Morphine-Induced Constipation Develops With Increased Aquaporin-3 Expression in the Colon via Increased Serotonin Secretion


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ABSTRACT

Aquaporin-3 (AQP3) is a water channel that is predominantly expressed in the colon, where it plays a critical role in the regulation of fecal water content. This study investigated the role of AQP3 in the colon in morphine-induced constipation. AQP3 expression levels in the colon were analyzed after oral morphine administration to rats. The degree of constipation was analyzed after the combined administration of HgCl2 (AQP3 inhibitor) or fluoxetine (5-HT reuptake transporter [SERT] inhibitor) and morphine. The mechanism by which morphine increased AQP3 expression was examined in HT-29 cells. AQP3 expression levels in rat colon were increased during morphine-induced constipation. The combination of HgCl2 and morphine improved morphine-induced constipation. Treatment with morphine in HT-29 cells did not change AQP3 expression. However, 5-HT treatment significantly increased the AQP3 expression level and the nuclear translocation of peroxisome proliferator-activated receptor gamma (PPARγ) 1 h after treatment. Pretreatment with fluoxetine significantly suppressed these increases. Fluoxetine pretreatment suppressed the development of morphine-induced constipation and the associated increase in AQP3 expression in the colon. The results suggest that morphine increases the AQP3 expression level in the colon, which promotes water absorption from the luminal side to the vascular side and causes constipation. This study also showed that morphine-induced 5-HT secreted from the colon was taken into cells by SERT and activated PPARγ, which subsequently increased AQP3 expression levels.

Key words: aquaporin-3; morphine; constipation; serotonin; fluoxetine
administration because morphine-induced constipation may deteriorate the patient's quality of life and disturb the continuous administration of morphine.

Currently, laxatives, such as osmotic and stimulant laxatives, are empirically used as a symptomatic therapy in patients with morphine-induced constipation (Camilleri, 2011; Herndon et al., 2002). However, treatment for morphine-induced constipation is often difficult with current laxatives (Larkin et al., 2008; McNicol et al., 2003). Morphine-induced constipation likely occurs because of an inhibition of peristaltic movements of the bowel (De Luca and Coupar, 1996; Kaufman et al., 1988; Manara et al., 1986), but other mechanisms remain poorly understood. It is very important to understand the causal factors for morphine-induced constipation and elucidate all of the aspects of these factors in the development of a therapeutic drug with a novel mechanism or in the proposal of novel therapies and prophylaxes.

Recently, aquaporins (AQPs), which are water channels, were identified as water transporters in the intestinal tract (Loo et al., 2002; Ma and Verkman, 1999). There are currently 13 types of AQPs in humans, AQP0 through AQP12, which are expressed in various tissues (King et al., 2004). Several AQPs are also expressed in the intestinal tract. The major AQPs in the colon, where it plays a critical role in the regulation of fecal water content, include AQP1, AQP2, AQP3, AQP4, and AQP8 (Gallardo et al., 2001; Koyama et al., 1999; MatsuZaki et al., 2004). We revealed that AQP3 is predominantly expressed in mucosal epithelial cells of the colon (Ikarashi et al., 2011c). We also showed that a decrease in AQP3 function or expression causes diarrhea because of decreased water absorption from the luminal side to the vascular side of the colon (Ikarashi et al., 2011a, c; 2012a, b, Kon et al., 2014). These reports indicate that AQP3 in the colon plays a critical role in the regulation of fecal water content.

This study focused on AQP3 and investigated the role of AQP3 in the colon during morphine-induced constipation. The mechanism of changes in AQP3 expression in the colon was also examined.

MATERIALS AND METHODS

Materials. Morphine hydrochloride was purchased from Shionogi & Co., Ltd. (Tokyo, Japan). Ketanserin tartrate, mercury (II) chloride (HgCl₂), and 4',6-diamidino-2-phenylindole (DAPI) solution were purchased from Wako Pure Chemicals (Osaka, Japan). Morphine-6-glucuronide (M-6-G), morphine-3-glucuronide (M-3-G), bovine serum albumin, and TRI reagent were purchased from Sigma-Aldrich Corp. (St. Louis, Missouri). Fluoxetine hydrochloride was purchased from LKT Laboratories, Inc. (St. Paul, Minnesota). RS39604 was purchased from Tocris (Ellisville, Missouri). Rabbit anti-rat serotonin (5-hydroxytryptamine; 5-HT) antibody was purchased from Alomone Labs (Jerusalem, Israel). Rabbit anti-rat AQP3 antibody was purchased from Tocris (Ellisville, Missouri). Alexa Fluor 488 donkey anti-rabbit IgG antibody and a DAPI solution. Immunohistochemistry. The colon was postfixed in 4% paraformaldehyde in PBS. The samples were immersed in 30% sucrose in PBS and subsequently embedded. The embedded blocks were frozen, and the frozen blocks were cut using a cryostat (Leica Microsystems, Tokyo, Japan) at 10 μm. The sections were reacted with a rabbit anti-rat AQP3 antibody and a rabbit anti-rat 5-HT antibody. The sections were treated with an Alexa Fluor 488 donkey anti-rabbit IgG antibody and a DAPI solution. The immunostained sections were observed under a fluorescence microscope (FV1200 IX83, Olympus Corporation, Tokyo, Japan).

Cell culture of HT-29 cells. HT-29 cells (DS Pharma Biomedical Co., Tokyo, Japan) were maintained in RPMI 1640 medium containing 10% fetal bovine serum. The cells were plated, and subconfluent cells were treated with morphine (0–1000 μM), M-3-G (0–1000 μM), M-6-G (0–10 μM), or 5-HT (0–10 μM) for 1, 3, or 6 h. HT-29 cells were treated with ketanserin (0.3 μM), Y-25130 (0.3 μM), RS39604 (0.3 μM), or fluoxetine (2.5–10 μM) for 1 h followed by 5-HT (1 μM) for an additional 1 h. The cells were passed 5–15 times before experiments.

Real-time RT-PCR. RNA was extracted from rat colon or HT-29 cells using the TRI reagent. A high-capacity cDNA synthesis kit was used to synthesize cDNA from 1 μg of RNA. Target gene expression was analyzed using real-time PCR using the primers listed in Table 1. Real-time PCR was conducted at 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. The amplification fluorescence intensity was monitored using the MyiQ single-color real-time RT-PCR detection system.
Preparation of fractions from colon for immunoblotting. Rat colon mucosa was homogenized in dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM ethylenediaminetetraacetic acid, 8.5 μM leupeptin, and 1 μM phenylmethylsulfonyl fluoride; pH 7.2). The homogenate was centrifuged (800 g at 4°C for 15 min), and the resulting supernatant (Supernatant A) was used. Supernatant A was centrifuged (200 000 g at 4°C for 1 h), and the precipitate included the crude membrane (CM) fraction that contained the total cell membrane. Supernatant A was centrifuged (17 000 g at 4°C for 30 min), and supernatant (Supernatant B) was removed. The precipitate included the plasma membrane (PM)-enriched fraction. Supernatant B was further centrifuged (200 000 g at 4°C for 1 h), and the precipitate included the intracellular vesicle (IV)-enriched fraction.

Preparation of nuclear extraction from HT-29 cells for immunoblotting. Nuclear protein extracts from HT-29 cells were prepared to examine PPARγ nuclear translocation. Proteins were extracted according to the NE-PER nuclear extraction kit protocol.

Electrophoresis and immunoblotting. The samples were separated using SDS-PAGE electrophoresis, and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked in blocking buffer and incubated with the following primary antibodies: rabbit anti-rat AQP3 antibody, rabbit anti-human PPARγ, or goat anti-human Lamin B. The membrane was incubated with secondary antibodies and detected by the ECL plus detection reagent. The proteins were visualized using an LAS-3000 Mini Lumino image analyzer (Fuji Film, Tokyo, Japan).

Statistical analyses. Numerical data are expressed as the mean ± standard deviation. The significance of differences was examined using Student’s t test for pairs of values and Dunnett’s or Tukey’s test for multiple comparisons. The results with a P < .05 were considered significant.

RESULTS

Changes in Fecal Water Content and AQP3 Expression Level in the Colon After Morphine Administration

The number of fecal pellets, pellet weight, and fecal water content up to 12 h after morphine administration were measured (Fig. 1). The number of fecal pellets after morphine treatment decreased significantly compared with the control group, and the weight of fecal pellets also decreased markedly in this group (Figs. 1A and 1B). Fecal water content decreased gradually 1 h after morphine administration, and it reached its lowest value 5 h after administration, which was ~60% of the control group (0 h) (Fig. 1C). These findings confirmed that morphine treatment caused constipation in rats.

AQP3 is predominantly expressed in mucosal epithelial cells of the colon, and it plays a critical role in the regulation of fecal water content (Ikarashi et al., 2012a). We analyzed AQP3 expression levels in the colon after morphine administration (Figs. 2 and 3). AQP3 mRNA expression level in the colon increased significantly 1 h after morphine treatment, and it increased ~2-fold of the expression before administration 5 h postadministration (Fig. 2A). Western blotting detected two AQP3 protein bands. One of these bands appeared at ~27 kDa and represented the deglycosylated form of AQP3. The other appeared at ~30-40 kDa and represented a glycosylated form of AQP3 (Silberstein et al., 1999; Spector et al., 2002). Glycosylation was associated with the stability and intracellular translocation of AQP3, but it does not influence water permeability (Baumgarten et al., 1998; Hendriks et al., 2004; Umenishi et al., 2005).

Therefore, the sum of these bands was measured as the protein expression level of AQP3 in this study. AQP3 protein expression level in the CM fraction of the colon showed a 2-fold increase 1 h after morphine administration, which persisted until 5 h after administration (Fig. 2B). AQP3 protein expression levels in the PM and IV fractions were also similar to the CM fraction (Figs. 2C and 2D). Increased AQP3 expression with morphine was observed at the apical and basal sides of mucosal epithelial cells of the colon (Fig. 3).

These findings revealed that AQP3 expression level in the colon increased during morphine-induced constipation.

Improved Effect of HgCl2 Co-administration on Morphine-induced Constipation

Mercury compounds, including HgCl2, inhibit the water permeability of AQP3 in vitro (Ishibashi et al., 1994; Kuwahara et al., 1997). In addition, we revealed that the intrarectal administration of HgCl2 to rats, at a concentration that inhibits ~70% of the water permeability of AQP3, also inhibits water transport from the intestinal tract into blood vessels and causes severe diarrhea within 1 h after administration (Ikarashi et al., 2012a). Therefore, we investigated whether increased AQP3 expression in the colon contributes to the onset of morphine-induced constipation using HgCl2 treatment. In brief, HgCl2 was administered intrarectally to rats 30 min after morphine administration, and the degree of constipation was studied up to 1.5 h after morphine administration. The results showed that the concomitant administration of morphine and HgCl2 significantly increased the number and weight of fecal pellets compared with morphine administration alone (Figs. 4A and 4B).

These findings confirmed that the increase in the AQP3 expression in the colon contributed to the onset of morphine-induced constipation.

Effects of Morphine and Morphine Metabolites on AQP3 mRNA Expression Levels in HT-29 Cells

UDP-glucuronosyltransferase in the liver metabolizes most orally administered morphine to nonanalgesic M-3-G and strongly analgesic M-6-G (Hoffman et al., 1997; Sawe et al., 1985).

| Table 1. Primer sequences for real-time PCR |
| --- | --- | --- |
| Gene | Forward (5′-3′) | Reverse (5′-3′) |
| rAQP3 | CCCCTTGTAGTGCCTCCTC | CCCTAGGTGGCGAGATTC |
| r18S rRNA | GCTCTGAGTACCCCTTACAGT | AGCTATGAGCCGCACTTAC |
| hAQP3 | AGACACGCCCTTCAGGATTTC | TCCCTGCGCTGAATATC |
| hGAPDH | ATGGGGAAGGTTGAGCTCG | GGCTCATATTGAGCGAACATA |

(Bio-Rad Laboratories). The mRNA gene expression levels were normalized to 18S rRNA or GAPDH gene expression levels.
FIG. 1. Effects of morphine on the constipation score and fecal water content. Rat fecal samples were collected for up to 12 h after morphine administration. The number of fecal pellets (A) and the weight of pellets (B) were measured. The fecal water content was measured (C). The mean fecal water content immediately after administration (0 h) was indicated as 100%. Data represent means ± SDs for 6 rats. Student’s t test or Dunnett’s test: *P < .05, **P < .01, and ***P < .001 versus control or 0 h.

FIG. 2. Changes AQP3 expression in the colon after morphine administration. Rat colons were harvested for up to 12 h, beginning immediately after morphine administration. The mRNA expression levels of AQP3 were measured using real-time RT-PCR, and normalized to 18 S rRNA (A). The CM (B), PM (C), and IV (D) fractions were prepared, and the protein expression levels of AQP3 were analyzed using Western blotting. Mean levels of AQP3 mRNA and protein expression immediately after administration (0 h) were indicated as 100%. Data represent means ± SDs for 6 rats. Dunnett’s test: *P < .05, **P < .01, and ***P < .001 versus 0 h.
However, M-6-G is scarcely produced in rats (Kuo et al., 1991; Yue et al., 1990). Therefore, this study assessed the effects of morphine and M-3-G on AQP3 expression using human colon cancer HT-29 cells. HT-29 cells are widely used in the study of AQP3 because these cells represent the normal physiological conditions of the colon (Dupont et al., 1980; Ikarashi et al., 2011b; Itoh et al., 2003, 2004; Laburthe et al., 1978).

AQP3 mRNA expression level was investigated after treatment with morphine or M-3-G at concentrations that are equivalent to concentrations in the blood or intestinal tract. However, no changes were observed up to 3 h after treatment (Fig. 5).

Relationship Between Increased AQP3 Expression Level and 5-HT After Morphine Administration

Morphine promotes the release of 5-HT from the intestinal wall, which induces constipation by inhibiting peristaltic movements in the bowel (Burks, 1973; Burks and Long, 1967). Therefore, we investigated whether 5-HT contributes to increased AQP3 expression after morphine administration.

5-HT was evaluated in an in vitro study to determine whether it increases AQP3 expression in the colon. 5-HT treatment in HT-29 cells increased AQP3 mRNA expression level at 1μM (Fig. 6A). The observed increase in AQP3 expression with 5-HT peaked 1 h after treatment, but returned to nearly control levels 6 h after administration (Fig. 6B). This change in AQP3 mRNA expression was similar to the observed changes after morphine administration (Fig. 2A).

Approximately 95% of 5-HT in the body exists in the gastrointestinal tract, and enterochromaffin (EC) cells, which are enteroendocrine cells in the colon, dominantly synthesize, store, and secrete 5-HT (Kim et al., 2001; Racke and Schworer, 1991). EC cells change their morphology from round to process forms during 5-HT secretion (Kuramoto et al., 2007). Therefore, we investigated changes in the location of 5-HT-positive EC cells and their morphology using immunostaining of the colon of rats treated with morphine. 5-HT-positive EC cells were dispersed in the colon in the control group, and many of these cells were located in the crypts of the lamina propria. In contrast, many 5-HT-positive EC cells in the morphine-treated group were located mainly in the mucous layer above the crypts. The EC cells in the morphine-treated group had a morphology with extended processes on the luminal side that differed from the round form in the control group (Fig. 7).

FIG. 3. The distribution of AQP3 protein expression in rat colon after morphine administration. Rat colons were removed 5 h after distilled water (control) or morphine administration. A, AQP3 (green) and nuclei (blue) were immunostained. B, Enlarged view of AQP3 in rat colon epithelial cells.

FIG. 4. Changes in the morphine-induced constipation score by HgCl2 administration. HgCl2 or saline was administered intrarectally to rats 30 min after treatment with oral morphine. The number (A) and the weight (B) of fecal pellets between morphine administration and 1.5 h after administration were measured. The data represent the means ± SDs for 5 rats. Tukey’s test: **P < .01 versus control. ††P < .01 versus morphine.
FIG. 5. Effects of morphine and M-3-G on AQP3 mRNA expression in HT-29 cells. HT-29 cells were treated with morphine (A) or M-3-G (B) for 3 h. AQP3 mRNA expression levels were measured using real-time RT-PCR, normalized to the GAPDH level, and presented with the mean of the control cells set to 100%. AQP3 mRNA expression levels of the control cells did not change 3 h posttreatment with original growth medium. The data represent the means ± SDs for 5 experiments.

FIG. 6. Effect of 5-HT on AQP3 mRNA expression in HT-29 cells. A, HT-29 cells were treated with 5-HT for 1 h. B, HT-29 cells were treated with 5-HT (1 μM) for up to 6 h. The expression level of AQP3 mRNA was measured using real-time PCR, normalized to the GAPDH level, and then presented with the mean of the control cells set to 100%. AQP3 mRNA expression levels of the control cells did not change 6 h posttreatment with original growth medium. The data represent the means ± SDs for 5 experiments. Dunnett’s test: **P < .01 and ***P < .001 vs. 0 μM or 0 h.

FIG. 7. The distribution of 5-HT-positive EC cells in rat colon after morphine administration. Rat colons were removed 1 h after distilled water (control) or morphine administration. 5-HT-positive EC cells (green) and nuclei (blue) were immunostained.
Mechanism of Increased AQP3 Expression by 5-HT

5-HT receptors and 5-HT reuptake transporter (SERT) are expressed in the colon (Beubler and Horina, 1990; Gill et al., 2005; Saksena et al., 2005; Wade et al., 1996). We investigated the mechanism by which 5-HT increases AQP3 expression using inhibitors of these receptors and transporter. Pretreatment with ketanserin (5-HT2 receptor antagonist) (Magro et al., 2007), Y-25130 (5-HT3 receptor antagonist), or RS39604 (5-HT4 receptor antagonist) (Gill et al., 2005) in HT-29 cells had no effect on the 5-HT-induced increase in AQP3 expression (Fig. 8A). However, pretreatment with fluoxetine (Stepulak et al., 2008), a SERT inhibitor, at least 2.5 μM almost completely suppressed the increase in AQP3 expression with 5-HT (Fig. 8B).

5-HT acts as a ligand for the nuclear receptor PPARγ after uptake into cells by SERT (Waku et al., 2010). PPARγ translocates to the nucleus after ligand binding to promote AQP3 transcription (Jiang et al., 2011). Therefore, we investigated the nuclear translocation of PPARγ after 5-HT treatment in HT-29 cells. The results showed that the nuclear expression level of PPARγ significantly increased, by ~2-fold, 1 h after 5-HT treatment. Fluoxetine pretreatment almost completely suppressed 5-HT-induced nuclear translocation of PPARγ (Fig. 8C).

These findings confirmed that 5-HT acted as a ligand for PPARγ after uptake into the cells by SERT, and 5-HT increased the nuclear translocation of PPARγ, which finally increased AQP3 expression.

Improved Effect of Co-administration with a SERT Inhibitor on Morphine-induced Constipation

We studied whether pretreatment with fluoxetine suppressed morphine-induced constipation and the increase in AQP3 expression in the colon. Fluoxetine was administered intraperitoneally to rats, and morphine was orally administered 15 min after fluoxetine treatment. The degree of constipation was evaluated up to 5 h after morphine administration. The colon was isolated to assess AQP3 protein expression levels.

The results showed that the concomitant administration of morphine and fluoxetine increased the number and weight of fecal pellets compared with morphine administration alone (Figs. 9A and 9B). However, these increases were smaller than those in the control group. AQP3 protein expression level in the PM fraction of the colon increased by ~2-fold after morphine administration. Fluoxetine co-administration showed a decrease of 30% from morphine alone (Fig. 9C). These findings confirmed that a SERT inhibitor suppressed the morphine-induced increase in AQP3 expression in the colon and constipation.

DISCUSSION

Water transport in the colon primarily occurs via the transcellular route through AQP3s because of the strong tight junctions of colonic epithelial cells (Loo et al., 2002; Phillips and Giller, 1973). AQP3 is predominantly expressed in mucosal epithelial cells in the colon, and a change in AQP3 expression or function affects fecal water content (Ikarashi et al., 2012a). This study assessed the role of AQP3 in the colon in morphine-induced constipation and revealed a novel pathogenesis.

We investigated the relationship between the degree of constipation and AQP3 expression level in the colon. The AQP3 expression level significantly increased 1 h after morphine administration, and this increase persisted up to 5 h post-administration (Fig. 2). The fecal water content gradually decreased with increasing AQP3 expression, and its lowest level was observed 5 h after morphine administration (Fig. 1C). These findings revealed that the AQP3 expression level in the colon increases during morphine-induced constipation.

Why does constipation develop when the AQP3 expression level in the colon increases? The following reason may underlie this effect. Water is transported from the luminal side to the vascular side through AQP3 because the osmotic pressure is lower in the colonic lumen than in the blood vessel, and the feces are concentrated. Too much water is absorbed from the intestinal contents when the AQP3 expression level increases. Consequently, feces within the colon harden and aggregate, which leads to difficult defecation and constipation. We studied...
above the crypts in rat colons after morphine administration. These cells were commonly distributed in the mucous layer, and 5-HT secretion increases during morphine-induced constipation (Kaufman et al., 1986). Accordingly, an increase in water absorption into the cells by SERT and 5-HT loses its physiological function (Coates et al., 2004). 5-HT is a ligand for nuclear receptor PPARγ, which contributes to epithelial cell proliferation and turnover (Waku et al., 2010). In contrast, PPARγ agonists increase the AQP3 expression level (Jiang et al., 2011). We accordingly hypothesized that 5-HT increases the nuclear translocation of PPARγ was confirmed in HT-29 cells. We also found that pretreatment with fluoxetine, a SERT inhibitor, suppressed PPARγ translocation. Fluoxetine pretreatment also suppressed the 5-HT-induced increase in AQP3 expression in an in vitro study (Figs. 8B and 8C). These findings strongly support our hypothesis.

We investigated whether fluoxetine pretreatment suppressed morphine-induced constipation. The results showed that fluoxetine pretreatment suppressed the morphine-induced...
increase in AQP3 expression in the colon (Fig. 9C), and it also significantly improved the degree of constipation (Figs. 9A and 9B). However, this improvement effect of fluoxetine on morphine-induced constipation was weak. The relatively small dose of fluoxetine in this study was unable to lower the increased AQP3 expression level in the colon in the morphine group compared with the control group (Ortiz and Artigas, 1992). This result is one reason why morphine-induced constipation could not be completely improved. The administration of an adequate dosage of a SERT inhibitor, such as fluoxetine, may improve morphine-induced constipation.

This study in rats could not evaluate M-6-G (Kuo et al., 1991; Yue et al., 1990). However, treatment with M-6-G in HT-29 cells increased AQP3 expression levels in a concentration-dependent manner (Supplementary Fig. S1). This result suggests that there may be a route in humans by which the production of M-6-G following morphine administration acts directly on the colon and increases AQP3 expression levels.

In summary, the results of this study suggest that morphine increases AQP3 expression levels in the colon, which promotes water absorption from the luminal side to the vascular side, hardens stools, and causes constipation. We considered that morphine promoted 5-HT secretion from EC cells, which subsequently inhibits peristaltic movements of the bowel and also increases AQP3 expression level to ultimately cause constipation. The proposed mechanism is that oversecreted 5-HT from EC cells is taken into the cells by SERT, which acts as a ligand for PPARγ, and consequently increases AQP3 mRNA expression (Fig. 10). The results of this study suggest that a substance that controls the function and expression of AQP3 in the colon can act as a new therapeutic drug for morphine-induced constipation. The results also show that a drug targeting SERT, which regulates the actions of 5-HT in the intestinal tract, may be an effective treatment for or prevention of morphine-induced constipation. These novel findings will be critical for the development of new drugs for constipation and in the proposal of new therapies.

ACKNOWLEDGMENT

We thank Ayako Mimura and Tomohiko Iizasa for their technical assistance.

FUNDING

Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (No. 25860195) and the Takeda Science Foundation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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