Effects of Magnesium Sulphate Administration on Aquaporin 3 in Rat Gastrointestinal Tract

Nobutomo Ikarashi, Takashi Ushiki, Toshihide Mochizuki, Takahiro Toda, Toshiyuki Kudo, Kohta Baba, Makoto Ishii, Kiyomi Ito, Wataru Ochiai, and Kiyoshi Sugiyama*

Department of Clinical Pharmacokinetics, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan.
Received August 25, 2010; accepted November 29, 2010; published online November 29, 2010

Aquaporin (AQP) 3 plays an important role in regulating faecal water content in the colon. We investigated the role of AQP3 in the colon in the laxative effect of magnesium sulphate ($MgSO_4$), a widely used osmotic laxative. Rats were administered $MgSO_4$, after which faecal water content, the colon mRNA expression levels of sodium *myo*-inositol transporter (SMIT) and taurine transporter (TauT), the colon protein expression levels of AQP3 were examined. Faecal water content increased over time after $MgSO_4$ administration, and severe diarrhoea was observed between 4 and 8 h after administration. The mRNA expression levels of SMIT and TauT, which are indicators of variations in osmotic pressure, were highest at 2 h after the administration of $MgSO_4$ and were still elevated at 8 h after administration when compared to immediately after the administration. The immunostaining analysis showed that AQP3 is a dominant AQP in the rat colon. The protein expression levels of AQP3 in the colon increased over time following the administration of $MgSO_4$ and at 8 h after administration were approximately 8 times higher than baseline levels. Previously, osmotic laxatives were believed to induce diarrhoea by elevating the osmotic pressure in the intestinal tract. The results of the present study suggest that the laxative effect of $MgSO_4$ is not simply caused by a change in the osmotic pressure in the intestinal tract, but could be a response to increased expression of AQP3.

Key words aquaporin; magnesium sulphate; laxative; water channel; sodium *myo*-inositol transporter; taurine transporter

In human, aquaporins (AQPs) are expressed in a variety of tissues, and there are currently 13 known types of AQPs: AQP0 through AQP12.[1] Several members of the AQP family are expressed in the intestinal tract, and at least 8 types are known to exist there: AQP1, AQP2, AQP3, AQP4, AQP7, AQP8, AQP9, and AQP10.[2—4]

The main types expressed in mucosal epithelial cells in the colon are AQP3, AQP4 and AQP8.[5] It has been reported that vasoactive intestinal polypeptide (VIP), a gastrointestinal hormone, is the causative agent in Verner–Morrison syndrome, a disease associated with diarrhoea[6]; that intravenous administration of VIP in healthy adults causes diarrhoea[7]; that serum VIP concentrations are elevated in rats with colitis[8]; and that VIP increases mRNA and protein expression levels of AQP3 in HT-29 cells, which are human colonic epithelial cells.[9] It has also been reported that following resection of the small bowel in rats, there is an increase in the mRNA expression of AQP3 in the colon as diarrhoea occurs.[9] Based on these findings, AQP3 appears to play a particularly important role in water transport in the colon. Despite suggestions of an apparent relationship between the occurrence of diarrhoea and AQP3 expression levels, the manner in which variations in AQP3 expression are related to the mechanism of action of laxatives has yet to be elucidated.

It is believed that commonly used osmotic laxatives, such as magnesium sulphate ($MgSO_4$) and magnesium oxide (MgO), induce diarrhoea by causing an increase in the osmotic pressure in the intestinal tract,[10] but no details are known about the relationship between these osmotic laxatives and water transport. In the present study, we investigated the role of AQP3 in the colon on the laxative effect of $MgSO_4$, a widely used osmotic laxative. First, rats were administered $MgSO_4$, and faecal water content was measured over time as an indicator of diarrhoea. Then, by analyzing the expression level of genes that sharply increase as a result of increased osmotic pressure, we investigated the relationship between diarrhoea and osmotic pressure. Next, protein expression levels of AQP3, AQP4, and AQP8 in the rat colon were analyzed by immunostaining in order to investigate the distribution and intensity of AQP expression. By analyzing the expression of AQP3 in the colons of rats administered $MgSO_4$, the relationship between the expression level and the laxative effects of $MgSO_4$ was then investigated.

MATERIALS AND METHODS

Animals Male Wistar rats (10 weeks old) were purchased from Sankyo Labo Service Corp., Inc. (Tokyo, Japan). Each rat was caged separately and 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, as adopted by the Committee on Animal Research at Hoshi University.

Treatment Rats were fasted for 18 h before $MgSO_4$ administration (water provided *ad libitum*). An aqueous solution of $MgSO_4$ (2 g/kg body weight) was administered orally to rats.[11,12] Rats were autopsied under ether anaesthesia immediately after treatment and at 2, 5, and 8 h after $MgSO_4$ administration, and the colons were removed. After washing the intestinal tract with phosphate-buffered solution (PBS; 140 mM NaCl, 20 mM Na_2HPO_4, 32 mM KCl, 1.5 mM KH_2PO_4, pH 7.4), samples were flash frozen with liquid nitrogen and stored at −80 °C. Faecal samples from the rats were collected for up to 8 h after the administration of $MgSO_4$ and were placed in silica gel, followed by drying for 24 h in a desiccator. Water content per gram of faeces was calculated based on the difference between wet and dry faecal weights.

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

Immunohistochemistry $MgSO_4$ non-treated 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 dissected out and post fixed in 4% PFA in PBS for 1 h at 4 °C. Samples were immersed in 30% sucrose/PBS overnight at 4 °C and embedded in OCT compound (Sakura Finetek U.S.A. 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 (Matsunami Glass, Osaka, Japan). Sections were washed with PBS and blocked with blocking solution (PBS containing 3% serum and 0.1% Triton X-100) for 1 h; subsequently, sections were incubated overnight at 4 °C in a mixture of primary antibody. Primary antibodies were as follows: rabbit anti-rat AQP3 (Alomone Labs, Jerusalem, Israel, 1 : 200); rabbit anti-rat AQP4 (Alomone Labs, 1 : 200); and rabbit anti-rat AQP8 (Alomone Labs, 1 : 200). After washing 3 times with PBS, sections were reacted with secondary antibody (Alexa Fluoro 488 anti-rabbit immunoglobulin G (IgG), Invitrogen Corp., Tokyo, Japan, 1 : 300) at room temperature for 1 h. After washing with PBS, sections were reacted with 4,6-diamidino-2-phenylindole (DAPI) solution (Dojindo Laboratories, Kumamoto, Japan, 1 : 2000) in PBS at room temperature for 30 min, washed 3 more times with PBS, and then coverslipped with vectashield (Vector Laboratories, Burlingame, CA, U.S.A.). Immunostained sections were observed under a fluorescence microscope FSX100 (Olympus Corporation, Tokyo, Japan).

RNA Preparation from Tissue Samples RNA was extracted from about 15 mg of frozen colon using the RNeasy Mini Kit (Qiagen GmBH, Hilden, Germany). RNA extraction was performed according to the protocol for the RNeasy Mini Kit. The resulting solution was diluted 50-fold using Tris/ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0) (TE buffer), and the purity and concentration (μg/ml) of RNA were calculated by measuring absorbances 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 (Applied Biosystems, Foster City, CA, U.S.A.) 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 (Bio-Rad Laboratories, Hercules, CA, U.S.A.), 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. With regard to 18S ribosomal RNA (rRNA), a housekeeping gene, 4 μl of a cDNA TE buffer solution that was prepared by diluting the above-mentioned solution 20-fold using TE buffer was used. Denaturation temperature was set at 95 °C for 15 s, annealing temperature at 56 °C for 30 s and elongation temperature at 72 °C for 30 s. Fluorescence intensity of the amplification process was monitored using the My iQ^{TM} Single Color Real-time RT-PCR Detection System (Bio-Rad Laboratories). mRNA expressions were normalized using 18S rRNA.

Preparation of Membrane Fraction for Immunoblotting Large intestinal mucosa scraped with a slide glass was homogenized using dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The resulting suspension was centrifuged for 15 min at 800×$\boldsymbol{g}$, and the supernatant was centrifuged for 1 h at 200000×$\boldsymbol{g}$. The precipitate was resuspended using dissecting buffer. All procedures were carried out at 4 °C. Protein concentrations were measured by the Lowry method[13] using bovine serum albumin (BSA) as a standard.

Electrophoresis and Immunoblotting Electrophoresis was performed by Laemmli's method.[14] Using the loading buffer (84 mM Tris, 20% glycerol, 0.004% bromophenol blue, pH 6.3, 4.6% sodium dodecyl sulfate (SDS), and 10% 2-mercaptoethanol), 6 μg of protein was diluted 2-fold and applied to a 12.5% polyacrylamide gel. After electrophoresis, the isolated proteins were transferred to a polyvinylidene difluorine (PVDF) membrane using CompactBLOT (AE-7500, ATTO Corp., Tokyo, Japan). After blocking 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 : 2000). After washing the membrane using 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-horse-radish peroxidase (HRP) antibody (Invitrogen Corp., 1 : 2000). After washing the membrane, the membrane was reacted with the ECL Plus detection reagent and visualized with LAS-3000 mini (Fuji Film, Tokyo, Japan), a luminoimage analyzer.

Statistical Analysis Numerical data are expressed as mean±standard deviation. Significance of the differences was examined by the ANOVA method, followed by Dunnett's test. Results with $p<0.05$ were considered significant.

RESULTS

Changes in Faecal Water Content Changes in faecal water content over time after administration of $MgSO_4$ to rats are shown in Fig. 1.

Faecal water content increased over time after $MgSO_4$ administration. Between 4 and 8 h after $MgSO_4$ administration, faecal water content increased significantly from approximately 5-fold to 13-fold *versus* baseline levels (0 h), and severe diarrhoea was observed during that time.

Table 1. Primer Sequences of Rat mRNA

Gene	Accession number	Forward (5′—3′)	Reverse (5′—3′)	Amplicon size (bp)
AQP3	NM_031703	CCCCTTGTGATGCCTCTC	CCCTAGCTGGCAGAGTTC	94
SMIT	NM_53715	AGGAGTCCTTGGGTTGGAAC	ACTGCAACAAGGCCTCCAG	116
TauT	NM_17208	GTTCTGGGAGCGCAACGT	ACCGAACACCCTTCCAGATG	93
18S rRNA	X00686	GTCTGTGATGCCCTTAGATG	AGCTTATGACCCGCACTTAC	117

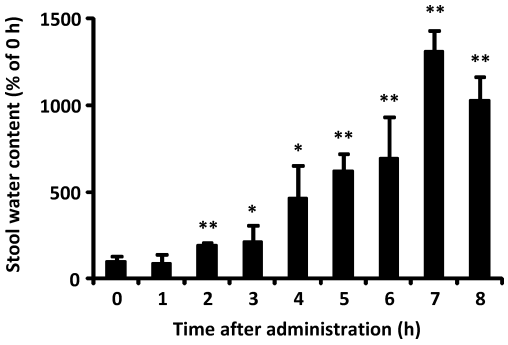

Fig. 1. Changes in Faecal Water Content after the Administration of $MgSO_4$

Rat faecal samples were collected at various times for up to 8 h beginning immediately after the administration of $MgSO_4$, and faecal water content was measured. The mean faecal water content immediately after the administration of $MgSO_4$ (0 h) was considered to be 100%. Data represent means±S.D.s for 6 rats. Dunnett's test: $*p<0.05$ and $**p<0.01$ *vs.* 0 h.

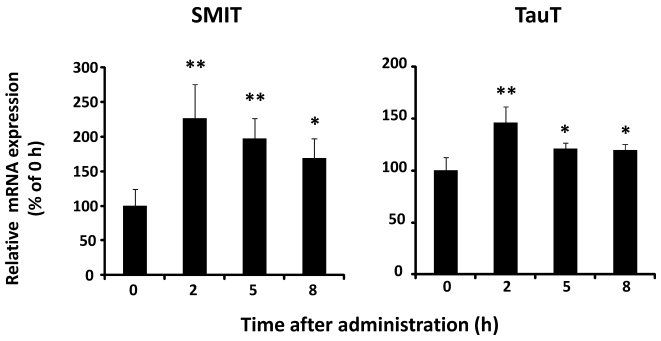

Fig. 2. Changes in Genes Associated with Osmotic Pressure in the Rat Colon after the Administration of $MgSO_4$

Immediately after the administration of $MgSO_4$ (0 h) and at 2, 5, and 8 h after administration, rat colons were harvested, and mRNA expression levels of SMIT and TauT were analyzed by real-time RT-PCR. Normalization was performed against 18S rRNA, and mean levels of mRNA expression immediately after the administration (0 h) were indicated as 100%. Data represent means±S.D.s for 6 rats. Dunnett's test: $*p<0.05$ and $**p<0.01$ *vs.* 0 h.

Changes in mRNA Expression of Genes Associated with Osmotic Pressure Regulation in the Colon Figure 2 shows the changes after administration of $MgSO_4$ in the mRNA expression of sodium *myo*-inositol transporter (SMIT) and taurine transporter (TauT), which are genes associated with the regulation of osmotic pressure in the colon.

In the colon, mRNA expression of SMIT increased significantly to approximately 2.2 times baseline levels at 2 h after the administration of $MgSO_4$ and then decreased over time. mRNA expression of TauT also increased significantly to approximately 1.5 times baseline levels at 2 h after administration of $MgSO_4$ and then subsequently decreased. However, even at 8 h after $MgSO_4$ administration, the mRNA expression levels of these genes were significantly higher than baseline levels immediately after administration.

Immunohistochemical Localization of AQP Protein in the Rat Colon AQP3, AQP4, and AQP8 are present in the colons of rats.[5] Distribution of AQP in the colon was confirmed by immunostaining analysis (Fig. 3). AQP3 was present in large quantities on the luminal side of the colon mucosa. There was little AQP4 on the luminal side, although large quantities were observed in the submucosal layer of the colon. Almost no expression of AQP8 was observed. Based on the results of immunostaining, it is clear that AQP3 is a dominant AQP in the rat colon. The absence of nonspecific reactivity was also confirmed by preparing a rat colon section reacted only with the secondary antibody (data not shown).

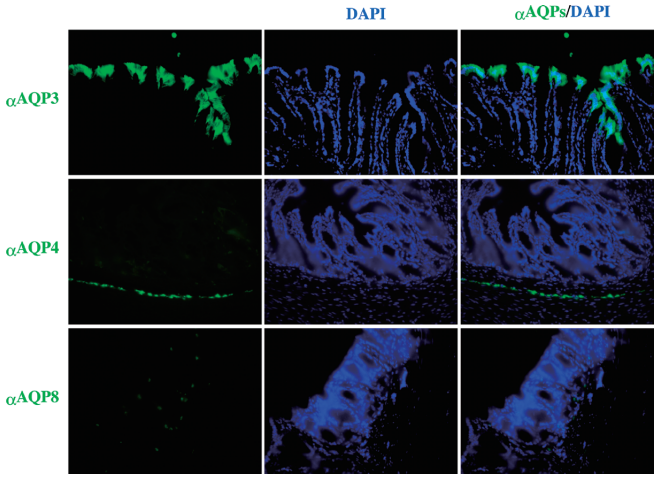

Fig. 3. Immunohistochemical Localization of AQP3, AQP4 and AQP8 Protein in the Rat Colon

Rat colons were harvested and AQP3, AQP4 and AQP8 (green), and cell nuclei (blue) were analyzed by immunostaining.

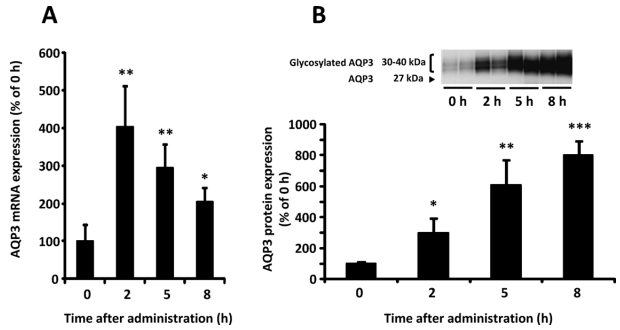

Fig. 4. Changes in mRNA Expression Levels (A) and Protein Expression Levels (B) of AQP3 in the Rat Colon after the Administration of $MgSO_4$

(A) Immediately after the administration of $MgSO_4$ (0 h) and at 2, 5, and 8 h after administration, rat colons were harvested, and mRNA expression levels of AQP3 were analyzed by real-time RT-PCR. Normalization was performed against 18S rRNA, and mean levels of mRNA expression immediately after administration (0 h) were indicated as 100%. (B) Immediately after the administration of $MgSO_4$ (0 h) and at 2, 5, and 8 h after administration, rat colons were harvested, crude membrane fractions were prepared, and the protein expression levels of AQP3 were analyzed by Western blotting. Mean levels of AQP3 protein expression immediately after administration (0 h) were indicated as 100%. Data represent means±S.D.s for 6 rats. Dunnett's test: $*p<0.05$, $**p<0.01$ and $***p<0.001$ *vs.* 0 h.

Changes in mRNA and Protein Expression Levels of AQP3 in the Colon Figure 4 shows the changes in the mRNA and protein expression of AQP3 in the colon after administration of $MgSO_4$ in rats.

mRNA expression of AQP3 was increased significantly at 2 h after the administration of $MgSO_4$, reaching approximately 4 times baseline levels and then subsequently decreasing over time.

Western blotting detected the AQP3 protein at 27 kDa and in the vicinity of 30 to 40 kDa. These are believed to represent non-glycosylated AQP3 and glycosylated AQP3, respectively.[15,16] The sum of these bands was analyzed as the protein expression level of AQP3. The protein expression levels of AQP3 in the colon increased over time following the administration of $MgSO_4$, and at 8 h after administration,

they were approximately 8 times higher than baseline levels.

DISCUSSION

$MgSO_4$ is known to increase osmotic pressure in the intestinal tract, causing bowel content to soften and increase in volume, eventually leading to a laxative effect.[10] Consequently, the mRNA expression levels of genes that regulate osmotic pressure in the rat colon were analyzed after the administration of $MgSO_4$, and the relationship between faecal water content and osmotic pressure was investigated. mRNA expression levels of SMIT and TauT were analyzed as genes involved in osmotic pressure regulation.[17] SMIT and TauT expression occurs in numerous tissues, primarily the kidneys and brain. The transcription of these osmoregulatory genes is known to be regulated along with changes in extracellular osmotic pressure, eventually leading to rapid changes in mRNA expression level.[18,19] mRNA expression levels have been reported to increase as early as 30 min after hypertonic stimulation, and decrease when osmotic pressure is reduced.[19,20] Therefore, the expression levels of these mRNAs can be used as indicators of variations in osmotic pressure.[20,21] mRNA expression of SMIT and TauT peaked at 2 h after the administration of $MgSO_4$ and subsequently decreased over time (Fig. 2). This is because the osmotic pressure in the colon was already high at 2 h after the administration of $MgSO_4$, and the osmotic pressure was thought to decrease over time due to the excretion of $MgSO_4$. The faecal water content was low at 2 h after administration, and there was no correlation between the increase in faecal water content due to $MgSO_4$ and the elevation of osmotic pressure in the colon. This suggests that the laxative effects of $MgSO_4$ cannot be fully explained by the increase in osmotic pressure.

It is known that AQP3, AQP4 and AQP8 are primarily expressed in the mucosal epithelial cells in the colon of rats.[5] Immunostaining for AQP3, AQP4, and AQP8 proteins in the colon showed that AQP3 was expressed in large quantities on the luminal side of the mucosa of the colon, and the intensity of expression was markedly higher than that of AQP4 and AQP8 (Fig. 3). These findings suggest that AQP3 plays a central role in water transport in the colon. Analysis of AQP3 expression levels found that mRNA expression of AQP3 peaked at 2 h after the administration of $MgSO_4$ and then gradually decreased (Fig. 4A). Western blot analysis showed that protein expression levels of AQP3 in the colon increased over time following $MgSO_4$ administration and were significantly higher at 8 h after administration, approximately 8 times as high as the baseline levels immediately after administration (Fig. 4B). The pattern of protein expression of AQP3 was correlated with the chronological changes in faecal water content. Previously, osmotic laxatives containing $MgSO_4$ were believed to induce diarrhoea by increasing the osmotic pressure in the intestinal tract. However, based on the results of mRNA expression analysis of SMIT and TauT, at 2 h after the administration of $MgSO_4$, osmotic pressure in the intestinal tract was already high, and yet diarrhoea did not occur. Despite the fact that water transfer from the vascular to the luminal side had occurred, AQP3 expression was still inadequate, and water transfer levels were insufficient for diarrhoea to occur. At 4 h after the administration of $MgSO_4$, although the osmotic pressure was somewhat lower than at 2 h after administration, diarrhoea occurred and is believed to be due to the transfer of large quantities of water from the blood vessels into the intestinal tract as a result of increases in the expression of AQP3.

In aqueous solution, $MgSO_4$ disassociates into Mg^{2+} and SO_4^{2-}, causing osmotic pressure to increase. Hypertonic stimulation is known to cause increases in AQP expression.[22,23] Hypertonicity induced increases in both AQP3 mRNA and AQP3 protein in MDCK epithelial cells. The amount of AQP3 mRNA was maximal 12 h after cells were exposed to hypertonic medium and decreased thereafter, whereas the amount of AQP3 protein was increased at 12 h, was maximal at 24 h, and decreased thereafter.[24] The time courses of the changes in both AQP3 mRNA and AQP3 protein in MDCK cells were similar to those of our results (Fig. 4) excluding the point that maximal times were different. Therefore, the increase in AQP3 protein expression in the colon following the administration of $MgSO_4$ can be attributed to the enhanced transcription of AQP3 due to an increase in the osmotic pressure in the colon. Moreover, magnesium acetate, which is used as an antacid, was recently found to increase the expression of AQP3 in Caco-2 cells, and findings suggest that this increase may be triggered by an increase in the intracellular Mg^{2+} level.[25] The time courses of the changes in both AQP3 mRNA and AQP3 protein in Caco-2 cells added magnesium acetate are similar to those in MDCK cells exposed to hypertonic medium.[24,25] As a result, it is believed that increases in the intracellular Mg^{2+} level due to the administration of $MgSO_4$ may also contribute to the increase in the expression levels of AQP3 in the colon. Consequently, the increase in AQP3 protein expression level despite the decrease of intraluminal Mg^{2+} concentration and osmotic pressure at 8 h after the administration of $MgSO_4$ can be attributed to the enhanced osmotic pressure and the increased intracellular Mg^{2+} level.

It is reported that the expression levels of AQP in the colon decrease in patients and model animals with ulcerative colitis associated with diarrhoea.[26] This fact indicates that the increase in AQP3 expression level cannot be fully explained by the increase of water volume in the lumen of the colon. Based on the results of the present study utilizing $MgSO_4$, the significance of the decrease in AQP expression level in ulcerative colitis is discussed as follows. In the case of ulcerative colitis, unlike the case of $MgSO_4$ administration, water is transferred from the luminal side to the vascular side of the colon due to lower osmotic pressure in the luminal side than the vascular side. Consequently, it is considered that the decreased level of AQP expression in the colon leads to the decreased level of water transfer from the intestinal tract, eventually causing diarrhoea. Currently, many types of laxatives such as sennoside, sodium picosulfate, and bisacodyl are used in clinical practice. Since these laxatives are not osmotic laxatives, AQP3 expression levels might be decreased if their laxative actions are associated with AQP3. We have been examining the relationship between AQP3 and laxatives other than $MgSO_4$.

Based on the results of the present study, the laxative effect produced by $MgSO_4$ is not simply a result of changes in osmotic pressure and is associated with the increased expression of AQP3 in the mucosal epithelial cells of the colon. AQP3 is expressed in the colon at higher levels than

any other member of the AQP family. Thus, new laxatives and antidiarrheals targeting intestinal AQP3 may be developed in the future.

REFERENCES

1) King L. S., Kozono D., Agre P., *Nat. Rev. Mol. Cell Biol.*, **5**, 687—698 (2004).
2) Gallardo P., Cid L. P., Vio C. P., Sepulveda F. V., *Am. J. Physiol. Gastrointest. Liver Physiol.*, **281**, G856—G863 (2001).
3) Laforenza U., Gastaldi G., Grazioli M., Cova E., Tritto S., Faelli A., Calamita G., Ventura U., *Biol. Cell*, **97**, 605—613 (2005).
4) Matsuzaki T., Tajika Y., Ablimit A., Aoki T., Hagiwara H., Takata K., *Med. Electron. Microsc.*, **37**, 71—80 (2004).
5) Koyama Y., Yamamoto T., Tani T., Nihei K., Kondo D., Funaki H., Yaoita E., Kawasaki K., Sato N., Hatakeyama K., Kihara I., *Am. J. Physiol.*, **276**, C621—C627 (1999).
6) Krejs G. J., *Am. J. Med.*, **82**, 37—48 (1987).
7) Kane M. G., O'Dorisio T. M., Krejs G. J., *N. Engl. J. Med.*, **309**, 1482—1485 (1983).
8) Kishimoto S., Kobayashi H., Shimizu S., Haruma K., Tamaru T., Kajiyama G., Miyoshi A., *Dig. Dis. Sci.*, **37**, 1729—1737 (1992).
9) Tsujikawa T., Itoh A., Fukunaga T., Satoh J., Yasuoka T., Fujiyama Y., *J. Gastroenterol. Hepatol.*, **18**, 803—808 (2003).
10) Izzo A. A., Gaginella T. S., Capasso F., *Magnes. Res.*, **9**, 133—138 (1996).
11) Galvez J., Zarzuelo A., Crespo M. E., Lorente M. D., Ocete M. A., Jimenez J., *Planta Med.*, **59**, 333—336 (1993).
12) Izzo A. A., Gaginella T. S., Mascolo N., Capasso F., *Br. J. Pharmacol.*, **113**, 228—232 (1994).
13) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
14) Laemmli U. K., *Nature* (London), **227**, 680—685 (1970).
15) Silberstein C., Kierbel A., Amodeo G., Zotta E., Bigi F., Berkowski D., Ibarra C., *Braz. J. Med. Biol. Res.*, **32**, 1303—1313 (1999).
16) Spector D. A., Wade J. B., Dillow R., Steplock D. A., Weinman E. J., *Am. J. Physiol. Renal Physiol.*, **282**, F1034—F1042 (2002).
17) Burg M. B., Ferraris J. D., Dmitrieva N. I., *Physiol. Rev.*, **87**, 1441—1474 (2007).
18) Handler J. S., Kwon H. M., *Am. J. Physiol.*, **265**, C1449—C1455 (1993).
19) Yamauchi A., Uchida S., Preston A. S., Kwon H. M., Handler J. S., *Am. J. Physiol.*, **264**, F20—F23 (1993).
20) Minami Y., Inoue K., Shimada S., Morimura H., Miyai A., Yamauchi A., Matsunaga T., Tohyama M., *Brain Res. Mol. Brain Res.*, **40**, 64—70 (1996).
21) Warskulat U., Wettstein M., Haussinger D., *Biochem. J.*, **321**, 683—690 (1997).
22) Hoffert J. D., Leitch V., Agre P., King L. S., *J. Biol. Chem.*, **275**, 9070—9077 (2000).
23) Storm R., Klussmann E., Geelhaar A., Rosenthal W., Maric K., *Am. J. Physiol. Renal Physiol.*, **284**, F189—F198 (2003).
24) Matsuzaki T., Suzuki T., Takata K., *Am. J. Physiol. Cell Physiol.*, **281**, C55—C63 (2001).
25) Okahira M., Kubota M., Iguchi K., Usui S., Hirano K., *Eur. J. Pharmacol.*, **588**, 26—32 (2008).
26) Hardin J. A., Wallace L. E., Wong J. F., O'Loughlin E. V., Urbanski S. J., Gall D. G., MacNaughton W. K., Beck P. L., *Cell. Tissue Res.*, **318**, 313—323 (2004).