Studies of Aloe. III. Mechanism of Cathartic Effect. (2)

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The mechanism of action of aloe-emodin-9-anthrone, a decomposition product of barbaloin, in causing a significant increase in the water content of the rat large intestine, was investigated. Aloe-emodin-9-anthrone inhibited rat colonic Na^+, K^+-adenosine triphosphatase (ATPase) *in vitro*, and increased the paracellular permeability across the rat colonic mucosa *in vivo*. Therefore, it seemed that the increase in water content of the rat large intestine produced by aloe-emodin-9-anthrone was due to both inhibition of absorption and stimulation of secretion without stimulation of peristalsis. Furthermore, pretreatment with loperamide, an antidiarrheal agent, completely prevented the increase of paracellular permeability induced by aloe-emodin-9-anthrone but did not completely reduce the concomitant increase in residual fluid volume. These findings suggest that aloe-emodin-9-anthrone has multiple mechanisms of action involved in the increase of water content in the rat large intestine.

Keywords aloe-emodin-9-anthrone; large intestine water content; Na^+, K^+-ATPase; paracellular permeability; barbaloin; cathartic effect mechanism; aloe; aloe-emodin; pontamine sky blue

In the previous study,[1] barbaloin, which is the main laxative component of aloe, was shown to undergo, when administered orally to rats, decomposition to aloe-emodin-9-anthrone (AE-anthrone) and aloe-emodin (AE) in the large intestine. Each of barbaloin, AE-anthrone and AE administered orally to rats was found to cause a marked increase in water content in the large intestine. Besides, it was shown that AE-anthrone, unlike the other two, caused the increase in water content in the rat large intestine through a mechanism not involving the stimulation of peristalsis. AE-anthrone also caused an increase in the Na^+ content of the rat colon segment. Therefore, it was considered that the increase in water content produced by AE-anthrone might result from an inhibition of electrolyte absorption or a stimulation of electrolyte secretion, *i.e.*, derangement of electrolyte transport in the rat large intestine. To elucidate the mechanism of this AE-anthrone-induced increase in water content in the rat large intestine, we examined the effects of AE-anthrone on rat intestinal Na^+,K^+-adenosine triphosphatase (ATPase) *in vitro* and on the paracellular permeability across the colonic mucosa of the rat *in vivo*. Moreover, we examined how the increase in paracellular permeability and the increase in water content produced by AE-anthrone were modified by loperamide, an agent which is known to affect water and electrolytes balance in the intestine.

Materials and Methods

Chemicals Barbaloin, AE-anthrone, AE and sennoside A used in this study were the same as described previously.[1] 1,8-Dihydroxyanthraquinone (DHA), hydroquinone, phenolphthalein and adenosine triphosphate (ATP) were purchased from Wako Pure Chemical Ind. (Tokyo, Japan) and sodium deoxycholate and pontamine sky blue (PSB) were obtained from Difco Laboratories (Detroit, Michigan, U.S.A.) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively.

Bovine serum albumin (BSA) and SEP-PAK® cartridges (SEP-PAK) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Japan Waters Co., Ltd. (Tokyo, Japan), respectively. Loperamide hydrochloride (Janssen) was a generous gift from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Animals Male Wistar rats were purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Before the start of the experiment, the rats were kept in an air-conditioned room (24±1 °C, 50—60% humidity) with a 12-h light-dark cycle (7:00—19:00) and had free access to commercial rat chow (Oriental-MF, Tokyo, Japan) and water.

Na^+,K^+-ATPase Assay The determination was carried out by the method of Hao.[2] Small and large intestinal mucosae were obtained from male Wistar rats (250—300 g) and homogenized in 5 ml of solution using a glass Potter-Elvehjem-type homogenizer with a Teflon pestle at 0 °C. The homogenate was treated according to the procedure shown in Fig. 1. The concentration of inorganic phosphorus (Pi) was estimated by the method of Fiske and Subbarow.[3] Protein assay of the homogenate was carried out by the method of Lowry *et al.*[4] using bovine serum albumin as a standard. Test substances were dissolved in water, or in 5% or 50% dimethylsulfoxide (DMSO) aqueous solution, and added to test tubes before addition of the homogenate. Each control tube received a volume of the solvent at the same concentration as in the test tube. The Na^+,K^+-ATPase activities of the small intestinal and colonic mucosae in the control group to which water was added were 11.24±1.15 and 9.77±0.91 μmol Pi/h/mg protein. The Na^+,K^+-ATPase activities of the colonic mucosa in the control groups to which DMSO was added at 5 and 50% were 9.51±1.14 and 4.93±0.53 μmol Pi/h/mg protein.

Mg^{2+}-ATPase Assay The above-described homogenate was treated

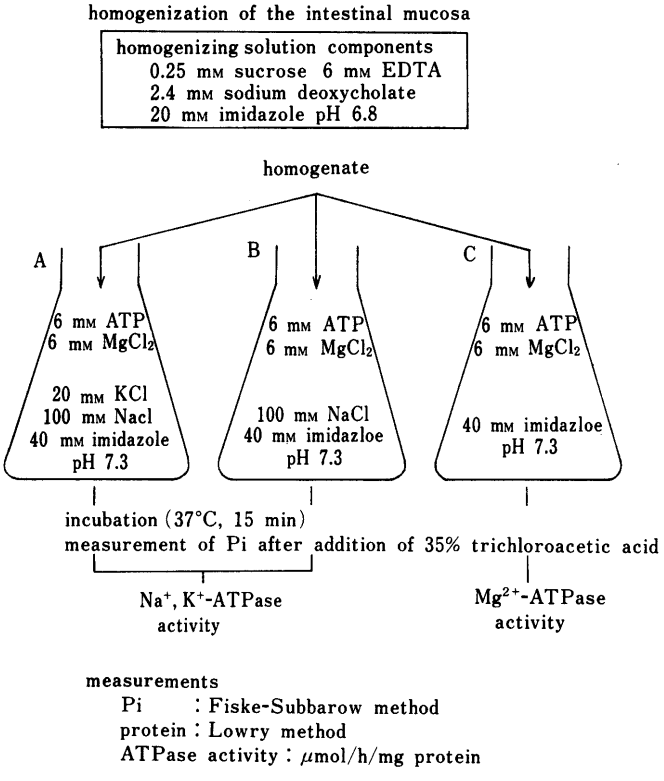

Fig. 1. Method Used for ATPase Activity Measurement

according to the procedure shown in Fig. 1 for the Na^+,K^+-ATPase assay.

Paracellular Permeability The biological assay methods used were modified versions of those by Nell *et al.*[5] and Sakurai *et al.*[6] Male Wistar rats (200—300 g), which had received only water for 18 h before the experiment, were anesthetized with pentobarbital sodium (30 mg/kg, i.p.). The abdomen was then opened and both ends of the colon were ligated. Small notches were made at both ends of the isolated segment. After the lumen had been moderately well rinsed with saline solution (20 ml), the solution remaining in the lumen was driven out as thoroughly as possible by a stream of air passed through it. Then, a colon segment (length, 8—11 cm) was made by ligating the inside of both notches. As soon as 2 ml of 5% gum arabic–saline solution, containing the test substance at 10^{-3} M, had been injected into the colon segment, the abdomen was closed. Then, 1 ml of 4% PSB saline solution was immediately administered intravenously (i.v.). One hour later, the colon segment was excised from the rat under ether anesthesia. The outside of the colon segment was rinsed with saline solution to remove adherent blood. The residual fluid content in the segment was collected and the volume measured. Then the amount of PSB contained in it was analyzed and used as an indicator of the paracellular permeability. In the control group, 2 ml of 5% gum arabic–saline solution was injected into the segment.

For loperamide treatment, 1 ml of 5% gum arabic–saline solution containing 0.02 mg of loperamide was injected into the segment before AE-anthrone injection (1 ml, 2×10^{-3} M, in 5% gum arabic–saline solution). The same volume of 5% gum arabic–saline solution was injected, instead of loperamide or AE-anthrone solution, into the control animals.

Determination of PSB The residual fluid in the colon segment mentioned above was homogenized ultrasonically with 3 ml of methanol. The supernatant was obtained by centrifugation at 3000 rpm for 10 min. The residue was treated twice with 3 ml of 70% methanol aqueous solution in a similar manner. The combined supernatants were evaporated to dryness under reduced pressure, and the residue, dissolved in 12 ml of a solvent mixture [AcOET–MeOH–H_2O (100 : 16.5 : 13.5)], was applied to a SEP-PAK cartridge. The SEP-PAK cartridge was then washed with 13 ml of the solvent mixture, and eluted with 10 ml of 50% aqueous methanol solution. The eluate was evaporated to dryness under reduced pressure, and the residue was redissolved in 5 ml of water. Finally, the absorbance of the solution was measured at 620 nm.

Results

Effects of Barbaloin and Related Substances on Rat Intestinal Na^+,K^+-ATPase The amount of Pi produced by non-enzymatic breakdown from ATP during incubation for 15 min was approximately constant. Table I shows the effects of barbaloin, AE and AE-anthrone on Na^+,K^+-ATPase obtained from rat intestinal epithelial cells. In this experiment, DHA, hydroquinone and phenolphthalein were used as reference substances,[7] and their effects on Mg^{2+}-ATPase were also examined simultaneously for comparison with their effects on Na^+,K^+-ATPase. The values in Table I represent percentages of the control.

Neither barbaloin nor AE had any effect on Na^+,K^+- or Mg^{2+}-ATPase in small intestinal and colonic mucosae of rats. However, AE-anthrone inhibited Na^+,K^+-ATPase by 30% in small intestinal mucosa and by 16% in colonic mucosa, and Mg^{2+}-ATPase in these mucosae by 36% and 30%, respectively. On the other hand, DHA inhibited neither of these enzymes, and hydroquinone inhibited only Na^+,K^+-ATPase (by 14% in small intestinal mucosa and by 35% in colonic mucosa). However, phenolphthalein caused a 70% inhibition of Na^+,K^+-ATPase in small intestinal mucosa and also over a 30% inhibition of Mg^{2+}-ATPase in both mucosae, having the strongest potency of all the agents examined. Among barbaloin and related substances, only AE-anthrone, which produced a clear increase in water content in the rat colon segment, inhibited both types of ATPases, as did phenolphthalein.

Effects of Barbaloin and Related Substances on the Paracellular Permeability across the Rat Colonic Mucosa Sodium deoxycholate and phenolphthalein were examined as positive references.[5] Figure 2 shows the amounts of residual fluid volume in the ligated colon segments at 1 h after injection of the test substances. Figure 3 shows the leakage of PSB into the colon segments. Barbaloin and AE had no effect on either the residual fluid volume or PSB leakage. However, AE-anthrone, as well as sodium deoxycholate and phenolphthalein, caused a significant increase in both parameters. This indicates that treatment with AE-anthrone markedly increased the paracellular permeability of PSB into the colon.

Effect of AE-anthrone and Loperamide on Paracellular Permeability across the Rat Colonic Mucosa Loperamide was injected into the colon segment 10 min before the

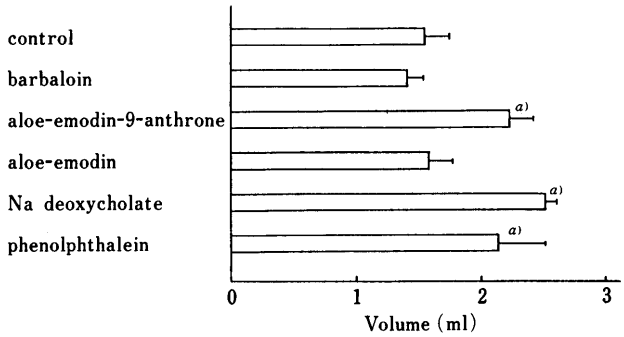

Fig. 2. Residual Fluid Volume in Colon Segments of Rats 1 h after Injection of Test Substance (10^{-3} M, 2 ml)

Each column represents the mean ± S.D. for 5—11 animals. *a)* $p<0.01$ as compared with controls.

TABLE I. Effect of Various Substances on Na^+,K^+-ATPase and Mg^{2+}-ATPase Activities in Rat Intestinal Mucosa

Substances	Conc. ($\times 10^{-4}$ M)	Na^+,K^+-ATPase[a] (% of control)		Mg^{2+}-ATPase[a] (% of control)	
		Small intestine	Colon	Small intestine	Colon
Barbaloin[b]	5	105.5 ± 7.4	109.3 ± 17.9	98.4 ± 12.8	96.7 ± 18.9
Aloe-emodin[c]	3	114.5 ± 22.0	128.7 ± 27.1	104.9 ± 9.3	93.5 ± 15.3
Aloe-emodin-9-anthrone[d]	3	69.5 ± 5.7[e]	83.9 ± 3.9[e]	63.5 ± 7.1[e]	70.5 ± 9.2[e]
1,8-Dihydroxyanthraquinone[c]	3	129.5 ± 25.7	107.1 ± 22.5	95.6 ± 6.2	104.4 ± 9.2
Hydroquinone[b]	3	86.1 ± 7.2	64.6 ± 2.3[e]	102.9 ± 6.0	112.4 ± 23.2
Phenolphthalein[d]	3	31.5 ± 15.9[f]	30.2 ± 6.0[f]	67.7 ± 8.3[e]	66.3 ± 7.8[e]

a) Each value represents the mean ± S.D. obtained from 5 experiments. *b)* Dissolved in water. *c)* Dissolved in 50% DMSO. *d)* Dissolved in 5% DMSO. *e)* $p<0.05$ as compared with controls. *f)* $p<0.01$ as compared with controls.

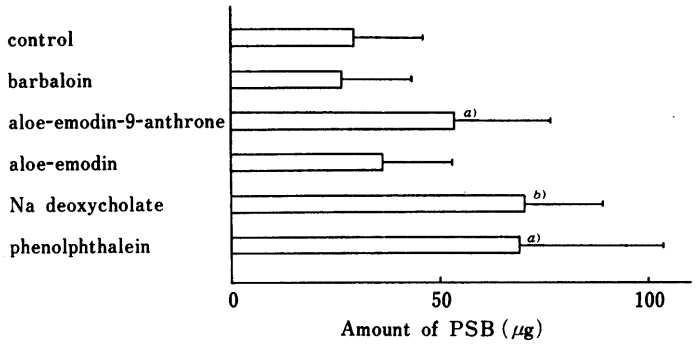

Fig. 3. PSB Leakage into Rat Colon Induced by Various Substances

Each colon segment was filled with 5% gum arabic solution (2 ml) along with test substance (10^{-3} M). Immediately, 4% PSB was administered i.v., and then leakage of PSB into the colon segment was determined after 1 h. Each column represents the mean ± S.D. for 5—11 animals. *a)* $p<0.05$ as compared with controls. *b)* $p<0.01$ as compared with controls.

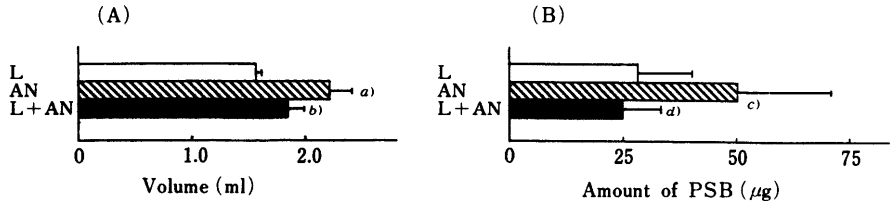

Fig. 4. Effect of Loperamide and Aloe-emodin-9-anthrone on Residual Fluid Volume (A) and PSB Leakage (B) into Rat Colon Segment

Each colon segment was filled with 5% gum arabic solution (1 ml) with or without loperamide (0.02 mg). After 10 min, 5% gum arabic solution (1 ml) with or without aloe-emodin-9-anthrone (2×10^{-3} M) was injected into the colon segment. Immediately, 4% PSB was administered i.v., and then residual fluid volume and leakage of PSB into the colon segment were determined after 1 h.

L: group injected with loperamide alone (negative control). AN: group injected with aloe-emodin-9-anthrone alone (positive control). L+AN: group injected with both loperamide and aloe-emodin-9-anthrone. *a)* $p<0.01$ as compared with L. *b)* $p<0.01$ as compared with L or AN. *c)* $p<0.05$ as compared with L. *d)* $p<0.01$ as compared with AN.

injection of AE-anthrone, and both the residual fluid volume and the leakage of PSB into the colon segment were determined. The results obtained from the group treated with loperamide alone (negative control) or AE-anthrone alone (positive control) are shown in Fig. 4. From a comparison with Figs. 2, 3 and 4, it was clear that loperamide had no effects on either normal residual fluid volume or normal PSB leakage into the rat colon segment. However, pretreatment with loperamide significantly reduced the effects of AE-anthrone on these parameters. In particular, PSB leakage stimulated by AE-anthrone was markedly reduced to the level of the negative control, *i.e.*, loperamide completely prevented the increase in paracellular permeability induced by AE-anthrone. Likewise, the effect of AE-anthrone on residual fluid volume was reduced, though not so remarkably, by pretreatment with loperamide ($p<0.01$).

Discussion

Microsomal Na^+,K^+-ATPase is well known to play an important role in the active transport of cations (Na^+ pump) across cell membranes. Chignell[7] showed that phenolphthalein, bisacodyl and DHA inhibited the microsomal Na^+,K^+-ATPase obtained from brush border (epithelial) cells of rat small intestine. On the basis of this, he suggested that inhibition of intestinal sodium transport by purgative drugs was responsible for the inhibition of intestinal Na^+,K^+-ATPase, resulting in water retention. Our present finding that Na^+,K^+-ATPase in rat intestinal mucosa was inhibited by hydroquinone and phenolphthalein, is consistent with this hypothesis. However, our result with regard to DHA differed from Chignell's. This might have been due to the difference in the solution used to dissolve the DHA.

In this study, we found that AE-anthrone inhibited Na^+,K^+-ATPase, suggesting that the significant increase in water content produced by AE-anthrone in the rat large intestine was partly caused by inhibition of Na^+ active transport. Furthermore, AE-anthrone inhibited not only Na^+,K^+-ATPase but also Mg^{2+}-ATPase, as was the case with phenolphthalein. Therefore, the inhibitory effect of AE-anthrone on Na^+,K^+-ATPase was non-specific.

On the other hand, it has already been shown that natural and synthetic anthraquinone laxatives not only inhibit the absorption of water and sodium from the intestine, but also cause an increase in the net transfer of fluid and electrolytes into the intestinal lumen.[8] Nell *et al.*[5] reported that under the influence of deoxycholate and oxyphenisatin, a diphenolic laxative, net transport of sodium and water from blood to the rat colonic lumen occurred mainly *via* the intercellular route. Therefore, they considered that deoxycholate and oxyphenisatin were able to increase the paracellular permeability across the colonic mucosa. Verhaeren *et al.*[9] reported that a significant increase in paracellular permeability across the colonic mucosa after pretreatment with DHA in the guinea pig could be responsible for the movement of water and electrolytes across the intestine. In contrast, an inhibitory effect of opiate agonists on the fluid secretion stimulated by several substances was reported by Beubler and Lembeck.[10] Verhaeren *et al.*[11] also reported that loperamide, an opiate agonist, blocked the DHA-stimulaled leakage of ^{99m}Tc-ethylenediaminetetraacetic acid (EDTA) (a marker of the extracellular space) through the mucosa. Therefore, he postulated that the antisecretory action of

loperamide, and the stimulating effect of non-osmotic laxatives such as DHA on fluid secretion have opposite effects on the paracellular permeability of the colonic mucosa.

Nell *et al.*[5] used ^{51}Cr-EDTA or [^{14}C] inulin as a tracer for the paracellular pathway and injected these substances i.v. or into ligated colon segments (*in situ*). Verhaeren *et al.*[9,11] injected ^{99m}Tc-EDTA into colon segments. In the present study, we used the pigment PSB, which is distributed only in the extracellular space after i.v. injection. Thus, the influence of AE-anthrone and loperamide on the paracellular permeability across the colonic mucosa was able to be determined by colorimetric measurement of pigment leakage into the colon segment. Indeed, an increase in the paracellular permeability was found to be induced by AE-anthrone in the rat colon segment. Furthermore, we observed that pretreatment with loperamide, an antidiarrheal agent, reduced the AE-anthrone-induced increases in both paracellular permeability and residual fluid volume. These findings imply that the effect of AE-anthrone on paracellular permeability may have something to do with the increase of water content indeed by this substance in the rat large intestine. However, the increase in residual fluid volume by AE-anthrone was not reduced completely by loperamide, in contrast to its complete reversal of the paracellular permeability increase. Therefore, it seems likely that AE-anthrone has multiple mechanisms for the increase of water content in the rat large intestine, such as inhibition of Na^+ active transport, increase of paracellular permeability, *etc.*

As to the cathartic mechanism of senna, which is a natural anthraquinone laxative like aloe, alteration of intestinal motility[12] and stimulation of intestinal secretion mediated by prostaglandins or a Ca-dependent mechanism[13] have been proposed. We have already suggested that the stimulation of peristalsis by AE might be involved in the cathartic activity of barbaloin, because AE caused contractions of isolated ileal strips from guinea pigs.[14] Therefore, we are planning to carry out a further study to elucidate the relationship between the stimulation of peristalsis and the increase in water content.

In conclusion, AE-anthrone inhibits Na^+,K^+-ATPase and increases paracellular permeability in the rat colon mucosa, and these actions are considered to play an important role in the cathartic activity of barbaloin.

Acknowledgements The authors are grateful to Dainippon Pharmaceutical Co. for their gift of loperamide hydrochloride. The authors also wish to thank Miss. E. Ohishi for carrying out the preliminary experiments for this study.

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