Goreisan regulates AQP3 expression and improves diarrhea

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ABSTRACT

Aim: Goreisan (GRS) is often used for gastrointestinal symptoms associated with bacterial and viral infections, to cure diarrhea and to prevent general dehydration. Although several clinical reports have shown the effectiveness of GRS, the pharmacological properties and underlining mechanism of GRS have not been clarified.

Methods: Lipopolysaccharide (LPS, 10 mg/kg, i.p.) was administered to ICR mice and the diarrhea score was measured every 15 min. Goreisan (GRS; 1 g/kg) was administered 30 min before LPS administration, and the tissues were collected 90 min later. Caco-2 cells were treated with tumor necrosis factor-α (TNF-α, 10 ng/mL) and GRS (0.1 mg/mL) for 24 h, and the aquaporin-3 (AQP3) expression level was examined by qPCR.

Results: In this in vivo study, GRS did not affect cytokine production, but markedly improved tissue injury and diarrhea scores. On the other hand, AQP3 mRNA and protein expression in the intestinal epithelium in the LPS-treated group were significantly reduced, and GRS inhibited this decrease in AQP3. In a similar experiment performed on AQP3-deficient mice, the diarrhea-improving and tissue-improving effects of GRS disappeared. Furthermore, in this in vitro study, GRS suppressed the decrease in AQP3 expression by TNF-α stimulation, and the mechanism was due to extracellular signal-regulated kinase (ERK) inhibition.

Conclusion: GRS has both an antidiarrhea effect and an intestinal tissue-protective effect, which it exerts through the inhibition of an inflammation-induced decrease in intestinal AQP3 expression. Our data strongly support the clinical usefulness of GRS to prevent dehydration in infectious gastroenteritis as well as the pharmacological rationale for using GRS to treat this disease.

KEY WORDS: aquaporin, diarrhea, goreisan (GRS), infectious gastroenteritis

INTRODUCTION

Infectious gastroenteritis is a disease caused by infection with bacteria or viruses and accompanied by symptoms that include abdominal pain, nausea/vomiting, and diarrhea. Approximately 75% of patients with infectious gastroenteritis are children under 10 years old, and approximately 1.8 million children die annually from this disease [1–5]. The main treatment for patients with infectious gastroenteritis is to prevent the dehydration associated with diarrhea and vomiting, and, in severe cases, rehydration by infusion is often performed. However, there are no effective drugs for treating the disease itself. Generally, when an infection occurs in the intestinal mucosa, the ensuing inflammatory reaction causes a breakdown of the intestinal mucosa barrier function [6–8], which disrupts the balance between water secretion and absorption in the intestinal tract, resulting in diarrhea and other symptoms.

Aquaporins (AQPs) are a family of transmembrane channels that allow the rapid movement of water and, in some cases, small molecules across the plasma membrane [9,10]. Currently, 13 mammalian AQPs (AQP0–12) have been identified [11]. AQP3 is expressed in epithelial cells in various organs, including the kidney, skin, and intestinal tract, and it controls water movement in those locations [12–14]. In the intestinal tract, AQP3 is expressed on the luminal side, and it contributes to water absorption on the vascular side. Additionally, AQP3 expression is reduced in rotavirus-induced diarrhea model mice [15]. Thus, a decrease in AQP3 expression appears to be involved in the exacerbation of diarrhea.

Goreisan (GRS) is a traditional Japanese herbal medicine (Kampo medicine) that is clinically used to treat edema because of its diuretic effect. In recent years, there have been
many clinical reports indicating the usefulness of GRS for treating diarrhea from infectious gastroenteritis, especially in children. However, the effect of GRS on diarrhea in inflammatory conditions has not been evaluated pharmacologically, and the related mechanism is still unknown.

In this study, we used lipopolysaccharide (LPS)-treated mice to examine the effects of GRS on diarrhea, inflammation, intestinal mucosal tissue injury, and AQP3 expression, as compared with the effects of the steroidal anti-inflammatory drug dexamethasone (DEX).

**METHODS**

**Reagents**

A spray-dried extract preparation of GRS (TJ-17, lot No. 2180017010) was kindly supplied by Tsumura & Co. (Tokyo, Japan). GRS doses for same-day use were prepared as follows: a mixture of Alismatis rhizoma (4 g, rhizomes of *Alisma orientale* (Sam.) Juz.), Atractylodis lanceae rhizoma (3 g, rhizomes of *Atractylodes lancea* DC.), Poly-porus (3 g, sclerotium of *Polyporus umbellatus* Fries), Hoelen (3 g, sclerotium of *Poria cocos* Wolf), and Cinnamomi Cortex (1.5 g, cortex of *Cinnamomum cassia* Blume) was added to water and extracted at 100°C for 1 h. The extracted solution was filtered and spray-dried to obtain dry extract powder. The chemical profile of GRS obtained by a three-dimensional HPLC analysis is shown in Figure S1. A rabbit polyclonal anti-AQP3 antibody was obtained from Sigma (St. Louis, MO, USA). The rabbit anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinase (ERK)-1/2] (Thr202/Thr204) and anti-p44/p42 MAPK (ERK1/2) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). U0126 was purchased from Wako (Saitama, Japan).

**Animals**

ICR mice (male, seven weeks old) was purchased from Japan SLC, Inc. (Shizuoka, Japan). The AQP3<sup>−/−</sup> mice generated by targeted gene disruption were a gift from Dr. A Verkman at University of California, San Francisco (CA, USA) [16,17]. All animal experiments were performed on mice aged 7–10 weeks old. These experiments were approved by the Presidents of Tokyo University of Science and Keio University, following consideration by the Institutional Animal Care and Use Committee (approval no. Y19017 and Y20010 at Tokyo University of Science, 16 075 at Keio University) and by the Genetic Modification Safety Committee, Keio University School of Medicine (approval no. 28-029), and were carried out in accordance with institutional procedures, national guidelines, and the relevant national laws on the protection of animals.

GRS (0.1–1 g/kg, p.o.) and DEX (1 mg/kg, i.p.) were administered 30 min before LPS administration.

**Cell culture and treatments**

Caco-2 (human colon adenocarcinoma) cells were obtained from JCRB cell bank (Osaka, Japan). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. GRS was dissolved in dimethylsulfoxide (DMSO), sonicated for 20 min, and then centrifuged (3000 g, 25°C, 5 min); the resulting supernatant was used in our experiments. Cells were serum-starved for 24 h before treatment with tumor necrosis factor-α (TNF-α; 10 ng/mL).

**Evaluation of diarrhea**

LPS (10 mg/kg) was administered intraperitoneally to mice, after which the mice were evaluated every 15 min, for a total of 90 min. The diarrhea score at each timepoint was assigned according to the following scale developed by Williams et al: (i) solid stools; (ii) loose stools; and (iii) watery stools [18].

**Tissue staining**

The ileum was fixed with 10% paraformaldehyde and then embedded in paraffin wax. Tissue sections (5 μm thickness) were prepared and stained either with hematoxylin and eosin (H&E) or used for immunohistochemistry. For H&E staining, the tissue sections were deparaffinized and stained with hematoxylin followed by eosin. For immunohistochemistry, the tissue sections were treated with a rabbit anti-mouse AQP3 antibody (1:500), then reacted with an appropriate secondary antibody. The antigen was visualized by treating the tissue sections with a solution of 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB)/0.05% H<sub>2</sub>O<sub>2</sub>/phosphate-buffered saline (PBS). The slides were dehydrated in alcohol, cleared in xylene, and covered for microscopic examination.

**Real-time quantitative polymerase chain reaction (RT-PCR)**

The cells were collected in 1 mL of RNAiso Plus<sup>®</sup> reagent (Takara, Shiga, Japan) and homogenized on ice, after which the total RNA was extracted from the homogenates in accordance with the manufacturer’s instructions. Reverse transcription was performed with PrimeScript<sup>®</sup> RT Master Mix (Takara). Real-time PCR (RT-PCR) was performed with SYBR<sup>®</sup> Premix Ex Taq™ (Takara) on a Chromo 4™ RT-PCR analysis system (Bio-Rad, Tokyo, Japan). Samples were run in duplicate. The thermal cycling program consisted of 95°C for 3 min for polymerase activation, followed by 40 cycles of denaturation (95°C for 15 s) and annealing and extension (60°C for 1 min). Reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected [threshold cycle (Ct)]. The resulting data were analyzed by the comparative Ct method. Primer sequences used are provided in Table 1.
Western blot analysis

Cells were washed with ice-cold PBS (pH 7.4) and then exposed to lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, and 1% (v/v) protease inhibitor cocktail] for 30 min at 4°C. Protein concentrations in the resulting lysates were measured using the bicinchoninic acid method with bovine serum albumin as the standard. Aliquots of each lysate (50 μg) were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with Blocking One-P, Blocking One (Nakarai tesque, Kyoto, Japan) or 5% skim milk powder in PBS containing 0.1% Tween-20 (tris-buffered saline, TBS) for 1 h at room temperature. After being blocked, the membrane was incubated with affinity-purified rabbit anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK) (1:1000), anti-p44/p42 MAPK (1:1000), or anti-AQP3 (1:1000) antibody at 4°C for 8 h. The membrane was then washed with TBS and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG for 1 h at room temperature. Immunocomplexes were detected using ECL chemiluminescent reagents (Amersham, Waukesha, WI, USA).

Statistics

Statistical analyses were performed by one-way or repeated-measures analysis of variance (ANOVA) followed by a Newman–Keuls test or Student’s two-tailed t-test for paired samples. A value of P < 0.05 was considered to be significant.

RESULTS

GRS suppressed LPS-induced diarrhea

LPS, the major component of the outer membrane of Gram-negative bacteria [19], is a potent activator of the innate immune system [20]. It is a common pathogen-associated molecular pattern (PAMP) [21,22], and its recognition by Toll-like receptor 4 (TLR4) initiates a systemic inflammatory response [23], through the activation of nuclear factor kappa B (NFkB) signaling pathways and subsequent cytokine

| Table 1 | Primer sequences for RT-PCR |
| --- | --- |
| **Target gene** | **Primer Sequence, 5’–3’ Forward** | **Reverse** |
| Human AQP3 | TGCTGGGGACCCCTCATC | GATCATATCCAAAGTGTC |
| Mouse AQP3 | AACTTGCCTGCACCAGCT | CGAAGACCCAGACATGGGAAC |
| Mouse TNF-α | TCCAGAAGCTCCAGGCGTGC | TGGTGTTGAGGTCTGGGCCAT |
| Mouse IL-1β | GTGGCTGGAGAAGCCTTGGG | TGGCTCCAGACAGCAGAGGC |
| Mouse IL-6 | TCCAGTGGCCTCCTGGGAC | GTGTAATTTAAGCTCCAGACTTG |
| Mouse IL-8 | CATGACTTCAAGCTGGCCG | TTATGAATCTCAGCCTC |
| Mouse IL-10 | GGGTGGCCAAGCGCTTATC | TCTACCCAGGGGATTCAAGTG |
| Mouse COX-2 | CAGGGCCCTCTCCGCTTAG | GCCTGGGGTCTCAGGGATGA |
| Mouse HO-1 | GGGTGACAGAGAAGCTTAAG | GTGTCTGGGATGACCTAGT |
| GAPDH | ACCATCTCCAGGAGCCGAG | CAGTCTTCTGGGTGGCAG |

Figure 1 | The diarrhea score was measured every 15 min after lipopolysaccharide (LPS; 10 mg/kg, i.p.) administration. Goreisan (GRS; 0.1–1 g/kg, p.o.) and dexamethasone (DEX; 1 mg/kg, i.p.) were administered 30 min before LPS administration. Each data point represent the mean ± SE (n = 9). *, **, and ***: P < 0.05, 0.01, 0.001 vs Control. #: P < 0.05 vs LPS.
production [24]. To determine whether GR3 has an antidiarrhea effect, mice were injected intraperitoneally with LPS (10 mg/kg), and their diarrhea score was calculated every 15 min. The mice were pretreated with GR3 (1 g/kg, administered orally) or DEX (1 mg/kg, administered intraperitoneally) 30 min before the administration of LPS. In the control mice, which were injected with LPS alone, the diarrhea score was time-dependently increased by 60 min, after which it remained high. In contrast, in both the GR3-treated mice and the DEX-treated mice, the diarrhea score was significantly lower at all timepoints (Fig. 1A). The effect of different doses of GR3 was examined at 90 min, and the results indicate that the antidiarrhea effect of GR3 is dose-dependent (Fig. 1B).

**Figure 2** Ninety minutes after lipopolysaccharide (LPS) administration, mucosal tissues were observed by hematoxylin and eosin staining and the expression level of inflammatory cytokines or aquaporin-3 (AQP3) was measured by quantitative polymerase chain reaction (qPCR). Each bar represents the mean ± SE (n = 9). *, **, and ***: P < 0.05, 0.01, 0.001 vs Control. #, ##: P < 0.05, 0.01 vs LPS.
GRS improved tissue damage without changing cytokine expression

DEX exerts a strong anti-inflammatory effect by inhibiting cytokine production. To determine whether GRS, like DEX, has an anti-inflammatory effect, we compared the characteristics of these two drugs. It is well known that an inflammatory reaction in intestinal mucosal tissue destroys the epithelial barrier, after which the balance of water secretion and absorption in the intestinal tract is disrupted, resulting in diarrhea. Therefore, we performed a histopathological examination of H&E-stained sections to characterize the amount of intestinal injury at 90 min. Marked villus shortening, clubbing, and blunting were observed in LPS-treated intestines. However, the amounts of intestinal epithelial cell damage in GRS- or DEX-treated mice were considerably lower (Fig. 2A), suggesting that these drugs protected the intestinal epithelial cells.

To determine the effect of GRS on inflammatory responses, the expression of inflammatory cytokines was also examined. We measured the levels of TNF-α, interleukin (IL)-1β, IL-6, IL-8, cyclooxygenase 2 (COX2), and IL-10 mRNA. The levels of all these mRNAs were markedly increased in LPS-treated intestines, and DEX significantly inhibited all these LPS-induced increases, except for the increase in IL-8 mRNA. In contrast, GRS did not affect the expression of these mRNAs, suggesting that GRS does not have anti-inflammatory effects (Fig. 2B). Interestingly, the expression of water channel AQP3, which is thought to play an important role in water absorption from the intestinal tract, was decreased by LPS, but GRS treatment considerably inhibited this decrease in AQP3 mRNA. Together, these findings suggest that the characteristic effect of GRS is different from that of DEX.
GRS abrogates LPS-induced decreases in AQP3 expression

To clarify the effect of GRS on AQP3 expression, the protein level of AQP3 was determined. In agreement with the mRNA data, the level of AQP3 protein expression in intestinal tissue, as assessed by western blot, was decreased by LPS administration (Fig. 3A). GRS inhibited this LPS-induced decrease in AQP3 expression, whereas DEX failed to inhibit it. In immunostained sections, AQP3 expression was observed on the surface of the villi in intestinal tissue from a healthy animal, and there was considerably less AQP3 expression in the villi tips of intestines from LPS-treated mice. In intestines from GRS-treated mice, the expression of AQP3 was similar to that of healthy mice, even though this drug does not inhibit cytokine production (Fig. 3B). In contrast, the level of AQP3 expression in intestines from DEX-treated mice was markedly lower, which is consistent with the western blot data (Fig. 3B). Furthermore, RT-PCR results revealed that GRS causes a dose-dependent increase in AQP3 mRNA expression (Fig. 3C).

GRS did not show an antidiarrhea effect in AQP3−/− mice

To determine whether the inhibition of an LPS-induced decrease in AQP3 expression by GRS was involved in the antidiarrhea effect of this drug, we examined the effect of GRS in LPS-treated AQP3−/− mice. Although GRS inhibited the LPS-induced increase in diarrhea score in wild-type mice, it had no such effect in AQP3−/− mice (Fig. 4A). Additionally, the corresponding histopathological results indicate that GRS also failed to protect against LPS-induced intestinal epithelial damage in AQP3−/− mice (Fig. 4B). These data clearly indicate that GRS inhibits LPS-induced diarrhea and intestinal epithelial cell damage by increasing the expression of AQP3.
GRS suppressed the LPS-induced decrease in AQP3 expression via the inhibition of MAPK

To determine the regulatory mechanism by which GRS controls AQP3 expression, we performed an in vitro experiment using intestinal epithelial cells. When Caco-2 cells were stimulated with TNF-α (10 ng/mL), their AQP3 mRNA expression was markedly decreased. The co-treatment of these cells with GRS dose-dependently inhibited the TNF-α-induced decrease in AQP3 expression (Fig. 5A). In contrast, co-treatment of these cells with DEX did not inhibit the TNF-α-induced decrease in AQP3 mRNA (Fig. 5B), which is consistent with the results from our in vivo experiments. We also examined the effect of co-treatment with U0126, an ERK1/2 inhibitor, and found that U0126 significantly inhibited the TNF-α-induced decrease in AQP3 expression (Fig. 5C), suggesting that the TNF-α-induced decrease in AQP3 expression was dependent on ERK1/2. To test this possibility, the phosphorylation level of ERK was measured. Co-treatment of TNF-α-treated Caco-2 cells with either GRS or U0126 considerably inhibited the phosphorylation of ERK (Fig. 5D). These data suggest that GRS may regulate the expression of AQP3 by inhibiting ERK phosphorylation.

DISCUSSION

This is the first study to verify the antidiarrhea effect of GRS in an animal model and to compare it with that of DEX. In cases of acute infectious gastroenteritis, the invasion of pathogens causes an inflammatory reaction, which destroys the intestinal epithelial barrier, thus impairing water absorption and resulting in diarrhea. To model this, the present study used LPS-treated mice [18]. Our data clearly indicate that both GRS and DEX have potent effects against inflammation-triggered diarrhea. The diarrhea score time courses of mice treated with GRS or DEX were similar, and the effect of GRS at a dose of 1 g/kg was as potent as that of DEX. Both drugs also protected against LPS-induced intestinal epithelial damage. Despite the similarities in their effects, the underlying mechanisms for the antidiarrhea effects of these two drugs appear to be different. DEX clearly inhibited LPS-induced increases in the expression of cytokine mRNA, suggesting that the antidiarrhea effect of DEX is a consequence of its anti-inflammatory effects. In contrast, GRS did not inhibit LPS-induced inflammatory responses in intestinal tissue.

Decreased AQP3 expression under inflammatory conditions has been previously reported from studies using various cell or tissue preparations, such as the intestinal tract and cultured keratinocytes [25–28]. Here, we found that GRS inhibited the LPS-induced decrease in intestinal AQP3 expression in mice. In agreement with previous reports, the expression of AQP3 was markedly decreased in both in vivo LPS-treated intestinal epithelial cells and in vitro TNF-α-treated cultured cells. AQP3 is believed to be a major route for water absorption from the intestinal tract. Additionally, it has been shown that AQP3 is important for maintaining the epithelial barrier function in the skin and intestinal mucosa [29–34]. Because of these previous findings, we hypothesized that the antidiarrhea effect of GRS was caused by its inhibition of a decrease in AQP3 expression. This idea is clearly supported by the data from our experiments with AQP3<sup>−/−</sup> mice. Both the antidiarrhea effect and the protective effect against epithelial damage exhibited by GRS in wild-type mice were completely absent in AQP3<sup>−/−</sup> mice. Notably, the possibility that GRS directly affects intestinal smooth muscle tone can be discounted because GRS did not inhibit acetylcholine-induced contraction in our preliminary study with isolated ileum strips (data not shown).

Our data suggest that both inflammation and a decrease in AQP3 expression are required for causing diarrhea in infectious gastroenteritis. DEX inhibited diarrhea by inhibiting the inflammatory response without changing the level of AQP3 expression, whereas GRS inhibited diarrhea by inhibiting the decrease in AQP3 expression without changing the inflammatory response. Notably, spontaneous diarrhea was not observed in AQP3<sup>−/−</sup> mice. This indicates that loss of AQP3 by itself does not cause diarrhea, and it also supports that inflammatory stimulation in the intestinal tract leads to diarrhea. Furthermore, Ikarashi et al. previously showed that the administration of bisacodyl, a stimulant laxative, to rats causes both a decrease in AQP3 expression and macrophage activation [35]. Inflammatory responses, such as cytokine production and macrophage activation, are part of a major defense system against infectious pathogens. Therefore, because the antidiarrhea effect of GRS does not involve changes in the inflammatory response, this drug may be more useful than DEX as a therapeutic treatment to prevent dehydration in infectious gastroenteritis.

Finally, we performed experiments in cultured cells to examine the mechanism by which GRS regulates AQP3 expression. The level of AQP3 mRNA expression was markedly decreased by TNF-α treatment, and co-treatment with GRS inhibited the TNF-α-induced decrease in AQP3 expression, which is consistent with results from our in vivo experiments. We previously found that TNF-α treatment decreased AQP3 expression in keratinocytes and that this decrease was mediated by MAP kinases, including ERK1/2 and p38 [28]. To investigate if MAP kinases were similarly involved in the effects of GRS, the present study compared the effect of MAP kinase inhibitors with that of GRS. Like GRS, the ERK1/2 inhibitor U0126 inhibited the TNF-α-induced decrease in AQP3 mRNA expression. Additionally, GRS was found to inhibit ERK1/2 phosphorylation after TNF-α treatment. Thus, the inhibitory effect of GRS on the TNF-α-induced decrease in AQP3 expression is probably a result of its inhibition of ERK1/2.

In conclusion, this study demonstrated that GRS has both an antidiarrhea effect and an intestinal tissue-protective effect, which it exerts through the inhibition of an
inflammation-induced decrease in intestinal AQP3 expression. Our data strongly support the clinical usefulness of GRS to prevent dehydration in infectious gastroenteritis as well as the pharmacological rationale for using GRS to treat this disease.

ACKNOWLEDGMENTS

We are grateful to Tsumura Corporation for providing the GRS extract and its HPLC analysis. We would like to thank Verkman AS for providing the genetically modified mice (AQP3<sup>−/−</sup> mice). This work was supported in part by a grant-in-aid for scientific research (20K07795) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

CONFLICTS OF INTEREST

Yoichiro Isohama received a research grant from Tsumura & Co. except for studies using AQP3 knockout mice.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1: Chemical profile of GRS obtained by the three-dimensional HPLC analysis.