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The protective and hormonal effects of proanthocyanidin against gastric mucosal injury in Wistar rats

Yoshikazu Iwasaki, Teruaki Matsui, and Yasuyuki Arakawa

Division of Gastroenterology and Hepatology, Department of Medicine, Nihon University School of Medicine, 30-1 Oyaguchi Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan

Background. Proanthocyanidin, a grape-seed polyphenol, has been reported to have protective properties against vascular injury and ulcers, preventive effects against atherosclerosis and cancer, and antioxidative effects, such as improving lipid metabolism and slowing aging. However, little has been reported on its antiulcer effects. We aimed to elucidate the antiulcer mechanism of proanthocyanidin. ***Methods.*** Gravinol, containing 89.3% proanthocyanidin, was used. Proanthocyanidin solution, in distilled water, at 0.002%, 0.02%, 0.2%, or 1%, was given to rats ad libitum for 2 weeks. Distilled water was given to control rats. The effect of proanthocyanidin on gastric mucosal injury was investigated with the water-immersion restraint stress model. The ratios of areas of hemorrhagic erosion were compared as the lesion index. Myeloperoxidase activities were also examined, as an index of tissue injury. Superoxide dismutase activity was measured to examine its antioxidative effect. Furthermore, serum gastrin, somatostatin, histamine, and prostaglandin E_2 levels were measured in this rat model. ***Results.*** Proanthocyanidin administration significantly suppressed gastric mucosal injury, induced by water-immersion restraint stress, in a dose-dependent manner. Myeloperoxidase activities were also significantly inhibited, whereas superoxide dismutase activities were significantly stimulated. As to gastrointestinal hormones, the secretion of gastrin, somatostatin, and histamine was significantly inhibited, while prostaglandin E_2 secretion was significantly stimulated. ***Conclusions.*** Proanthocyanidin was shown to have a protective effect on the gastric mucosa. The mechanisms underlying the effect of proanthocyanidin were considered to be the following: anti-gastrin and anti-histamine effects to prevent attacks by water-immersion restraint stress, and mucoprotective properties, bestowed by increased prostaglandin and increased superoxide dismutase activities in the gastric mucosa.

Key words: proanthocyanidin, water-immersion restraint stress, isolated stomach infusion model, gastrin, somatostatin, histamine, prostaglandin E_2

Introduction

Wine drinking has been appreciated for a long time all over the world, and recent health-oriented trends have heightened interest in the physiological effect of polyphenols contained in wine. "Polyphenol" is a generic designation of elements in plants that contain two or more phenolic hydroxy residues in a molecule. These elements are important constituents for the protection and maintenance of plant species. Proanthocyanidin (PA), a major constituent of grape-seed polyphenols contained in wine, is a chemical compound made by the condensation and polymerization of flavan-3-ol or flavan-3, 4-diols at the 4′–6′ or the 4′–8′ positions, and it is a condensed tannin. PA has been discussed in relation to the famous French paradox,[1,2] and has been reported to improve ischemic cardiovascular disease[3] and to help prevent atherosclerosis.[4] It has already been used as a vascular remedy for the prevention of lifestyle-related diseases in Europe. Its beneficial effects on the gastrointestinal tract include enhancement of bacterial flora in the intestine, preventive action against colorectal cancer,[5,6] a decrease in the prevalence of *Helicobacter pylori* in wine-producing areas,[7] and antiulcer effects.[8] However, PA's antiulcer effects have not been elaborated in previous reports.

Therefore, in an attempt to clarify the mechanism of PA's inhibitory effect on gastric mucosal injury, we used a water-immersion restraint (WIR) stress model in rats. In addition, we investigated the mechanism

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Reprint requests to: Y. Iwasaki

from the aspect of antioxidative properties, and effects on prostaglandin (PG) and gastrointestinal tract hormones.

Materials and methods

Reagents

PA was provided in the form of crude PA (Gravinol S; lot GVL0003) as a gift from Kikkoman (Tokyo, Japan). Gravinol S contains 89.3% PA. The composition of PA included: dimers, 6.6%; trimers, 5.0%; tetramers, 2.9%; oligomers, 74.8%; and pentamers, 6.6% or more.

Elements other than PA included: monomeric flavonols (2.53% (+)-catechin, 2.17% (−)-epicatechin, 1.37% (−)-epigallocatechin, and 0.53% (−)-epigallocatechin gallate); 2.24% moisture; 1.06% protein; and 0.8% ash.

Gravinol S was diluted with distilled water to prepare solutions at concentrations of 0.002%, 0.02%, 0.2%, and 1%.

Methods

Male Wistar rats, weighing 130–160 g (Charles River Japan, Tokyo, Japan), were kept in a 12-h light-and-dark cycle environment. Room conditions were temperature of 23°C and 50% humidity. The animals were fed a commercial diet (Oriental Yeast, Tokyo, Japan). Distilled water was given to a control group, and a 0.002%, 0.02%, 0.2%, or 1% PA solution was given to each of four PA groups. The five groups were compared. Distilled water with or without PA was given ad libitum for 2 weeks. In the PA groups, the intake was measured by a metabolic gauge. On average, the intake of PA in its crude form was as follows: 0.694 ± 0.347 mg/body per day in the 0.002% PA group; 7.34 ± 2.45 mg/body per day in the 0.02% PA group; 66.02 ± 11.5 mg/body per day in the 0.2% PA group; and 341.4 ± 74.7 mg/body per day in the 1% PA group. There were no significant differences in body weight among the five groups after 2 weeks. All experiments were performed after 24-h fasting, during which distilled water with or without PA was given ad libitum. The WIR model was prepared only to induce gastric mucosal injury.

All animal experiments were performed with the approval of the Ethics Committee of Nihon University School of Medicine.

Preparation of gastric mucosal injury and measurement of the lesion index

A gastric mucosal injury model induced by WIR stress was prepared according to the method of Takagi and Okabe.[9] Rats were immobilized in a restraint cage and were immersed, up to the xiphoid process, for 4 h in a water bath controlled at 23°C. Later, under anesthesia with pentobarbital sodium, the abdomen was incised and the stomach was extracted. After dissection along the greater curvature of the stomach, the extent of gastric mucosal injury was observed. Photographs of the resected stomach were taken with a digital camera (COOLPIX990; Nikon, Tokyo, Japan). The ratio of the total area of hemorrhagic erosion in the corpus to the total mucosal area in the corpus was measured with Mac SCOPE, which is an image analysis software program (Mitani, Chiba, Japan). The ratio was designated as the lesion index.

In the same model, gastric mucosal tissue was collected and homogenized. Myeloperoxidase (MPO) activity was measured using the COBAS-FARA method,[11,12] and superoxide dismutase (SOD) activity in the gastric mucosal tissue was measured using a nitrite-kit method.[10]

The resected gastric mucosal tissue was snap-frozen, and a 25-fold volume of 0.5% hexadecyltrimethylammonium bromide (HTAB) in a potassium phosphate buffer, at a pH of 6.0, was added later. The tissue was homogenized on a beaker full of ice. Ultrasound treatment (for 30 s at 20 kHz) was repeated five times. After three freeze-and-thaw cycles at 37°C, the homogenate was centrifuged at 25°C and 23 000 rpm for 15 min for extraction.

MPO measurement

We added 2.9 ml of 50-nM phosphate buffer at pH 6.0 (containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide) to 0.1 ml of sample. The mixture was incubated for 15 min and the optical density (OD) was then measured at a wavelength of 460 nm.

SOD measurement

Distilled water, or 0.1 ml of 10 mM KCN and 0.4 ml of distilled water, was added to 0.1 ml of sample. To this, 0.2 ml of the reaction reagent, prepared by the addition of 10 ml of distilled water, was added, and the mixture was incubated at 37°C for 5–10 min. Then, 0.2 ml of the enzyme solution was added and the mixture was incubated at 37°C for 30 min. Color developer was added, and the mixture was incubated at room temperature for 30 min. The OD was measured at 550 nm.

Preparation of isolated rat stomach infusion model and measurement of gastrointestinal hormones

Figure 1 shows the isolated stomach infusion method[13] used. The rats were anesthetized with pentobarbital sodium to prepare for the isolated stomach infusion model. The stomach, duodenum, and associated vessels

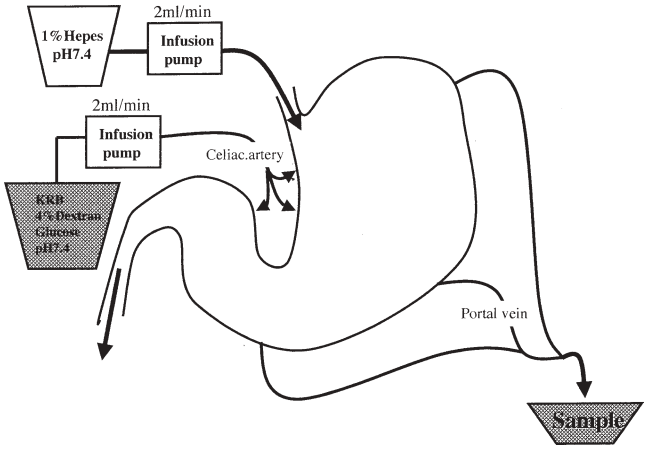

Fig 1. Isolated rat stomach infusion model. *KRB*, Krebs-Ringer bicarbonate; *Hepes*, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]

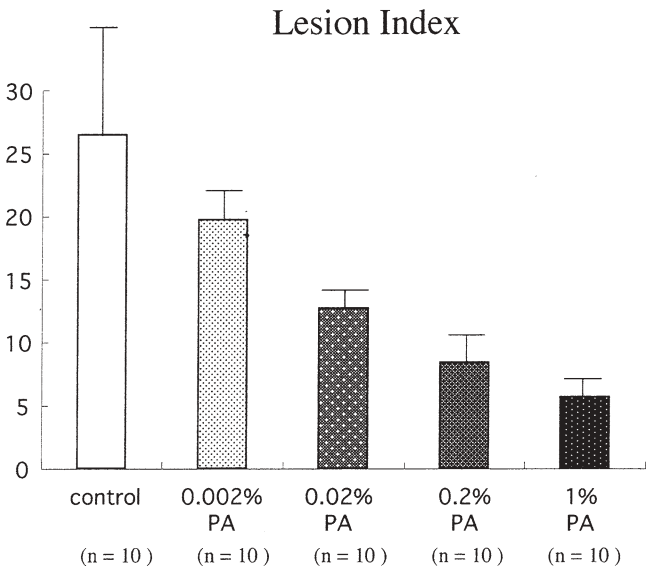

Fig 2. Protective effects of proanthocyanidin (*PA*) on gastric mucosal lesions induced by water-immersion restraint stress. The lesion index was determined as the ratio of the area of mucosal hemorrhagic erosion to the stomach body area. In comparision with findings in the control group ($n = 10$), the lesion index was significantly (**$P < 0.01$; analysis of variance [ANOVA]) decreased in the 0.002% proanthocyanidin-treated group ($n = 10$), the 0.02% proanthocyanidin-treated group ($n = 10$), the 0.2% proanthocyanidin-treated group ($n = 10$), and the 1% proanthocyanidin-treated group ($n = 10$)

were resected. Polyethylene tubes were inserted and fixed into the celiac artery and the portal vein. Other arteries, veins, and the pancreas were ligated and excised. After ligation, polyethylene tubes were inserted and fixed to the cardia and pyloric regions of the resected stomach. Subsequently, the resected stomach was bathed in Krebs Ringer-bicarbonate buffer (KRBB) supplemented with 4% dextran and 0.8 g/dl glucose, gassed with 95% O_2 and 5% CO_2, and immersed in the solution at 37°C, pH 7.4 for the following experiment. To exclude the effect of gastric acid, the extracted stomach canal was perfused at a rate of 2 ml/min with NaOH solution adjusted to a pH of 7.4 with the addition of 1% HEPES. The vascular system was also perfused from the celiac artery, at a rate of 2 ml/min, with KRBB supplemented with 4% dextran, 0.8 g/dl glucose, and bovine serum albumin (Sigma Chemical, St Louis, USA), and the solution was resuspended at 0.2 g/dl, bubbled with 95% O_2 and 5% CO_2, and adjusted to pH 7.4. The temperature of both solutions (i.e., the extracted stomach canal and the vascular system) was maintained at 37°C. The perfusate draining from the portal vein was sampled every 2 min and fractionated. To exclude the influence of these procedures, the sample was collected after 20 min of perfusion. Gastrin, somatostatin, and histamine levels in the collected samples were measured by radioimmunoassay (RIA), and PGE_2 was measured by radioimmunoassay with polyethylene glycol (RIA-PEG).

Statistical analysis

The values for obtained data were presented as means ± SD, and dose-dependency was statistically analyzed by analysis of variance (ANOVA). When a P value was less than 0.05, it was considered statistically significant.

Results

Effects on gastric mucosal injury induced by WIR stress

The lesion index (Fig. 2)

The lesion index was 26.44 ± 10.52 in the control group, 19.67 ± 2.41 in the 0.002% PA group, 12.67 ± 1.73 in the 0.02% PA group, 8.38 ± 1.14 in the 0.2% group, and 5.62 ± 1.23 in the 1% PA group. The lesion index decreased in a dose-dependent manner, and the inhibition of gastric mucosal injury was significant (ANOVA; $P < 0.01$).

MPO activities (Fig. 3)

The MPO activity in the gastric mucosal tissue was 39.22 ± 10.7 U/mg protein in the control group, 37.47 ± 11.26 U/mg protein in the 0.002% PA group, 30.65 ± 9.5 U/mg protein in the 0.02% PA group, 24.37 ± 4.50 U/mg protein in the 0.2% PA group, and 17.05 ± 6.50 U/mg protein in the 1% PA group. The MPO activities were significantly inhibited in a dose-dependent manner (ANOVA; $P < 0.01$).

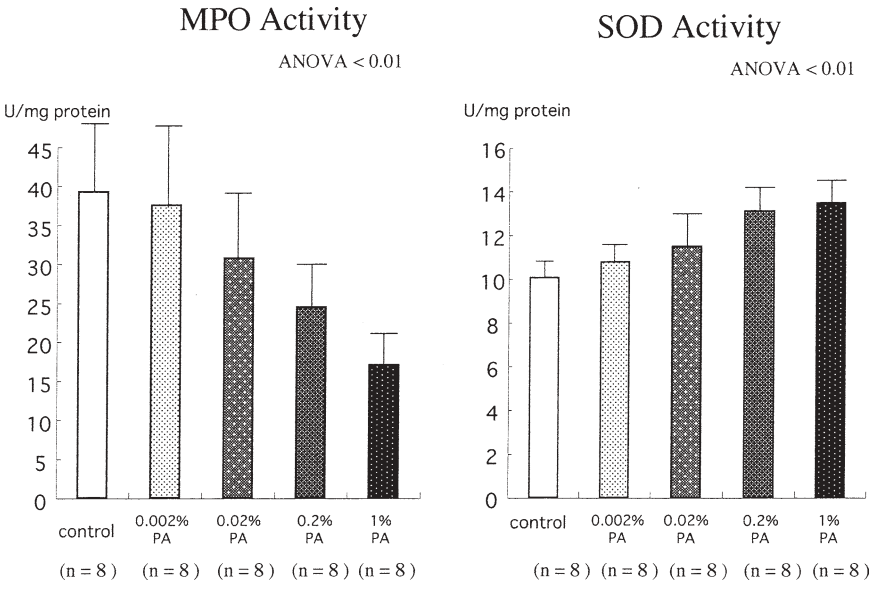

Fig 3. Effects of proanthocyanidin on myeloperoxidase (*MPO*) activity and superoxide dismutase (*SOD*) activity in the gastric mucosal tissue. In comparison with findings in the control group ($n = 8$), the secretion of MPO was significantly ($^{**}P < 0.01$) inhibited, and that of SOD was significantly stimulated, in the 0.002% proanthocyanidin-treated group ($n = 8$), the 0.02% proanthocyanidin-treated group ($n = 8$), the 0.2% proanthocyanidin-treated group ($n = 8$), and the 1% proanthocyanidin-treated group ($n = 8$)

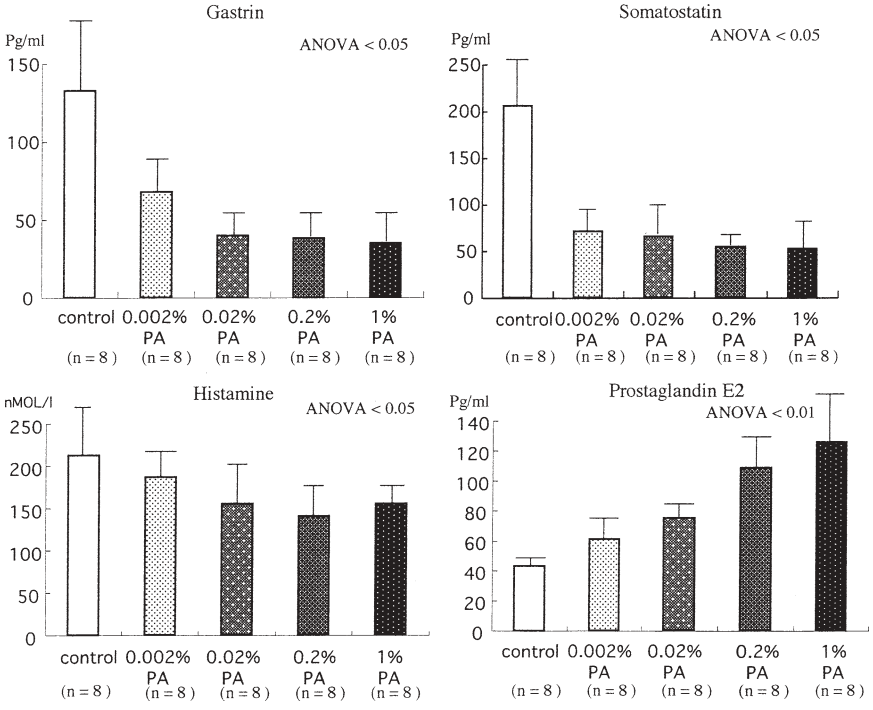

Fig 4. Effects of proanthocyanidin on gastrin, somatostatin, histamine, and prostaglandin E_2 release in the perfusate. In comparison with findings in the control group ($n = 8$), the secretion of gastrin, somatostatin, and histamine was significantly ($^{*}P < 0.05$) inhibited in the 0.002% proanthocyanidin-treated group ($n = 8$), the 0.02% proanthocyanidin-treated group ($n = 8$), the 0.2% proanthocyanidin-treated group ($n = 8$), and the 1% proanthocyanidin-treated group ($n = 8$). In comparison with findings in the control group ($n = 8$), the secretion of prostaglandin E_2 was significantly ($^{**}P < 0.01$) stimulated in the 0.002% proanthocyanidin-treated group ($n = 8$), the 0.02% proanthocyanidin-treated group ($n = 8$), the 0.2% proanthocyanidin-treated group ($n = 8$), and the 1% proanthocyanidin-treated group ($n = 8$)

SOD activities (Fig. 3)

The SOD activity in the gastric mucosal tissue was 10.05 ± 0.92 U/mg protein in the control group, 10.76 ± 1.19 U/mg protein in the 0.002% PA group, 11.45 ± 1.71 U/mg protein in the 0.02% PA group, 13.08 ± 1.42 U/mg protein in the 0.2% PA group, and 13.43 ± 1.40 U/mg protein in the 1% PA group. The SOD activities were significantly stimulated in a dose-dependent manner (ANOVA; $P < 0.01$).

Effects on gastrointestinal hormones and PGE_2

Effects on gastrin secretion (Fig. 4)

The gastrin level in the gastric mucosal tissue was 132.50 ± 58.25 pg/dl in the control group, 67.62 ± 19.32 pg/dl in the 0.002% PA group, 39.62 ± 14.43 pg/dl in the 0.02% PA group, 37.87 ± 17.98 pg/dl in the 0.2% PA group, and 34.71 ± 21.92 pg/dl in the 1% PA group. Gastrin secretion was significantly inhibited in a dose-dependent manner (ANOVA; $P < 0.05$).

Effects on somatostatin secretion (Fig. 4)
The somatostatin level in the gastric mucosal tissue was 205.30 ± 52.06 pg/dl in the control group, 70.87 ± 18.88 pg/dl in the 0.002% PA group, 65.50 ± 25.37 pg/dl in the 0.02% PA group, 54.12 ± 7.93 pg/dl in the 0.2% PA group, and 51.75 ± 19.08 pg/dl in the 1% PA group. Somatostatin secretion was significantly inhibited in a dose-dependent manner (ANOVA; $P < 0.05$).

Effects on histamine secretion (Fig. 4)
The histamine level in the gastric mucosal tissue was 212.5 ± 77.08 nM/l in the control group, 186.25 ± 30.67 nM/l in the 0.002% PA group, 155.12 ± 53.22 nM/l in the 0.02% PA group, 140.75 ± 42.46 nM/l in the 0.2% PA group, and 155 ± 21.32 nM/l in the 1% PA group. Histamine secretion was significantly inhibited in a dose-dependent manner (ANOVA; $P < 0.05$).

Effects on PGE_2 secretion (Fig. 4)
The PGE_2 level in the gastric mucosal tissue was 43 ± 7.38 pg/dl in the control group, 60.87 ± 19.14 pg/dl in the 0.002% PA group, 75.12 ± 12.42 pg/dl in the 0.02% PA group, 108.25 ± 27.23 pg/dl in the 0.2% PA group, and 127.75 ± 47.46 pg/dl in the 1% PA group. PGE_2 secretion was significantly stimulated in a dose-dependent manner (ANOVA; $P < 0.01$).

Discussion

We focused on the antiulcer effects of PA, and examined the lesion index in a gastric mucosal injury model created by WIR stress.

In this model, the lesion index was lowered in a dose-dependent manner in the PA groups, and PA evidently had protective properties against gastric mucosal injury.

The protective properties were confirmed by the results regarding MPO, which is a marker for tissue mucosal injury. The administration of PA significantly suppressed MPO activity in the gastric mucosal tissue. MPO is known to be a marker for neutrophil infiltration into mucosal tissue and is increased in mucosa when stress ulcers appear.[14]

Active oxygen has also been considered important in tissue injury because of its association with the hypoxanthine-xanthine oxygenase (HIX-XO) system and the neutrophil system.[15–17] There have been reports that tissue injury with neutrophil infiltration is associated with free radicals, active oxygen, and adherence of neutrophils to the mucosa.[18,19] It has been reported that PGs have potent antioxidative action[20–22] and free-radical scavenger activity.[23–28] As a result of the scavenging of active oxygen and free radicals, adherence of neutrophils to the mucosa was indirectly inhibited, and MPO activity was also suppressed.

Reported mechanisms of acute stress-induced gastric mucosal injury include decreased in situ PGs,[29] increased acid secretion,[30] disturbed microcirculation in the gastric mucosa,[31] suppressed cell cycles,[32] and increased motility of the stomach.[33] In particular, ischemia-reperfusion injury is considered important when there is disturbed blood flow or reperfusion, because active oxygen is produced in the tissue or by neutrophils. Furthermore, disturbed microcirculation is induced by factors such as increased in situ permeability of vessels in the mucosa.[34]

As previously mentioned, PA has antioxidative action and free-radical scavenger activity. According to reports that examined gastric mucosal injury from the aspect of antioxidative action, ischemia-reperfusion injury was related to the presence of free radicals and active oxygen that is produced from oxygen molecules.[35–37] Indeed, there was a report that SOD activity was decreased in injured mucosal tissue.[38]

Yoshikawa et al.[39] reported that SOD, a free-radical scavenger, could remarkably inhibit gastric mucosal injury induced by WIR stress. Hirota et al.[40] prepared SOD bound to albumin to maintain the concentration of SOD in the blood for a long time, which contributed to their obtaining the same result. In our study, SOD activities in the gastric mucosa were stimulated in a dose-dependent manner in the PA groups.

Altogether, it was concluded that PA scavenged free radicals and active oxygen produced by ischemia-reperfusion injury, which led to extended and increased SOD activity. The increase in SOD activity was assumed to have played an important role in the inhibition of gastric mucosal injury. PA's antioxidative action was elucidated to play a critical role as an inhibitory factor against gastric mucosal injury.

We also examined the effect of PA on acid secretion by measuring gastrointestinal hormones related to acid secretion, such as gastrin, somatostatin, and histamine.

In the isolated rat stomach infusion model employed in this study, changes in gastrointestinal hormone levels in the stomach alone can be examined while maintaining the interaction among G cells, D cells, enterochromaffin-like cells (ECL) cells, and parietal cells. The effects of hormones and nerves in the whole body are excluded. By using this model, the effect of PA administration alone on the changes in gastrointestinal hormone levels could be examined. The secretion of gastrin, somatostatin, and histamine was significantly inhibited. There have been reports on the relationship between hormone dynamics and acid secretion in the isolated rat stomach infusion model,[13,41] which further support the finding that PA evidently inhibits acid secretion.

However, the mechanisms of inhibiting acid secretion have not been investigated in great detail. The mecha-

nisms of inhibiting acid secretion with catechin, a tea-leaf polyphenol, have been well investigated. The inhibited acid secretion was ascribable to the inhibition of histamine synthesis and secretion by decreased histidine carboxylase (HDC) activities.[42,43] Furthermore, Sato *et al*.[44] reported that HDC activity was decreased via a pathway involving G cells. They used the isolated rat stomach infusion model, and their results in regard to the changes in gastrointestinal hormone levels were very similar to ours with PA administration. Based on these results, it is presumed that PA first stimulates G cells to mainly inhibit the secretion of gastrin, and subsequently the secretion of histamine and somatostatin.

It has been speculated that the inhibition of gastrin secretion reduces HDC activities, and thereby decreases histamine synthesis in ECL cells. As a mechanism of the inhibition of somatostatin secretion, it was presumed that PA inhibited gastrin secretion, and reduced somatostatin secretion via gastrin receptors on D cells.

We investigated PGE_2 in an attempt to examine the effects of PA as a protective factor for the stomach. PGE_2 increases blood flow in the gastric mucosa, and has mucoprotective effects in the isolated rat stomach infusion model. As to the relationship between the paucity of PGs and gastric mucosal injury, the administration of neutralizing antibody against PGs caused gastric ulcers.[45,46] In the present study, by measuring PGE_2 in the isolated rat stomach infusion model, we observed stimulation of PGE_2 secretion with the administration of PA. Ogino et al.[47] reported that SOD could also increase PGE_2. Accordingly, the increased SOD activity in the gastric mucosa induced by PA administration seemed to influence the increase of PGE_2. Because PGE_2 increases gastric mucosal blood flow and is mucoprotective, increased PGE_2 secretion inhibited the acute stress-induced gastric mucosal injury. Therefore, it was assumed that PA inhibited gastric mucosal injury by strengthening mucosal protection.

In conclusion, the inhibition of mucosal injury by PA was considered to be attributable to (i) the inhibition of acid secretion via G cells to circumvent attacks of WIR stress, (ii) increased PGE_2 secretion, and (iii) increased SOD activity to augment mucosal protection. These interactions appear to inhibit acute stress-induced gastric mucosal injury.

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