Inhibition of Aquaporin-3 Water Channel in the Colon Induces Diarrhea

Nobutomo Ikarashi, Risako Kon, Tomohiko Iizasa, Noriko Suzuki, Reiko Hiruma, Kiyomi Suenaga, Takahiro Toda, Makoto Ishii, Motohiro Hoshino, Wataru Ochiai, and Kiyoshi Sugiyama*

Department of Clinical Pharmacokinetics, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan.
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Aquaporin (AQP) 3, which is predominantly expressed in the colon, is considered to play an important role in regulating the fecal water content in the colon. In this study, the role of AQP3 in the colon was examined using $HgCl_2$ and $CuSO_4$, which are known to inhibit AQP3 function. The fecal water content was measured up to 1 h after the rectal administration of $HgCl_2$ or $CuSO_4$ to rats. The results showed that the fecal water content in the $HgCl_2$ administration group increased significantly to approximately 4 times that in the control group, and severe diarrhea was observed. However, no changes were observed in the mRNA expression level of the osmoregulatory genes (sodium *myo*-inositol transporter and taurine transporter) and the level and distribution of AQP3 protein expression, as determined 1 h after the administration of $HgCl_2$. Comparable results were observed in the $CuSO_4$ administration group. The results of this study indicated that the inhibition of AQP3 function in the colon caused diarrhea. Therefore, it has been revealed that the fecal water content in the colon is controlled by the transport of water from the luminal side to the vascular side, which is mediated by AQP3. Our findings suggest that a drug that modulates the function or expression of AQP3 in the colon may represent a new target for the development of laxatives.

Key words aquaporin-3; colon; laxative

In recent years, it has become increasingly clear that aquaporins (AQPs), which are water channels, are involved in water transport in the intestinal tract.[1] AQPs are membrane channels that transport water within the human body and are therefore important for the regulation of water homeostasis. There are currently thirteen known types of AQPs in humans, AQP0 through AQP12, that are expressed in a variety of tissues.[2] Several members of the AQPs family 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.[3–6]

The major AQPs expressed in the colon are AQP1, AQP2, AQP3, AQP4, and AQP8.[3,6,7] Among these, AQP3 is the most dominantly expressed AQP in the colon and plays an important role in the absorption of water.[8,9] Previously, we suggested the possibility that the laxative effect produced by $MgSO_4$, which is an osmotic laxative, is not simply a result of changes in the osmotic pressure and is associated with the increased expression of AQP3 in the mucosal epithelial cells of the colon.[10,11] In addition, we have revealed that bisacodyl, which is classified as a stimulant laxative, decreases the expression of AQP3 in the mucosal epithelial cells of the colon, which results in the inhibition of water transfer from the intestinal tract to the vascular side and eventually leads to the development of diarrhea.[12,13] Based on these results, it has become clear that the changes in AQP3 expression cause changes in the water transport ability of the colon. However, there have been no reports that examined the effect of changes in the function of AQP3 on the transport of water in the colon in detail. In this study, the water transport ability of the colon when the function of AQP3 is inhibited was investigated *in vivo*. The fecal water content was examined after the rectal administration of $HgCl_2$[14] or $CuSO_4$,[15] which are known to inhibit AQP3 function, to rats. In addition, to examine whether the actions of these compounds are limited to the inhibition of AQP3, the effect of these compounds on the osmotic pressure and AQP3 expression in the colon were also investigated.

MATERIALS AND METHODS

Materials $HgCl_2$, $CuSO_4 \cdot 5H_2O$, and 4′,6-diamidino-2-phenylindole (DAPI) solution were purchased from Wako Pure Chemicals (Osaka, Japan). Bovine serum albumin (BSA) and TRI reagent were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). Rabbit anti-rat AQP3 antibody was purchased from Alomone Labs (Jerusalem, Israel). Alexa Fluoro 488 donkey anti-rabbit immunoglobulin G (IgG) and primers were purchased from Invitrogen Corp. (Tokyo, Japan). Anti-rabbit IgG-horse-radish peroxidase (HRP) antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). An enhanced chemiluminescence (ECL) system plus Western blotting detection reagents was purchased from GE Healthcare (Chalfont St. Giles, U.K.). A high capacity cDNA synthesis kit was purchased from Applied Biosystems (Foster City, CA, U.S.A.) and iQ SYBR green supermix was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other reagents were of the highest commercially available grade.

Animals Male Wistar rats (10 weeks old) were purchased from Japan SLC, Inc. (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 Animal, as adopted by the Committee on Animal Research at Hoshi University.

Treatment The rats were anesthetized with ether, and a saline solution containing 1.5 mM of $HgCl_2$ or 3.0 mM of $CuSO_4$ (1 mg/kg body weight) was intrarectally administered to rats. A saline solution without any added compounds was intrarectally administered to the control group. Rats were

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: sugiyama@hoshi.ac.jp

autopsied under ether anesthesia at 1 h after the administration of the $HgCl_2$ and $CuSO_4$ solutions, and the colons were removed. After washing the colons with phosphate-buffered saline (PBS: 140 mM NaCl, 20 mM Na_2HPO_4, 32 mM KCl, and 1.5 mM KH_2PO_4; pH 7.4), the samples were flash frozen in liquid nitrogen and stored at −80°C. Fecal samples from the rats were collected for up to 1 h after the administration of the $HgCl_2$ and $CuSO_4$ solutions 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 the wet and dry fecal weights.

RNA Preparation from Tissue Samples RNA was extracted from approximately 15 mg of frozen colon tissue using TRI reagent. The resulting solution was diluted 50-fold using Tris/ethylenediaminetetraacetic acid (EDTA) buffer (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 Reverse Transcription-Polymerase Chain Reaction (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 the following were added: 12.5 μL of iQ SYBR green supermix, 1.5 μL of forward primer of the target gene (5 pmol/μL), 1.5 μL of reverse primer (5 pmol/μL), 2 μL of cDNA TE buffer solution, and 7.5 μL of RNase-free water. For 18S ribosomal RNA (rRNA), a housekeeping gene and 2 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^{TM} single-color real-time RT-PCR detection system (Bio-Rad Laboratories). The mRNA expressions were normalized using 18S rRNA.

Preparation of Fraction for Immunoblotting from Colon Tissue The large intestinal mucosa was scraped with a slide glass and homogenized (1250 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; pH 7.2) using the digital homogenizer (Iuchi Co., Osaka, Japan) 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) fractions. Supernatant A was centrifuged (200000×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 Co., Ltd., Tokyo, Japan). This homogenate contained the CM fraction, which contained the cell membrane. Supernatant A was centrifuged (17000×g at 4°C for 30 min), and the supernatant (Supernatant B) 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.

Electrophoresis and Immunoblotting Protein concentrations were measured with the bicinchoninic acid assay (BCA) method[16] using BSA as a standard. Electrophoresis was performed using Laemmli's method.[17] Using the loading buffer (0.1 M Tris, 20% glycerol, 0.004% bromophenol blue, 4% sodium dodecyl sulfate, and 10% 2-mercaptoethanol; pH 6.8), 6 μg of protein was diluted 2-fold and was applied to a polyacrylamide gel. After electrophoresis, the isolated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 1 h using 1% skim milk, rabbit anti-rat AQP3 antibody (1/500) was applied to the resulting membrane for 1 h at room temperature. After washing the membrane 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 (1/5000). After washing the membrane was reacted with the ECL plus detection reagent and visualized with the LAS-3000 mini (FUJIFILM, Tokyo, Japan).

Immunohistochemistry The rats were anesthetized with ether, and their hearts were perfused first with PBS and then with 50 mL of ice-cold 4% paraformaldehyde (PFA) in PBS. The colon was removed and post-fixed in 4% PFA in PBS for 1 h at 4°C. The colon samples were then immersed in 30% sucrose in PBS overnight at 4°C and embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA, U.S.A.). Frozen sections were cut with a cryostat (Leica Microsystems, Tokyo, Japan) at 10 μm and mounted onto MAS-coated glass slides. The sections were washed with PBS, blocked with a blocking solution (PBS containing 3% fetal bovine serum and 0.1% Triton X-100) for 1 h, and incubated overnight at 4°C in a solution containing rabbit anti-rat AQP3 antibody (1/200). After washing 3 times with PBS, the sections were reacted with Alexa Fluoro 488 donkey anti-rabbit IgG (1/200) at room temperature for 1 h. After washing with PBS, the sections were reacted with DAPI solution (1/500) in PBS at room temperature for 30 min, washed 3 more times with PBS, and covered with a Vectashield mounting medium (Vector Laboratories, Burlingame, CA, U.S.A.). The immunostained sections were observed under a microscope (BZ-9000, Keyence Corporation, Tokyo, Japan).

Hematoxylin-Eosin Staining A colon section embedded

Table 1. Primer Sequences of Rat mRNA

Gene	Accession number	Forward (5′–3′)	Reverse (5′–3′)	Amplicon size (bp)
SMIT	NM_53715	AGGAGTCCTTGGGTTGGAAC	ACTGCAACAAGGCCTCCAG	116
TauT	NM_17208	GTTCTGGGAGCGCAACGT	ACCGAACACCCTTCCAGATG	93
18S rRNA	X00686	GTCTGTGATGCCCTTAGATG	AGCTTATGACCCGCACTTAC	117

SMIT; sodium *myo*-inositol transporter, TauT; taurine transporter.

in OCT compound was frozen. The frozen specimens were cut at a thickness of 10 μm with a cryostat, fixed on a MAS-coated glass slide, and stained with hematoxylin-eosin solution for microscopic examination.

Statistical Analysis Numerical data are expressed as the means±standard deviation. Significance of the differences was examined with the Student's *t*-test. Results with $p<0.05$ were considered significant.

RESULTS

Effect of $HgCl_2$ and $CuSO_4$ on Fecal Water Content Feces were collected up to 1 h after rectal administration of rats with $HgCl_2$ or $CuSO_4$ for calculation of the fecal water content (Fig. 1).

The fecal water content after $HgCl_2$ administration increased to approximately 4 times that in the control group, and severe diarrhea was observed. The fecal water content in the $CuSO_4$ administration group also increased to approximately 4 times the content in the control group.

Effect of $HgCl_2$ and $CuSO_4$ on the Genes Associated with Osmotic Pressure Regulation in the Rat Colon Sodium *myo*-inositol transporter (SMIT) and taurine transporter (TauT), which are genes associated with the regulation of osmotic pressure, are expressed in numerous tissue. The transcription of these osmoregulatory genes is known to be regulated in response to changes in the extracellular osmotic pressure, eventually leading to rapid changes in the mRNA expression level.[18–20] The mRNA expression levels of SMIT and TauT in the colon of the rats 1 h after administration of $HgCl_2$ or $CuSO_4$ were measured to investigate changes in the osmotic pressure in the lumen of the colon.

No significant differences were observed in the mRNA expression levels of SMIT and TauT between the $HgCl_2$

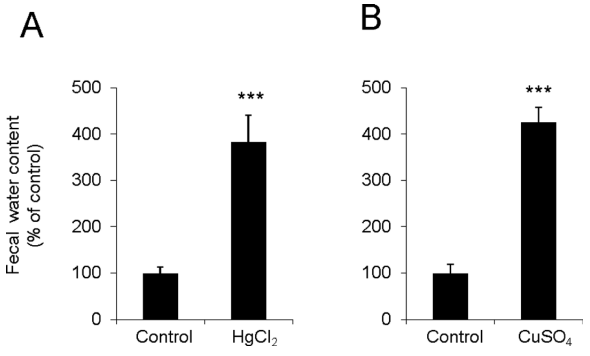

Fig. 1. Effect of $HgCl_2$ and $CuSO_4$ on Fecal Water Content

Rats were intrarectally administered $HgCl_2$ (1 mg/kg, A) or $CuSO_4$ (1 mg/kg, B). Saline solution was intrarectally administered to the control group. Feces were collected 1 h after administration, and the fecal water content was measured. The data are shown using the mean value of fecal water content of the control group was indicated as 100%. The data represent the means±S.D. for 6 rats. Student's *t*-test: ***$p<0.001$ *vs.* Control.

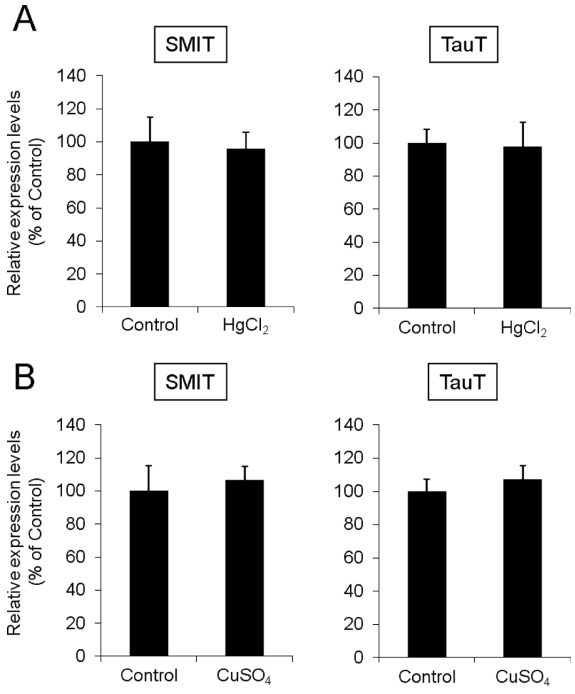

Fig. 2. Effect of $HgCl_2$ and $CuSO_4$ on SMIT and TauT mRNA Expression Levels in the Rat Colon

Rats were intrarectally administered $HgCl_2$ (1 mg/kg, A) or $CuSO_4$ (1 mg/kg, B). Saline solution was intrarectally administered to the control group. Rat colons were harvested 1 h after treatment. The mRNA expression levels of SMIT and TauT were analyzed by real-time RT-PCR. Normalization was performed against 18S rRNA, and the mean levels of mRNA expression of the control group were indicated as 100%. The data represent the means±S.D. for 6 rats.

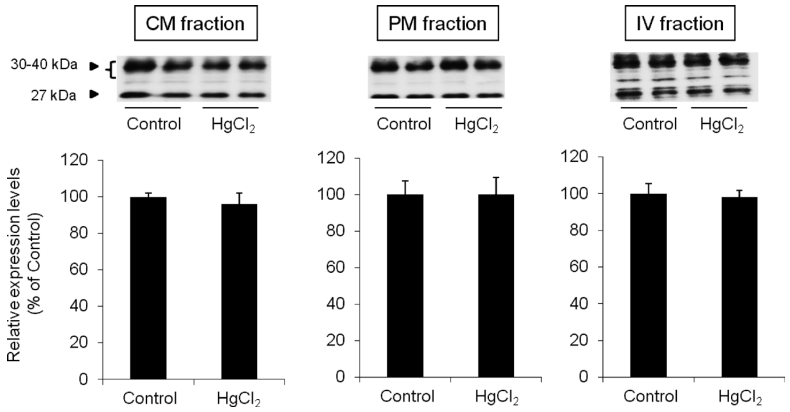

Fig. 3. Effect of $HgCl_2$ on AQP3 Protein Expression Level in the Rat Colon

Rats were intrarectally administered $HgCl_2$ (1 mg/kg). Saline solution was intrarectally administered to the control group. Rat colons were harvested 1 h after treatment. The crude membrane (CM), plasma membrane (PM), and intracellular vesicle (IV) fractions were prepared, and the protein expression levels of AQP3 were analyzed by Western blotting. Mean levels of AQP3 protein expression of the control group were indicated as 100%. The data represent the means±S.D. for 6 rats.

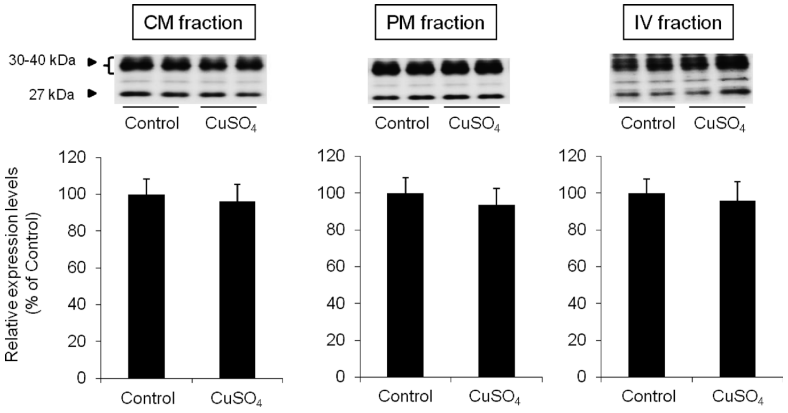

Fig. 4. Effect of $CuSO_4$ on AQP3 Protein Expression Level in the Rat Colon

Rats were intrarectally administered $CuSO_4$ (1 mg/kg). Saline solution was intrarectally administered to the control group. Rat colons were harvested 1 h after treatment. The crude membrane (CM), plasma membrane (PM), and intracellular vesicle (IV) fractions were prepared, and the protein expression levels of AQP3 were analyzed with Western blotting. Mean levels of AQP3 protein expression of the control group were indicated as 100%. The data represent the means±S.D. for 6 rats.

administration group and the control group (Fig. 2A). The levels of expression of the mRNA of SMIT and TauT in the $CuSO_4$ administration group were also almost comparable to that in the control group (Fig. 2B).

Effect of $HgCl_2$ and $CuSO_4$ on the Level of AQP3 Protein Expression in the Rat Colon AQP3, which is predominantly expressed in the colon, is considered to play an important role in regulating fecal water content in the colon.[12] We measured the AQP3 protein expression level in the colon after rectal administration of $HgCl_2$ or $CuSO_4$. Two bands of AQP3 protein were detected. One of these bands appeared at approximately 27 kDa and represented the deglycosylated form of AQP3, and the other appeared at approximately 30–40 kDa and represented a glycosylated form of AQP3.[21,22] The glycosylation is associated with the stability and intracellular translocation of AQPs but has no influence on water permeability.[23–25] In this study, therefore, the sum of these bands was analyzed as the protein expression level of AQP3 (Figs. 3, 4).

The protein expression level of AQP3 in the CM fraction of the colon at 1 h after the administration of $HgCl_2$ was almost the same as that in the control group. Regarding the PM fraction and the IV fraction, no differences in the protein expression level of AQP3 was observed between the $HgCl_2$ administration group and the control group (Fig. 3).

The protein expression level of AQP3 in the CM fraction of the colon at 1 h after the administration of $CuSO_4$ was almost the same as that in the control group. Regarding the PM fraction and the IV fraction, no differences in the protein expression level of AQP3 were observed between the $CuSO_4$ administration group and the control group (Fig. 4).

The Effect of $HgCl_2$ and $CuSO_4$ on the Distribution of AQP3 Expression and Mucosal Damage in the Rat Colon The results of immunochemical staining using an AQP3-specific antibody revealed that AQP3 is localized to the epithelial cells of the colonic mucosa and is expressed both on the apical and basal surfaces of the epithelial tissues (Fig. 5). Changes in these expression characteristics of AQP3 in the colon were not observed after the administration of $HgCl_2$ or $CuSO_4$ (Fig. 5).

Hematoxylin-eosin staining of the colon tissue sections showed no mucosal damage to the colon attributable to the administration of $HgCl_2$ or $CuSO_4$ (Fig. 6).

DISCUSSION

Each AQP monomer consists of six transmembrane domains. The AQPs functions as a tetramer and selectively transport water, glycerin, and other compounds. AQPs are identified based on two highly conserved asparagine–proline–alanine (NPA) motifs that are important for the formation of a water-permeating pore.[26,27] Mercury reagents inhibit water permeability through AQP3 by binding to Cys-11, which exists near the NPA motifs.[14] Copper reagents inhibit water permeability through AQP3 by binding to 3 amino acid residues of Trp-128, Ser-152, and His-241.[15] In this study, the fecal water content after the inhibition of AQP3 by rectal administration of $HgCl_2$ or $CuSO_4$ was measured to evaluate the function of AQP3 in the colon.

It has been reported that 0.3 mM of $HgCl_2$ and 1.0 mM of $CuSO_4$ inhibit approximately 50 to 70% of the water permeability of AQP3 *in vitro*.[14,15,28] In this study, the solutions of 1.5 mM of $HgCl_2$ and 3.0 mM of $CuSO_4$ were rectally administered (1 mg/kg). It has been revealed that the rectal administration of $HgCl_2$ or $CuSO_4$ to rats significantly increases the fecal water content to a level approximately 4 times that in the control group, leading to severe diarrhea (Fig. 1). These results suggest that the inhibition of AQP3 in the colon may suppress water transport from the luminal side to the vascular side of the colon, leading to diarrhea.

We have revealed that a decrease in the AQP3 expression level in the colon inhibits water transport from the luminal side to the vascular side, leading to diarrhea.[12,13] The expression of AQP3 was examined to determine whether is decreased with the administration of $HgCl_2$ and $CuSO_4$. The results confirmed that the level and distribution of AQP3 expression in the colon are not affected by the administration of $HgCl_2$ or $CuSO_4$ (Figs. 3 to 5). These results suggested that diarrhea after the administration of $HgCl_2$ and $CuSO_4$ is not attributable to a decrease in the AQP3 expression level.

It is known that mucosal damage in the colon causes diarrhea.[29,30] As shown in Fig. 6, no mucosal damage was observed after the administration of $HgCl_2$ or $CuSO_4$. Therefore, it has been suggested that diarrhea after the administration of $HgCl_2$ or $CuSO_4$ is not attributable to mucosal damage in the colon.

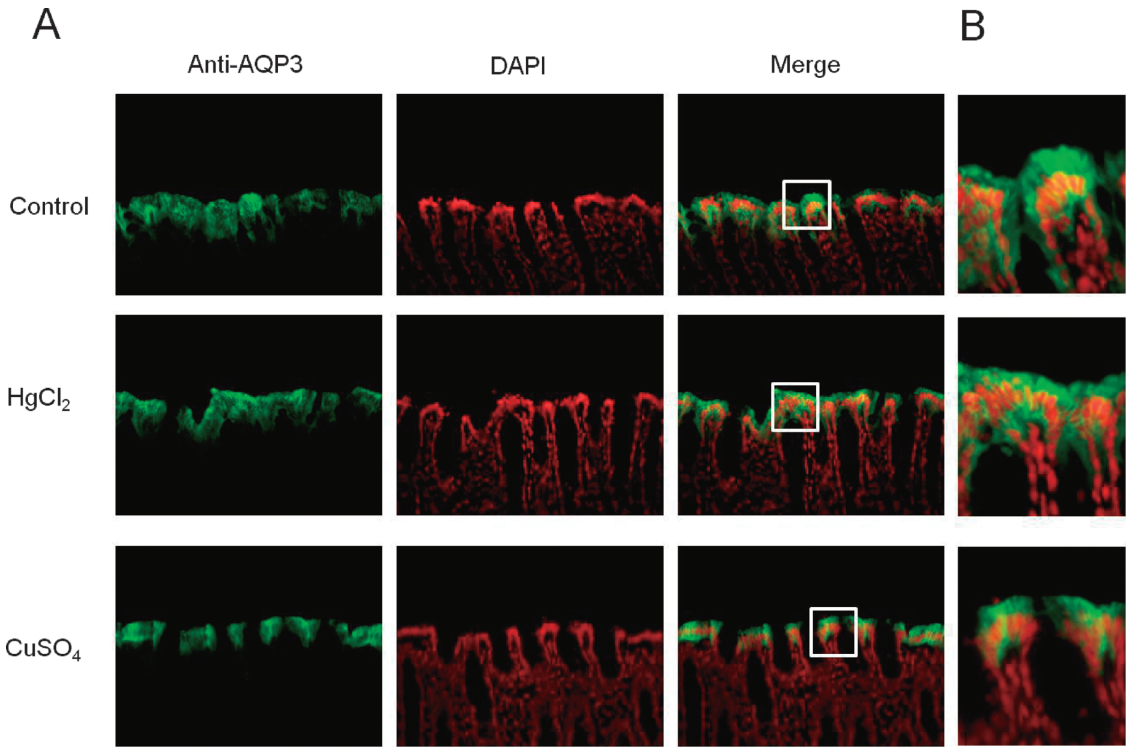

Fig. 5. The Distribution of AQP3 Expression in the Rat Colon after the Administration of $HgCl_2$ and $CuSO_4$

Rats were intrarectally administered $HgCl_2$ (1 mg/kg) or $CuSO_4$ (1 mg/kg). Saline solution was intrarectally administered to the control group. (A) Rat colons were harvested 1 h after treatment. AQP3 (green) and nuclei (red) were immunostained. (B) Enlarged view of AQP3 in the mucosal epithelial cells in the colon of rats.

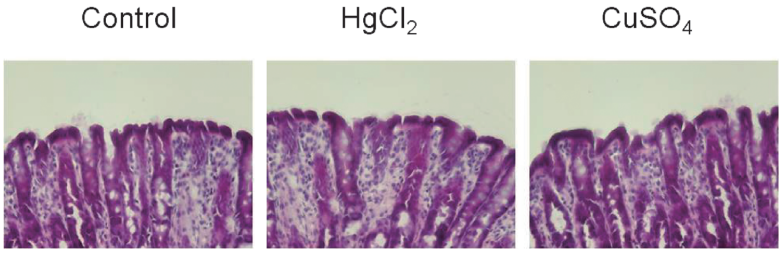

Fig. 6. Representative Histological Sections of Colon Stained with Hematoxylin–Eosin

Rats were intrarectally administered $HgCl_2$ (1 mg/kg) or $CuSO_4$ (1 mg/kg). Saline solution was intrarectally administered to the control group. The colon was removed and stained using hematoxylin–eosin.

AQP3 facilitates the transport of water in accordance with the osmotic pressure gradient. Therefore, when the osmotic pressure on the luminal side of the colon increases, water is transported from the vascular side to the luminal side, which is opposite to the direction under normal physiological conditions, and diarrhea develops.[11] To evaluate the luminal osmotic pressure after rectal administration of $HgCl_2$ or $CuSO_4$, the mRNA expression levels of osmoregulatory genes, SMIT and TauT, which are known to swiftly react to changes in osmotic pressure, were examined.[18–20] The results revealed that the administration of $HgCl_2$ or $CuSO_4$ did not alter the mRNA expression levels of these genes (Fig. 2). Based on these findings, it has been suggested that diarrhea after the administration of $HgCl_2$ and $CuSO_4$ is not attributable to an increase in the osmotic pressure in the colon.

In summary, it has become clear that fecal water content is controlled by water transport from the luminal side to the vascular side of the colon, which is mediated by AQP3, and the inhibition of the action of AQP3 causes the inhibition of water transport from the luminal side to the vascular side, eventually leading to diarrhea.

In recent years, due to various factors such as irregular meals/lifestyles, insufficient intake of dietary fiber and water, excess stress, overuse of laxatives, lack of exercise, reduced gastrointestinal tract function with age, and the adverse effect of opioids such as morphine, constipation has become a major health problem worldwide.[31,32] It is thought that a drug that modulates the function or expression of AQP3 in the colon may become a new target for the development of laxatives.

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