mechanism by which conventional NSAIDs (excluding ASA) induce small intestinal mucosal injury has recently been clarified. Two pathways appear to be involved, cyclooxygenase (COX)-dependent and COX-independent pathways. In both pathways, the first step of NSAID-induced enteropathy is the increase of small intestinal epithelial cell permeability (6, 7, 16, 26, 36, 38).

Most NSAIDs act as a COX inhibitor. COX catalyzes the formation of prostaglandin (PG) from arachidonic acid (38). Because PG is involved in the regulation of small-intestinal blood flow and various mucosal functions such as increasing mucus secretion and mucosal tight junction (TJ) barrier, NSAID-induced suppression of PG formation results in a severe mucosal injury. Because TJs are located on the most apical side of intercellular junctions and act as an intercellular seal or gate (1, 2), the reduction of TJ proteins, such as claudin family proteins, occludin, and zonula occludens-1 (ZO-1), allows easy penetration of bile acid, intestinal bacteria, or toxins between epithelial cells, resulting in unfavorable mucosal injury (16, 36, 38). On the other hand, the COX-independent pathway was proposed as a three-hit theory. Initially, NSAIDs directly damage small intestinal epithelial mitochondria. Next, the mitochondrial damage results in the depletion of intracellular energy and the induction of free radicals, which disrupts the intercellular junctions of the small intestinal epithelium. Finally, weakened mucosal TJ barriers result in the easy penetration of bile acid, intestinal bacteria, or toxins (6).

In our previous study, we showed that indomethacin (IND), a conventional NSAID, significantly induces small intestinal epithelial cell apoptosis via oxidative stress-dependent and COX-independent pathways (26). On the contrary, only a few studies have been done investigating ASA-induced small intestinal mucosal injury. Maiden et al. (19) and Smecuol et al. (34) both reported that ASA increases intestinal epithelial cell permeability as well as conventional NSAIDs in human studies (19, 34). However, the precise mechanism of this phenomenon is still unclear. Therefore, in this study, we investigated the molecular mechanism by which ASA induces increased small intestinal epithelial cell permeability (6, 7, 16, 26, 36, 38).
permeability by using a small intestine in vitro model, focusing particularly on oxidative stress.

MATERIALS AND METHODS

Cell line and culture medium. Caco-2, a well-differentiated human colorectal adenocarcinoma cell line, was grown for 3 wk in culture medium. After confluence, Caco-2 differentiates and exhibits a phenotype similar to human small intestinal epithelium both structurally and functionally and is the most commonly used cell line for in vitro studies of intestinal epithelial permeability (14, 22, 33). The Caco-2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 mg/ml glucose, 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% CO2. Differentiated Caco-2 were used in all experiments.

Reagents. DMEM/high glucose, PBS, and FBS were purchased from Invitrogen (Carlsbad, CA). ASA, prostaglandin E2 (PGE2), and fluorescein isothiocyanate (FITC)-labeled dextran (molecular mass of 4,000 kDa, FD4) were purchased from Sigma-Aldrich (St. Louis, MO). Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) and catalase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies specific for TJ proteins (claudin-1, claudin-2, claudin-4, occludin, and ZO-1) and the anti-actin antibody catalase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Cell viability.

Differentiated Caco-2 cells were grown in 96-well plates and incubated with ASA (0–20 mM) for 0–24 h. Cell viability was quantified using a methyldiathyryl tetrazolium (MTT)-based 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt assay kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, the cells were washed two times with PBS and incubated with MTT solution for 1 h at 37°C; the absorbance was measured at 450 nm using a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA).

Transport studies. For transport studies, Caco-2 cells (2 × 10^5 cells/well) were cultured on permeable filter membranes with a pore size of 0.4 μm and a surface area of 4.7 cm^2 in clusters of 24 wells (Millicell Hanging Cell Culture, Billerica, MA) until confluence and cultured for 3 wk. Direction of clearance was defined as apical to basolateral side. We measured epithelial cell permeability of Caco-2 using two different methods: transepithelial electrical resistance (TEER) and the clearance of FD4.

TEER was measured using a Millicell-ERS meter (Millipore, Bedford, MA) that connected to a pair of chokestick electrodes to ensure the integrity of the monolayer formed on the filters. TEER measurements were performed during the experiment to check the effect of ASA and the other reagents through the transcellular pathway and paracellular pathway (12, 17) at 0, 3, 6, and 12 h.

The transport of FD4 across the Caco-2 monolayer demonstrates the activity of paracellular transport (12, 17). FD4 solution (100 μl; concentration 500 μg/ml) was added to the apical chamber, and the fluorescent intensity of FD4 in the apical and basal chambers was measured at 0, 3, 6, and 12 h by a fluorescent microplate reader (Molecular Devices).

Western blot. After being treated with 10 mM ASA for 0, 3, 6, and 12 h, the differentiated Caco-2 cells were immediately rinsed with ice-cold PBS two times, and the cell pellet was lysed with Lysis Buffer (Cell Lytic M; Sigma-Aldrich) and collected with a cell scraper. Cellular debris was removed by centrifugation at 12,000 rpm/min at 4°C for 15 min. Supernatant was collected, and the protein concentration was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Berkeley, CA). Protein from each sample (20 μg) was electrophoresed on 10% SDS-PAGE gels for 30 min at 250 V and transferred to the nitrocellulose membrane (Invitrogen Japan, Tokyo, Japan) using a semidy transfer system (Invitrogen). The membrane was incubated for 30 min with blocking solution (10% Ez blocking; ATTO, Tokyo, Japan) in 10 mM TrisCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 (TBS-T) at room temperature. Next, it was incubated with appropriate primary antibody for 1 h at room temperature. After being washed with TBS-T three times, the membrane was incubated in appropriate secondary antibody for 1 h at room temperature. The immunocomplexes were detected using a Western blot luminol reagent kit (ECL plus; GE Health Bio-Sciences, Tokyo, Japan), and quantification was performed using Image J software (National Institutes of Health, Bethesda, MD).

Assessment of oxidative stress. Differentiated Caco-2 cells grown on 96-well black plates were labeled with a redox-sensitive fluorogenic probe, Redox Sensor Red CC-1 (Molecular Probes, Eugene, OR), that recognizes superoxide and hydrogen peroxide and treated with ASA (10 mM) for 6 h. The effect of ASA on reactive oxygen species (ROS) production in Caco-2 can be quantified using a fluorescent microplate reader (SpectraMax M2; Molecular Devices). To further quantify ROS, we used a redox-sensitive MitoSOX Red reagent (Molecular Probes), which is a specific superoxide marker in mitochondria.

In addition, to qualify the ROS production, differentiated Caco-2 cells grown on a 35-mm μ-dish (iBidi, Martinsried, Germany) were incubated with 1 μM of Redox Sensor Red CC-1 and 1 μM of Mitotracker Green FM (Molecular Probes) for 10 min at 37°C before ASA (10 mM) treatment for 30 min. Fluorescent intensity and distribution were also observed using a laser-scanning confocal microscope (FV10i; Olympus, Tokyo, Japan).

Antioxidant treatment. We used an antioxidant treatment to investigate the relationship between ASA-induced ROS production and ASA-induced increase of epithelial cell permeability. As an antioxidant, we used Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP), which, as a superoxide dismutase (SOD) mimetic, catalyzes the dismutation of superoxide in mitochondria. Thus, this molecule can potentially influence ROS production and ROS-mediated epithelial cell permeability.

**Fig. 1.** Acetyl salicylic acid (ASA) increased cell permeability of Caco-2 in a time-dependent manner. Differentiated Caco-2 cells were incubated with 10 mM of ASA, and intercellular permeability was measured by transepithelial electrical resistance (TEER, A) and by fluorescein isothiocyanate-conjugated dextran (FD4. B) transport studies at 0, 3, 6, and 12 h. The data are expressed as means ± SE of three separate experiments. *P < 0.01 compared with 0 h. **P < 0.001 compared with 0 h.
exhibit the beneficial and synergistic effects of SOD (18, 23). In some experiments, we added 100 μM of MnTMPyP. At this concentration, MnTMPyP does not induce cell death before stress application or during the period of stress as previously reported (18, 23). We also used 100 μM of catalase to attenuate hydrogen peroxide accumulation. Caco-2 was exposed to ASA with or without MnTMPyP or catalase, and we measured the ROS production and epithelial cell permeability of Caco-2.

Immunocytochemistry of the ZO-1 and occludin (TJ protein). Junctional localization of ZO-1 and occludin was assessed by immunofluorescent antibody labeling. Hoechst 33342 was used as a nuclear stain just before analysis by a laser-scanning confocal microscope (FV10i; Olympus). Differentiated Caco-2 cells were incubated with ASA for 0, 3, and 6 h at 37°C in a 35-mm μ-dish. The Caco-2 cells were washed two times in cold PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. The cells were then incubated in a 10% Ez-blocking solution for 30 min at room temperature and then incubated with primary antibody for 1 h (also at room temperature). After being washed with PBS three times, the cells were incubated in FITC-conjugated secondary antibody for 1 h at room temperature without light. Immunolocalization of ZO-1 or occludin was visualized using a laser-scanning confocal microscope.

Immunoprecipitation. Immunoprecipitation was used to assess ASA-induced, ROS-modified TJ protein expression. Differentiated Caco-2 cells were incubated with 10 mM of ASA for 0 or 3 h with or without MnTMPyP. Protein lysates were immunoprecipitated with 2 μg of anti-ZO-1 antibodies for 2 h at 4°C. The immunocomplexes were precipitated by incubation with protein G-Sepharose for 3 h at 4°C. Immunopellets were washed four times in PBS, and each sample was electrophoresed on 10% SDS-PAGE gels for 30 min at 250 V and transferred to the nitrocellulose membrane using a semidry transfer system. The membrane was incubated with anti-ZO-1 antibodies or anti-cysteine sulfenic acid (Millipore) for 1 h at room temperature. After being washed with TBS-T three times, the membrane was incubated in appropriate secondary antibody for 1 h at room temperature. The immunocomplexes were detected using a Western blot luminol reagent kit.

Statistical analysis. All data were presented as means ± SE with at least three separate experiments. Statistical analyses were performed using Bonferroni’s multiple-comparison tests; \( P < 0.05 \) was considered statistically significant.

RESULTS

ASA increased cell permeability of Caco-2 in a time-dependent manner. Because epithelial cell death might be one of the reasons for increased cellular permeability, we tried to exclude the factor of cell death in this study. To this end, we added 10 mM of ASA to Caco-2 and incubated for 24 h; there was no induction of Caco-2 cell death (data not shown). As shown in Fig. 1, this concentration of ASA significantly reduced TEER and increased FD4 flux, indicating the cell death-independent effect of ASA on epithelial cell permeability. In addition, to
exclude the factor of COX inhibition, PGE₂ was used, since PG derivatives are clinically useful for the prevention of NSAID-induced gastroenteropathy and ulcer (2, 16, 26). Although ASA has been reported to decrease PGE₂ production via COX inhibition, the supplementation of 10 μM of PGE₂ to ASA-stimulated Caco-2 did not inhibit ASA-increased cellular permeability in this study (data not shown), suggesting that the ASA-induced effect on cell permeability is independent of PGE₂ suppression. Interestingly, ASA significantly reduced TEER (at 3 h) before the increase of paracellular permeability (6 h), as shown in Fig. 1.

ASA specifically decreased ZO-1 expression of Caco-2. TJ (claudin-1, -2, and -4, occludin, and ZO-1) have been implicated in the paracellular barrier function of small intestinal mucosa (27). To elucidate the target protein among the TJ complex, we assessed the expression of claudin-1, -2, and -4, occludin, and ZO-1 by Western blot. Surprisingly, ASA did not affect the expression of the claudin family proteins or occludin within 12 h; however, ASA specifically decreased the expression of ZO-1 in a time-dependent manner (Figs. 2A, 2B, and 6A), which was correlated with increased permeability as assessed by FD4 flux.

Immunofluorescent cytochemistry showed that ZO-1 was continuously localized in Caco-2 intercellular space; however, ASA clearly decreased the continuity of the ZO-1 protein in a time-dependent manner (see Fig. 6C).

ASA-induced ROS production in Caco-2. Because we previously reported that high concentrations of ASA induce ROS production, which results in the apoptosis of small intestinal epithelial cells (28), we examined whether or not a low concentration of ASA had the same effect. We found that a low concentration of ASA significantly and rapidly induced ROS production in Caco-2 within 30 min as assessed by Redox Sensor Red CC-1 using a fluorescent microplate reader (Fig. 3A) and laser-scanning confocal microscope (Fig. 3B).

ASA-induced superoxide production in Caco-2. Redox Sensor Red CC-1 detects superoxide and hydrogen peroxide production but cannot distinguish which reactive species are involved in this phenomenon. A superoxide-specific fluorogenic probe (MitoSOX Red) combined with MnTMPyP (MnSOD mimic) and catalase to determine the species of ASA-induced ROS. As shown in Fig. 4, A and D, ASA significantly increased the production of superoxide as assessed by the fluorescent intensity of MitoSOX Red. Moreover, we found that MnTMPyP (but not catalase) significantly reduced the ASA-increased fluorescent intensity of MitoSOX Red (Fig. 4, B and C), suggesting the clear involvement of superoxide in this phenomena. In addition, a merged image of MitoSOX Red with mitochondria selective fluorescent probe Mitotracker Green FM showed that ASA might induce superoxide production in mitochondria (Fig. 4D).

MnTMPyP suppressed ASA-induced increase of Caco-2 paracellular permeability. To investigate the involvement of superoxide production in ASA-increased epithelial cell permeability, we measured TEER and FD4 flux with the supplementation of MnTMPyP. As shown in Fig. 5B, MnTMPyP signi-

![Fig. 3. ASA induced reactive oxygen species (ROS) production in Caco-2. Differentiated Caco-2 cells were incubated with 10 mM of ASA for 6 h, and the fluorescent intensity of Redox Sensor Red CC-1 was measured by a fluorescent microplate reader (A) and qualified by the confocal laser-scanning microscope (B). A representative fluorescent image of three independent experiments is shown (×100). The data of fluorescent intensity of Redox Sensor are expressed as means ± SE of three separate experiments. *P < 0.05 compared with 0 mM. **P < 0.001 compared with 0 mM.](http://ajpgi.physiology.org/)}
Fig. 4. ASA-induced superoxide production in Caco-2. Differentiated Caco-2 cells were incubated with 10 mM of ASA for 1 h, and the fluorescent intensity of MitoSOX Red was measured by a fluorescent microplate reader in the absence (A) or the presence (B) of Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) or catalase (C). Data are expressed as means ± SE of three separate experiments. A: *P < 0.05 compared with 0 mM. **P < 0.001 compared with 0 mM. B: *P < 0.05 compared with 10 mM ASA. C: NS, not significant. D: differentiated Caco-2 cells were incubated with 100 μM MnTMPyP for 6 h before ASA stimulation (10 mM for 0.5 h). The image of mitochondria (MitoTracker Green: green fluorescence) in the panel on top, ROS production (MitoSOX Red: red fluorescence) in the panel in the middle, and merged (yellow fluorescence) images in the panel on the bottom were obtained using the confocal laser-scanning microscope. Representative images of three independent experiments are shown (×100).
above, we used anti-sulfenic acid antibody in a ZO-1-specific expression in Caco-2. occludin protein within Caco-2 (Fig. 7).

To further elucidate the involvement of superoxide production on ASA-decreased ZO-1 expression, we measured the expression of TJ proteins with (Fig. 6, A and C) and without (Fig. 6, B and D) the supplementation of MnTMPyP and found that MnTMPyP suppressed the ASA-induced decrease of ZO-1 expression as assessed by Western blot (Fig. 6B) and a laser-scanning confocal microscope (Fig. 6D). This result means that ASA-induced superoxide production might either: 1) reduce the expression of ZO-1; or 2) modify the ZO-1 protein such that anti-ZO-1 antibody cannot recognize its binding site on the ZO-1 protein. ASA-induced superoxide production ultimately disturbs the function of TJ proteins and increases the cell permeability of Caco-2.

ASA did not affect redistribution of occludin in Caco-2. To exclude the possibility of ASA-induced redistribution of occludin protein, we performed the immunocytochemistry for occludin and found that ASA did not induce redistribution of occludin protein within Caco-2 (Fig. 7).

ASA-induced superoxide production might modify ZO-1 expression in Caco-2. To validate the latter possibility described above, we used anti-sulfenic acid antibody in a ZO-1-specific immunoprecipitation study. Sulfenic acid is the simplest organosulfur oxyacid (in contrast to sulfenic acids and sulfonic acids). Sulfenic acid formation is a reversible posttranslational modification that may be used to monitor protein oxidation of reactive cysteine within target proteins (5). An immunoblot (Fig. 8) showed that cysteine residues of ZO-1 protein were specifically modified by oxidative stress. In addition, MnTMPyP clearly suppressed the expression of ASA-induced sulfenic acid of ZO-1 protein.

**DISCUSSION**

Although the mechanism by which conventional NSAIDs, excluding ASA, induce small intestinal mucosal injury has been extensively investigated, there has been very little research on the mechanism by which ASA induces small intestinal mucosal injury. Previously, we investigated the mechanism of ASA-induced small intestinal mucosal injury focusing on ASA-induced epithelial cell apoptosis. However, ASA does not always cause epithelial cell apoptosis in human small intestine. Rather, it is more likely that ASA increases small intestinal epithelial cell permeability as a first step. Secondary factors such as bile acid, bacteria, or foods, or other unknown factors, might induce mucosal injury. Therefore, in this study, we clarified the mechanism by which ASA induces small intestinal epithelial cell injury focusing mainly on epithelial cell permeability.

There have been no reports on the mucosal concentration of ASA in small intestine so far. Studies on the oral administration of ASA in rats have shown that ASA causes gastric injury in the stomach at concentrations between 10 and 100 mM. Therefore, the concentration of ASA used in this study (10 mM) might be physiologically relevant to those used in an in vivo study (37). Because ASA induced Caco-2 cell death in a concentration- and time-dependent manner, we selected 10 mM of ASA and added it to Caco-2 for 24 h, which did not induce cell death (data not shown). Another group has also reported that ASA does not induce Caco-2 cell death at concentrations ranging from 0.1 to 10 mM (32).

First, we assessed ASA-induced increase of epithelial cell permeability by measuring TEER and FD4 flux. We found that a nontoxic concentration of ASA increased epithelial cell permeability in a time-dependent manner (Fig. 1). Interestingly, ASA increased epithelial permeability at 3 h as assessed by TEER; however, it did not increase epithelial permeability until 6 h. This result might indicate that ASA first affects transcellular permeability before affecting paracellular permeability. Because we found that ASA clearly damaged the continuity of the ZO-1 protein (which is the major player of the TJ strand) at 3 h (Fig. 6C), another possibility is that TEER detects the loss of epithelial integrity more sensitively than FD4 flux.

Next, we assessed the involvement of COX in ASA-increased epithelial cell permeability; COX catalyzes the formation of PG from arachidonic acid (16). COX inhibition is one of the major mechanisms by which ASA increases epithelial cell permeability. ASA suppresses not only the inducible isoform of COX (COX-2) in inflammatory sites, but also the constitutive isoform (COX-1) in gastrointestinal mucosa, which has a pivotal role in mucosal cytoprotection (13). To exclude the involvement of COX in ASA-increased epithelial
cell permeability, we used PGE$_2$ in the permeability study and found that the PGE$_2$ supplementation did not suppress the increased permeability (data not shown). Although PGE$_2$ has been reported to protect paracellular permeability (8, 9), it could not suppress ASA-induced increased cell permeability in our study. The reason for this discrepancy might be dependent on the difference of stimulants, cell type, or experimental settings. Further study is needed to elucidate this issue.

Because epithelial cell permeability greatly depends on the integrity of the TJ complex, in which ZO-1 establishes a link between the transmembrane claudin family proteins, occludin, and the cytoskeleton (40), we investigated TJ protein change in ASA-stimulated Caco-2 cells. At 6 h after ASA administration, only ZO-1 expression was decreased among TJ proteins (Figs. 2A, 2B, and 7C). This is consistent with previous studies as follows. Marchiando et al. (20) reported that remarkable redistribution of ZO-1 defines TJ barrier maintenance during pathological intestinal epithelial shedding. Guan et al. (15) also reported that redistribution of ZO-1 served as an early marker of the TJ movement that sustains continual epithelial barrier function during epithelial shedding.

It has been reported that the total levels of occludin following stimulation of epithelial cells with various proinflammatory mediators did not reduce the overall levels of occludin; however, it induced membrane-to-cytoplasm redistribution of occludin, resulting in a “weakening” of the TJs (39). Therefore,
we performed the immunochemistry for occludin in ASA-stimulated Caco-2 and found that ASA did not induce redistribution of occludin protein within Caco-2 (Fig. 7), suggesting the specific modification of ZO-1 in ASA-stimulated Caco-2.

We (24) and another group (25) have reported the involvement of oxidative stress in conventional NSAID-induced gastric epithelial cell injury, and it is now a widely accepted mechanism. In regards to the small intestine, Basivireddy et al. (4) reported that IND induced functional disorder of mitochondria and ROS production in crude enterocytes obtained from the rat small intestine. We also have reported that IND-increased ROS production induces apoptosis of small intestinal epithelial cells (26). However, the involvement of ROS on ASA-increased small intestinal epithelial cell permeability is not yet clear. In this study, we found that a nontoxic concentration of ASA significantly increased ROS production (Figs. 3A, 3B, and 4A) that was suppressed by MnSOD mimetic (Fig. 4, B and D) but not catalase (Fig. 4C), suggesting the involvement of superoxide from the mitochondria.

To further investigate the relationship between ASA-induced ROS production and increased paracellular permeability, we used MnTMPyP, a SOD mimetic, which possesses the catalytic activity of enzymes and is permeable to cells. This molecule can potentially exhibit the beneficial and synergistic effects of SOD toward an excess of superoxide (18, 23). In this study, MnTMPyP significantly reduced the level of ROS production in Caco-2 (Fig. 4, B and D) and suppressed ASA-induced increase of paracellular flux (Fig. 5, A and B), suggesting that ASA-induced superoxide production might have an effect on the paracellular pathway.

In addition, we found that MnTMPyP suppressed the ASA-induced decrease of ZO-1 expression as assessed by Western blot (Fig. 6B) and a laser-scanning confocal microscope (Fig. 6D). This result means that ASA-induced superoxide production might either: 1) reduce the expression of ZO-1; or 2) modify the ZO-1 protein such that anti-ZO-1 antibody cannot recognize its binding site on the ZO-1 protein. ASA-induced superoxide production ultimately disturbs the function of TJ proteins and increases the cell permeability of Caco-2.

Rao et al. (29–31) have reported that oxidative stress induced a rapid increase in tyrosine phosphorylation of occludin and ZO-1 and increased the paracellular permeability of Caco-2 without affecting cell viability. In addition, it has been reported that cysteine can be modified by superoxide (10). Therefore, we hypothesized that ASA-induced increases in superoxide production might modify the cysteine residue of the ZO-1 protein. To clarify this issue, we measured the expression of sulfenic acid by immunoprecipitation of the ZO-1 protein. Among the amino acid residues of protein, cysteine residues are the most vulnerable to a variety of oxidative injuries. Oxidation of such cysteine is converted into signals that control cell regulatory pathways and induction of gene expression. Cysteine sulfenic (-SOH) acids were first described as transient intermediates in the further oxidation of thiols to sulfinic (-SO₂H) and sulfonic (-SO₃H) acids and in the formation of disulfides from free thiols. However, steric properties and unique electronic and intramolecular hydrogen-bonding effects have been implicated in the stabilization of sulfenic acids (11). Therefore, the stabilized sulfenic acid can be gradually accumulated in Caco-2 after the attack of oxidative stress. In this study, the cysteine residues of ZO-1 were specifically oxidized by ASA-induced superoxide increases in Caco-2, and this might have resulted in the increased permeability.

In general, the lifetime of ROS is considered to be very short, and there must be a time lag between ROS production (within 1 h) and the accumulation of sulfenic acid (within 3 h).
Accumulated sulfenic acid of ZO-1 protein might ultimately lead to the chemical modification of ZO-1 after three or more hours. However, this is our speculation, and further study is needed to prove it.

Taken together, ASA-induced changes in cell permeability and superoxide-induced modification of ZO-1 function might be mechanisms by which ASA induces small intestinal mucosal injury. Therefore, drugs that have anti-oxidative properties might be useful for the prevention of ASA-induced mucosal injury in the small intestine.

REFERENCES


