The laxative effect of bisacodyl is attributable to decreased aquaporin-3 expression in the colon induced by increased PGE2 secretion from macrophages

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Ikarashi N, Baba K, Ushiki T, Kon R, Mimura A, Toda T, Ishii M, Ochiai W, Sugiyama K. The laxative effect of bisacodyl is attributable to decreased aquaporin-3 expression in the colon induced by increased PGE2 secretion from macrophages. Am J Physiol Gastrointest Liver Physiol 301: G887–G895, 2011. First published August 25, 2011; doi:10.1152/ajpgi.00286.2011.—The purpose of this study was to investigate the role of aquaporin3 (AQP3) in the colon in the laxative effect of bisacodyl. After oral administration of bisacodyl to rats, AQP3, macrophages, cyclooxygenase 2 (COX2), and prostaglandin E2 (PGE2) were examined in the colon. The mechanism by which bisacodyl decreases the expression of AQP3 was examined using HT-29 and Raw264.7 cells. When diarrhea occurred, a significant increase in the expression of PGE2 and a decrease in AQP3 expression were observed. Immunostaining showed COX2 expression only in macrophages. The PGE2 concentration increased significantly 30 min after the addition of bisacodyl to Raw264.7 cells. Thirty minutes after PGE2 addition to HT-29 cells, the AQP3 expression level decreased to 40% of the control. When pretreated with indomethacin, bisacodyl did not induce an increase in the colon PGE2 level, a decrease in the AQP3 expression level, or diarrhea. The results suggest that bisacodyl may decrease the expression of AQP3 in the colon, which inhibits water transfer from the luminal to the vascular side and leads to a laxative effect. This study also showed that direct activation of colon macrophages by bisacodyl increases the secretion of PGE2, which acts as a paracrine factor and decreases AQP3 expression in colon mucosal epithelial cells.

AQP3; PGE2; bisacodyl; COX2

IN RECENT YEARS, IT HAS BECOME increasingly clear that aquaporins (AQPs), water channels, are involved in water transport in the intestinal tract (20). There are currently 13 known types of AQPs in humans, AQP0 through AQP12, which are expressed in a variety of tissues (15). Several AQPs are expressed in the intestinal tract, and at least the following eight types are known to exist there: AQP1, AQP2, AQP3, AQP4, AQP7, AQP8, AQP9, and AQP10 (5, 7, 17, 23). The main AQPs expressed in the colon are AQP1, AQP2, AQP3, AQP4, and AQP8 (5, 16, 23). Of these, extensive research has been conducted on AQP3, which is considered to play an important role in the colon, especially regarding water transfer (13, 34). We have found that the administration of the osmotic laxative magnesium sulfate (MgSO4) to rats increases the expression of AQP3 in the colon, and an increase in the expression of AQP3 plays an essential role in the laxative effect of MgSO4 (11, 12).

Bisacodyl is classified as a stimulant laxative and is widely used to treat constipation. Bisacodyl increases the production of prostaglandin E2 (PGE2) in intestinal epithelial cells and inhibits the activity of Na+-K+-ATPase, and, as a result, the osmotic pressure in the intestinal tract increases. It is believed that this increase in osmotic pressure causes an increase in the secretion of electrolytes, such as Na+ and K+, and water in the intestinal tract, which contributes to the laxative effect of bisacodyl (28, 31). However, many details regarding water transfer in the colon have not yet been elucidated. In this study, the following examinations were conducted to investigate the role of AQPs in the colon in the laxative effect of bisacodyl. The distribution and localization of expression of each AQP in the colon of rats were first examined. By analyzing the expression of AQP3 in the colons of rats that were administered bisacodyl, the relationship between the expression level of AQP3 and the laxative effects of bisacodyl was investigated. In addition, the mechanism of the changes induced by bisacodyl in the expression of AQP3 in the colon was examined.

MATERIALS AND METHODS

Materials. Bisacodyl, tumor necrosis factor (TNF)-α, indomethacin, and 4’,6-diamidino-2-phenylindole (DAPI) solution were purchased from Wako Pure Chemicals (Osaka, Japan). BSA and TRI reagent were purchased from Sigma-Aldrich (St. Louis, MO). PGE2 was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-rat AQP1 antibody, rabbit anti-rat AQP2 antibody, rabbit anti-rat AQP3 antibody, rabbit anti-rat AQP4 antibody, and rabbit anti-rat AQP8 antibody were purchased from Abcam (Cambridge, UK). Mouse anti-rat macrophage/dendritic cell monoclonal antibody was purchased from Trans Genic (Kobe, Japan). Alexa Fluoro 488 donkey anti-rabbit IgG, Alexa Fluoro 555 donkey anti-mouse IgG, and primers were purchased from Invitrogen (Tokyo, Japan). Anti-rabbit IgG-horseradish peroxidase (HRP) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An enhanced chemiluminescence (ECL) system plus Western blotting detection reagent was purchased from GE Healthcare (Chalfont St. Giles, UK). A high-capacity cDNA synthesis kit was purchased from Applied Biosystems ( Foster City, CA), and iQ SYBR green supermix was purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents were of the highest commercially available grade.

Animals. Male Wistar rats (10 wk old) were purchased from Japan SLC (Shizuoka, Japan). Rats were kept at room temperature (24 ±
1°C and 55 ± 5% humidity with 12 h of light (artificial illumination: 08:00–20:00). The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and was approved by the Committee on Animal Research at Hoshi University.

Treatment. Rats were fasted for 18 h before bisacodyl administration (water was provided ad libitum). An aqueous solution of bisacodyl (20 mg/kg body wt) was administered orally to rats. Rats were autopsied under ether anesthesia immediately after treatment and at 1, 2, 3, 5, 8, and 12 h after bisacodyl administration, and the colon and spleens were removed. In addition, indomethacin (10 mg/kg) or saline was administered intraperitoneally to rats 15 min before oral administration of bisacodyl, and the colons were removed 2 h after bisacodyl administration. After the colon was washed with PBS (140 mM NaCl, 20 mM NaH2PO4, 32 mM KCl, and 1.5 mM KH2PO4, pH 7.4), samples were flash-frozen with liquid nitrogen and stored at −80°C. Fecal samples from the rats were collected for up to 8 h after the administration of bisacodyl and were placed in silica gel followed by drying for 24 h in a desiccator. The water content per gram of feces was calculated based on the difference between wet and dry fecal weights.

Immunohistochemistry. The rats were anesthetized with ether, and their hearts were perfused with PBS. They were then perfused with 50 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. The colon was removed and postfixed in 4% PFA in PBS for 1 h at 4°C. Samples were immersed in 30% sucrose in PBS overnight at 4°C and embedded in optimum cutting temperature compound (Sakura Finetek, Torrance, CA). Frozen sections were cut with a cryostat (Leica Microsystems, Tokyo, Japan) at 10 μm and mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan). The sections were washed with PBS, blocked with a blocking solution (PBS containing 3% FBS and 0.1% Triton X-100) for 1 h, and incubated overnight at 4°C in a mixture of primary antibodies. The following primary antibodies were used: rabbit anti-rat AQP1 (1:200), rabbit anti-rat AQ2P (1:200), rabbit anti-rat AQP3 (1:200), rabbit anti-rat AQP4 (1:200), rabbit anti-rat COX-2 (1:100), and mouse anti-rat macrophage/dendritic cells (1:25). After being washed three times with PBS, the sections were reacted with secondary antibodies [Alexa Fluoro 488 donkey anti-rabbit IgG (1:200) and Alexa Fluoro 555 donkey anti-mouse IgG (1:200)] at room temperature for 1 h. After being washed with PBS, the sections were reacted with DAPI solution (1:500) in PBS at room temperature for 30 min, washed three more times with PBS, and covered with a Vectashield (Vector Laboratories, Burlingame, CA). The immunostained sections were observed under a microscope (BZ-9000; Keyence, Tokyo, Japan).

Cell culture of Raw264.7 cells. The Raw264.7 cells (DS Pharma Biomedical, Tokyo) were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin G potassium, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were plated on a 100-mm dish at a density of 2 × 10⁶ cells/cm², incubated in a CO2 incubator at 37°C for 36 h, and treated with bisacodyl (10 μg/ml), TNF-α (10 μg/ml), and PGE2 (10 μM) dissolved in culture medium for 30 min, 2 h, or 6 h. The cells were collected using a cell scraper, and the expression of AQP3 protein was measured using Western blotting. Cells passed 5 to 15 times were used in the experiments.

Cell culture of HT-29 cells. The HT-29 cells (DS Pharma Biomedical) were maintained in RPMI (1640 medium supplemented with 10% FBS, 100 U/ml penicillin G potassium, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were plated on a 100-mm dish at a density of 2 × 10⁶ cells/cm², incubated in a CO2 incubator at 37°C for 36 h, and treated with bisacodyl (10 μg/ml), TNF-α (10 μg/ml), and PGE2 (10 μM) dissolved in culture medium for 30 min, 2 h, or 6 h. The cells were collected using a cell scraper, and the expression of AQP3 protein was measured using Western blotting. Cells passed 5 to 15 times were used in the experiments.

RNA preparation from tissue samples. RNA was extracted from ~15 mg of frozen colon or Raw264.7 cells using TRI reagent. The resulting solution was diluted 50-fold using TE buffer, and the purity and concentration (μg/ml) of RNA were calculated by measuring the absorbance at 260 and 280 nm using a U-2800 spectrophotometer (Hitachi High Technologies, Tokyo, Japan).

Real-time RT-PCR. A high-capacity cDNA synthesis kit was used to synthesize cDNA from 1 μg of RNA. TE buffer was used to dilute the cDNA 20-fold to prepare the cDNA TE buffer solution. The expression of target genes was detected by preparing the primers listed in Table 1 and by performing real-time RT-PCR. To each well of a 96-well PCR plate, 25 μl of iQ SYBR green supermix, 3 μl of forward primer of the target gene (5 pmol/μl), 3 μl of reverse primer (5 pmol/μl), 4 μl of cDNA TE buffer solution, and 15 μl of RNase-free water were added. For 18S rRNA, a housekeeping gene, 4 μl of a cDNA TE buffer solution, which was prepared by diluting the above-mentioned solution 20-fold using TE buffer, was used. A denaturation temperature of 95°C for 15 s, an annealing temperature of 56°C for 30 s, and an elongation temperature of 72°C for 30 s were used. The fluorescence intensity of the amplification process was monitored using the My iQ single-color real-time RT-PCR detection system (Bio-Rad Laboratories). The mRNA expressions were normalized using 18S rRNA or GAPDH.

Preparation of fraction for immunoblotting from colon. The large intestinal mucosa was scraped with a slide glass and homogenized (1,250 rpm, 5 strokes) in dissecting buffer [0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 μM phenylmethylsulfonyl fluoride (PMSF), pH 7.2] on ice. The homogenate was centrifuged (800 g at 4°C for 15 min), and the resulting supernatant (supernatant A) was used to prepare the crude membrane (CM), plasma membrane (PM), and intracellular vesicle (IV)

Table 1. Primer sequences of mRNA

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<th>Gene</th>
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<th>Reverse (5'→3')</th>
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<td>rIL-6</td>
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r. Rat; TNF-α, tumor necrosis factor-α; IL, interleukin; COX, cyclooxygenase; m, mouse.

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fractions. Supernatant A was centrifuged (200,000 g at 4°C for 1 h), and the supernatant was removed. Dissecting buffer was added to the precipitate, and the precipitate was homogenized using an ultrasonic homogenizer (UH-50; SMT, Tokyo, Japan). This homogenate contained the CM fraction, which contained the cell membrane. Supernatant A was centrifuged (17,000 g at 4°C for 30 min), and the supernatant was removed. Dissecting buffer was added to the precipitate, which was homogenized using an ultrasonic homogenizer, to yield the plasma membrane-enriched PM fraction. Supernatant B was further centrifuged (200,000 g at 4°C for 1 h), and the supernatant was removed. Dissecting buffer was added to the precipitate and homogenized using an ultrasonic homogenizer to yield the intracellular vesicle-enriched IV fraction.

Preparation of the CM fraction for immunoblotting HT-29 cells. HT-29 cells were recovered with a cell scraper and suspended in lysis buffer (10 mM Tris, 150 mM NaCl, 8.5 μM leupeptin, 1 μM PMSF, and 0.5% Nonidet P-40, pH 7.2). The cell suspension was homogenized using an ultrasonic homogenizer on ice, and the homogenate was centrifuged (15,000 g for at 4°C for 10 min). The supernatant was collected as the CM fraction.

Electrophoresis and immunoblotting. Protein concentrations were measured by the BCA method using BSA as a standard. Electrophoresis was performed using Laemmli’s method. With the use of the loading buffer (84 mM Tris, 20% glycerol, 0.004% bromphenol blue, 4.6% SDS, and 10% 2-mercaptoethanol, pH 6.3), 6 μg of protein were diluted twofold and were applied to a polyacrylamide gel. After electrophoresis, the isolated proteins were transferred to a polyvinylidene difluoride membrane. After being blocked for 1 h using 1% skim milk, the resulting membrane was reacted for 1 h at room temperature with rabbit anti-rat AQP3 antibody (1:500 for rat colon; 1:100 for HT-29 cells). After the membrane was washed with TBS-Tween (20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween 20, pH 7.6), the resulting membrane was reacted for 1 h at room temperature with anti-rabbit IgG-HRP antibody (1:5,000). After the membrane was washed, the membrane was reacted with the ECL Plus detection reagent and visualized with the LAS-3000 mini (Fuji Film, Tokyo, Japan) luminoimage analyzer.

Measurement of the PGE2 concentration in the rat colon. The PGE2 concentration in the rat colon was analyzed using the prostaglandin E2 EIA kit (Cayman Chemical). The extraction of PGE2 was performed according to the protocol for the PGE2 EIA kit. Briefly, ~200 mg of rat colon were homogenized in 1 ml of homogenate buffer (PBS containing 1 mM EDTA and 10 μM indomethacin, pH 7.4) on ice. The homogenate was centrifuged (8,000 g at 4°C for 10 min), and the supernatant was analyzed.

Statistical analysis. Numerical data are expressed as means ± SD. The significance of the differences was examined using Student’s t-test for pairs of values and Dunnett’s or Tukey’s test for multiple comparisons. Results with P < 0.05 were considered to be significant.

RESULTS

Immunohistochemical localization of AQP proteins in the rat colon. It is known that AQP1, AQP2, AQP3, AQP4, and AQP8 are present in the colons of rats (5, 16, 23). We confirmed the distribution of AQPs in the rat colon using immunostaining. In the colon, AQP3 was expressed most dominantly in mucosal epithelial cells. AQP1 was expressed around lymphatic and blood vessels, whereas AQP4 was expressed in the submuscular layer. The expressions of AQP2 and AQP8 were low in the colon (Fig. 1A).

It was previously known that AQP3 are expressed in large quantities on the basal side of mucosal epithelial cells in the colon (22); however, the results of this study show that AQP3 is expressed on both the apical and basal sides of mucosal epithelial cells in the colon (Fig. 1B).

Changes in the fecal water content and the expression of AQP3 in the colon caused by bisacodyl administration. The changes in the fecal water content over time after the administration of bisacodyl to rats are shown in Fig. 2A. The fecal water content increased over time after bisacodyl administration and reached statistical significance 2 h after administra-

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**Fig. 1.** Analysis of the expression distribution of aquaporins (AQPs) in the colons of rats. A: colons were removed from the rats. AQPs (green) and nuclei (blue) were immunostained. B: enlarged view of AQP3 in the mucosal epithelial cells of rats.
tion. Between 4 and 8 h after bisacodyl administration, the fecal water content increased remarkably from ~8 to 14 times higher than baseline levels (0 h), and severe diarrhea was observed during that time.

The time course of the change in the protein expression of AQP3, which was most dominantly expressed in mucosal epithelial cells in the colon, was examined by Western blotting (Fig. 2, B–D). Two AQP3 protein bands were detected. One of these appeared at ~27 kDa and represented the deglycosylated form of AQP3, whereas the other appeared at ~30–40 kDa and represented a glycosylated form of AQP3 (32, 33). This glycosylation is associated with the stability and intracellular translocation of AQP3 but has no influence on water permeability (1, 8, 35). Therefore, in this study, the sum of these bands was analyzed as the protein expression level of AQP3. The protein expression level of AQP3 in the colon CM fraction began to decrease significantly 2 h after the administration of bisacodyl and decreased by ~80% 5 h after administration relative to the initial expression (0 h). The expression level of AQP3 was ~50% lower than immediately after administration, even at 8 h after administration (Fig. 2B). The changes in the protein expression levels of AQP3 in the colon PM and IV fractions were similar to the CM fraction (Fig. 2, C and D). The result of the immunostaining analysis also showed that the level of AQP3 expressed in mucosal epithelial cells in the colon decreased over time. In addition, it was shown that a decrease in the expression of colon AQP3, which was attributed to bisacodyl administration, was observed on both the apical and basal sides (Fig. 3).

Time course of the changes in the expression level of inflammatory cytokines and COX in the colon caused by bisacodyl administration. The mRNA expression level of TNF-α in the rat colon increased significantly to ~2.3 times higher than the baseline levels 2 h after the administration of bisacodyl. The mRNA expression level of interleukin (IL)-1β and IL-6 also increased significantly to approximately two times higher than the baseline level 2 and 3 h after the administration of bisacodyl (Fig. 4).

No changes were observed in the mRNA expression level of COX1 in the colon of rats. In contrast, the mRNA expression levels of COX2 increased significantly to ~2.2 and 3 times higher than the baseline levels at 2 and 3 h after the administration of bisacodyl, respectively (Fig. 4).

The expression and localization of COX2 in the colon after the administration of bisacodyl were examined by double-staining COX2 protein and macrophages. The result indicated that the macrophages, which are localized in the mucosa of the colon, predominantly expressed COX2 2 h after the administration of bisacodyl (Fig. 5).

Effect of bisacodyl on Raw264.7 cells. When macrophages are activated, the expression and secretion of TNF-α increase, which causes an increase in the expression of COX2 and eventually leads to the secretion of PGE2 (6, 18). The effect of bisacodyl on the activation of macrophages was examined using Raw264.7 cells (Fig. 6).

The mRNA expression levels of TNF-α at 0.5 and 2 h after the addition of bisacodyl to Raw264.7 cells increased significantly to ~2.2 and 6 times higher than baseline levels (0 h), respectively (Fig. 6A). The concentrations of TNF-α in the
culture supernatant at 0.5 and 2 h after the addition of bisacodyl increased significantly to ~1.8 and 8 times higher than baseline levels, respectively (Fig. 6C).

The mRNA expression levels of COX2 at 0.5 and 2 h after the addition of bisacodyl to Raw264.7 cells increased significantly to ~1.8 and 6 times higher than baseline levels, respectively (Fig. 6B).

The concentrations of PGE2 in the culture supernatant at 0.5 and 2 h after the addition of bisacodyl to Raw264.7 cells were ~1.6 and 2.2 times higher than baseline levels, respectively (Fig. 6D).

Effects of bisacodyl, TNF-α, and PGE2 on the protein expression level of AQP3 in HT-29 cells. Bisacodyl, TNF-α, and PGE2 were added to HT-29 cells, which were derived from
human colon cancer and are widely used to investigate the mechanism of laxatives and diarrhea development (14, 26) to examine the effects on the protein expression level of AQP3 (Fig. 7).

The protein expression levels of AQP3 at 0.5 and 2 h after the addition of bisacodyl to HT-29 cells did not show a significant difference compared with baseline (0 h) (Fig. 7A).

The protein expression levels of AQP3 at 0.5 and 2 h after the addition of TNF-α to HT-29 cells were not significantly different compared with baseline levels. Alternatively, a significant decrease in the protein expression level of AQP3 was observed 6 h after the addition of TNF-α (Fig. 7B).

The protein expression level of AQP3 decreased significantly to ~40% of the levels immediately after the addition of PGE2 to HT-29 cells as early as 0.5 h after the addition (Fig. 7C).

Changes in the fecal water content, colon PGE2 concentration, and protein expression level of AQP3 caused by bisacodyl administration to rats pretreated with indomethacin. The changes in the laxative effect of bisacodyl caused by pretreatment with indomethacin, which is a COX inhibitor, were examined (Fig. 8).

The fecal water content of rats 2 and 5 h after bisacodyl administration were almost equal to that immediately after the administration (0 h), and no occurrence of diarrhea was observed (Fig. 8A).

The PGE2 concentration in the rat colon 2 h after bisacodyl administration alone was ~1.8 times higher than the control group. When pretreated with indomethacin, little PGE2 production was observed in the colon (Fig. 8B).

In the rats pretreated with indomethacin, the protein expression level of AQP3 in the CM fraction of the colon 2 h after bisacodyl administration was nearly equal to the control group (Fig. 8C).

DISCUSSION

Because tight junctions in the epithelial cells of the colon are rigid, AQPs play an important role in water transfer in the colon (20). AQP1, AQP2, AQP3, AQP4, and AQP8 are known to be expressed in the mucosal epithelial cells of the colon in rats (5, 16, 23). Immunostaining analysis revealed that AQP3 is predominantly expressed in the mucosal epithelial cells of the colon in rats on both the apical and basal sides of the cells (Fig. 1). Based on this result, it was considered that AQP3 is the most important AQp for water transfer in the mucosal epithelial cells of the rat colon. Therefore, we examined the role of AQP3 in the laxative effect of bisacodyl. When bisacodyl was administered orally to rats, the protein expression levels of AQP3 in the CM, PM, and IV fractions all began to decrease 2 h after administration (Fig. 2), and these decreases were observed on both the apical and basal sides of the cells (Fig. 3).

Furthermore, a good correlation was observed between this decrease in the AQP3 expression level and an increase in fecal water content. These results suggest the possibility that bisacodyl administration causes a decrease in the level of AQP3 in the mucosal epithelial cells in the colon, which eventually leads to diarrhea.

Why does a decrease in the expression level of AQP3 lead to diarrhea? Under normal physiological conditions, the osmotic pressure in the lumen of the colon is lower than on the vascular side (27). Therefore, water is transferred from the intestinal tract to the vascular side of the cells, which results in fecal concentration. As mentioned earlier, water transfer is primarily mediated by AQP3. Therefore, when the expression level of AQP3 decreases, water transfer from the intestinal tract to the vascular side of the cells decreases, resulting in diarrhea. As mentioned earlier, water transfer is primarily mediated by AQP3. Therefore, when the expression level of AQP3 decreases, water transfer from the intestinal tract to the vascular side of the cells decreases, resulting in diarrhea.
which causes water retention in the intestinal tract and leads to diarrhea. Wang et al. (37) reported that, in knockout mice deficient in AQP4, which is predominantly expressed in the mucosal epithelial cells of the mouse colon, the fecal water content was higher than that of wild-type mice, even under normal physiological conditions. This indicates the possibility that a decrease in AQPs causes an increase in fecal water content, which supports the view suggested by this study.

We examined the mechanism by which bisacodyl decreases the expression level of AQP3. The possibility that bisacodyl acted directly on the mucosal epithelial cells of the colon and decreased the expression level of AQP3 was examined using HT-29 cells derived from human colon cancer cells. HT-29 cells have been widely used to study the mechanisms of diarrhea and laxative effects because HT-29 cells represent the normal physiological condition of the colon despite the fact that they are cancer cell lines derived from human colon cancer (4). The addition of bisacodyl to HT-29 cells exerted no effect on the protein expression level of AQP3, even 2 h after the addition (Fig. 7A). Therefore, it is unlikely that bisacodyl acts directly on the mucosal epithelial cells of the colon and decreases the expression level of AQP3.

We examined the possibility that bisacodyl indirectly decreases the expression level of AQP3 in the colon. Bisacodyl administration to rats pretreated with indomethacin increased the fecal water content, colon PGE2 concentration, and protein expression level of AQP3 (Fig. 8). The fecal water content and protein expression level of AQP3 are shown with the mean value immediately after administration (0 h) as 100%. Data represent means ± SD for 6 rats. Tukey’s test: **P < 0.01 and ***P < 0.001 vs. 0 h or control. ##P < 0.01 and ###P < 0.001 vs. rats treated with bisacodyl alone at each hour.
AQP3 was observed within 30 min of PGE2 addition (Fig. 9). Furthermore, TNF-α (9, 19, 38) and PGE2 (25, 39) decrease the expression levels of AQPs. Based on this, we examined whether bisacodyl directly activated macrophages and caused the secretion of TNF-α and PGE2 using Raw264.7 cells. When bisacodyl was added to Raw264.7 cells, the secretion of TNF-α, the expression level of COX2, and the secretion of PGE2 increased (Fig. 6). When bisacodyl was administered to rats and diarrhea occurred, the mRNA expression levels of inflammatory cytokines such as TNF-α, the mRNA expression level of COX2 (Fig. 4), and the secretion of PGE2 (Fig. 8B) increased in the colon. The increase in COX2 expression was observed predominantly in macrophages when diarrhea occurred (Fig. 5). These results indicated that bisacodyl activated macrophages and increased the secretion of TNF-α and PGE2.

We proceeded to examine whether TNF-α and PGE2 acted on the mucosal epithelial cells of the colon as paracrine factors and decreased the expression of AQP3. Horie et al. (9) reported that the protein expression level of AQP3 started to decrease 6 h after the addition of TNF-α to keratinocytes. Our results showed that the expression level of AQP3 did not change 2 h after the addition of TNF-α and started to decrease as late as 6 h after addition to HT-29 cells (Fig. 7B). The increase in TNF-α and the decrease in AQP3 in the colon were observed at nearly the same time (Figs. 2 and 4). Therefore, it is considered that, although TNF-α, which was secreted by macrophages, is involved in the increased expression of COX2 as an autocrine factor, the probability that TNF-α acted on the mucosal epithelial cells of the colon as a paracrine factor and caused an immediate decrease in the expression of AQP3 is low. When PGE2 was added to the HT-29 cells, the expression level of AQP3 decreased dramatically. This decrease in the expression of AQP3 was observed within 30 min of PGE2 addition (Fig. 7C). Based on these observations, PGE2, which was secreted by macrophages, acted on the mucosal epithelial cells of the colon as a paracrine factor and decreased the expression of AQP3.

Finally, we examined whether the laxative effect of bisacodyl is decreased by pretreating the rats with the COX inhibitor indomethacin. The results showed that the laxative effect of bisacodyl was inhibited by the administration of indomethacin along with the decrease in the protein expression of AQP3 in the mucosal epithelial cells in the colon (Fig. 8). This result indicates the possibility that PGE2 is involved in the drastic and immediate decrease in AQP3 in the mucosal epithelial cells in the colon induced by bisacodyl administration. Although the mechanism by which PGE2 decreases AQP3 is not yet clear, it may increase the endocytosis and degradation of AQP3 (25, 39).

Previously, the following possibilities were suggested as the mechanism of the development of diarrhea caused by bisacodyl. Because bisacodyl increases the production of PGE2, the activity of Na⁺-K⁺-ATPase in the mucosal epithelial cells in the colon is inhibited, and the concentrations of Na⁺ and K⁺ in the lumen increase. As a result, the osmotic pressure in the lumen increases, and water is transferred from the vascular side to the luminal side of the cells, which eventually leads to the laxative effect (28, 31). However, even if the osmotic pressure in the colon increases via the administration of bisacodyl, as indicated in the above-mentioned mechanism, water transfer from the luminal side of the cells is decreased under the condition of decreased expression level of AQP3 in the colon. This occurs because the rate of water transfer via AQPs depends more on the expression level of AQPs on the cell surface than on the difference in the osmotic pressure between the inside and outside of the cells (21, 36). Therefore, it is more appropriate to regard the mechanism of the laxative effect of bisacodyl as inhibited water absorption caused by a decrease in the expression of AQP3 in the colon than increased water secretion caused by an increase in the osmotic pressure in the intestinal tract.

In summary, bisacodyl may exhibit its laxative effect by inhibiting water transfer from the intestinal tract to the vascular side of the cells by decreasing the expression of AQP3 in the colon. Bisacodyl may directly activate macrophages and increase the production and secretion of PGE2. Furthermore, PGE2 may act on the mucosal epithelial cells in the colon as a paracrine factor and decrease the expression of AQP3 (Fig. 9). The results of this study suggest that compounds that decrease the expression of AQP3 in the colon or activate macrophages may exhibit a laxative effect by increasing the fecal water content. The efficacy of compounds that activate macrophages is decreased by continued use (2, 3). Therefore, the continued use of bisacodyl may lead to a decrease in its efficacy. Nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and celecoxib, inhibit COX. Therefore, the concomitant use of bisacodyl and NSAIDs may decrease the efficacy of bisacodyl. The novel findings presented in this study will be important in the proper use and development of laxatives.

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![Suggested main mechanisms of the laxative effect of bisacodyl.](http://ajpgi.physiology.org/
EFFECT OF BISACODYL ON AQP3 EXPRESSION IN COLON

DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES


