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Gastroprotections of escins Ia, Ib, IIa, and IIb on ethanol-induced gastric mucosal lesions in rats

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Abstract

Effects of escins Ia, Ib, IIa, and IIb isolated from horse chestnuts on ethanol-induced gastric mucosal lesions and the roles of capsaicin-sensitive afferent neurons, endogenous nitric oxide (NO), sulfhydryls, prostaglandins, as well as gastric secretion and the sympathetic nervous system, were investigated in rats. Test samples were given orally to fasted rats 1 h before ethanol (1.5 ml/rat, p.o.) treatment or ligation of the pylorus. Escins Ia–IIb (10–50 mg/kg) potently inhibited ethanol-induced gastric mucosal lesions, whereas desacylescins I and II (50 mg/kg) showed no such effect. These active saponins (10 and 20 mg/kg) did not decrease the gastric secretion. The gastroprotections of escins Ia–IIb were attenuated by the pretreatment with capsaicin, N^G-nitro-L-arginine methyl ester, and indomethacin, but not by *N*-ethylmaleimide. The effects of escins Ia–IIb were also attenuated in streptozotocin-induced diabetic rats, in which the activity of the sympathetic nervous system was abnormal. These results suggest that the gastroprotections of escins Ia–IIb on ethanol-induced gastric mucosal lesions are acid-independent, whereas endogenous prostaglandins, NO, capsaicin-sensitive afferent neurons, and the sympathetic nervous system participate.

Keywords: Escin; Ethanol-induced gastric lesion; Capsaicin-sensitive afferent neuron; Nitric oxide (NO); Prostaglandin; Sympathetic nervous system; Gastroprotection

1. Introduction

'Escin', one of the most important saponin constituents, is known as a mixture of saponins occurring in horse chestnut, the seed of *Aesculus hippocastanum* L. (Hippocastanaceae). The saponin mixture 'escin' is reported to have a potent anti-inflammatory activity against different types of edema (Di Rosa et al., 1971) and a great capacity to raise the vascular tone, and also to help in the regulation of the microcirculation (Antoni et al., 1979). This effect is attributable to the formation of prostaglandin $F_{2\alpha}$ in the vascular tissue and can be suppressed by non-steroid anti-inflammatory drugs (Longiave et al., 1979). Experimentally, 'escin' prevented the development of gastric lesions in various ulcers (Marhuenda et al., 1993), and reduced gastric secretion (Marhuenda et al., 1994). Recently, we isolated 12 acylated polyhydroxyolean-12-ene 3-*O*-monodesmosides, escins Ia, Ib, IIa, IIb, IIIa, IIIb, IV, V, and VI and isoescins Ia, Ib, and V, from horse chestnuts and determined their chemical structures (Yoshikawa et al., 1994, 1996, 1998). We have also reported on both the inhibitory activity of escins Ia–IIb on the increase of blood glucose or ethanol concentration in oral glucose- or ethanol-loaded rats (Yoshikawa et al., 1994, 1996) and their anti-inflammatory effects (Matsuda et al., 1997). Furthermore, investigation of the modes of action using principal escins for the hypoglycemic activity revealed that they potently inhibited gastric emptying in rats, and additionally, they showed weak inhibitory activity on glucose uptake in the small intestine in vitro (Matsuda et al., 1998). We now describe the effects of escins Ia–IIb and their desacyl derivatives (desacylescins I and II) on ethanol-induced gastric mucosal lesions in rats. We also discuss the roles of capsaicin-sensitive afferent neurons, endogenous nitric oxide (NO), sulfhydryls, prostaglandins, as well as gastric secretion and the sympathetic nervous system in the gastroprotection by escins Ia–IIb in conscious rats.

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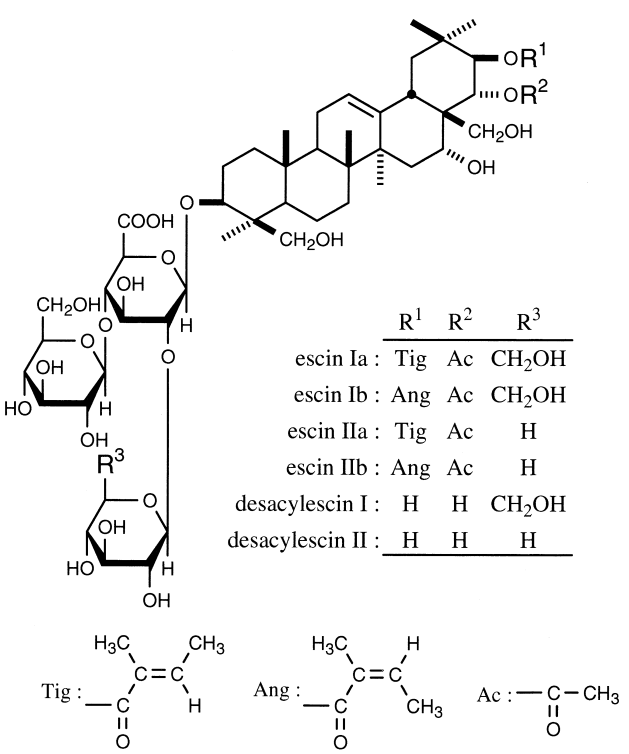
2. Materials and methods

2.1. Chemicals

Escins Ia, Ib, IIa, and IIb were isolated from the seeds of European *Aesculus hippocastanum*, and desacylescins I and II were obtained by alkaline hydrolysis of escins Ia and IIa according to our previous report (Fig. 1) (Yoshikawa et al., 1996). Omeprazole was obtained from Fujisawa Pharmaceutical, Japan. Other reagents were purchased from Wako Pure Chemical, Japan.

2.2. Animals

Male Sprague–Dawley rats were purchased from Kiwa Laboratory Animal, Japan. The animals were maintained at a constant temperature of 23 ± 2°C and were fed a standard laboratory chow (MF, Oriental Yeast, Japan) for a week. The animals were fasted for 24–26 h prior to experiments, but were supplied with water ad libitum. The saponins dissolved in phosphate buffered saline (PBS) or omeprazole suspended in 0.5% carboxylmethyl cellulose sodium (CMC-Na)-PBS was given orally at 5 ml/kg in each experiment, while PBS or CMC-Na-PBS was given orally in the corresponding control group at 5 ml/kg. The experiments were performed with conscious animals unless otherwise noted.



	R^1	R^2	R^3
escin Ia :	Tig	Ac	CH_2OH
escin Ib :	Ang	Ac	CH_2OH
escin IIa :	Tig	Ac	H
escin IIb :	Ang	Ac	H
desacylescin I :	H	H	CH_2OH
desacylescin II :	H	H	H

Fig. 1. Chemical structures of escins Ia, Ib, IIa, and IIb and desacylescins I and II.

2.3. Ethanol-induced gastric mucosal lesions in rats

The acute gastric lesions were induced by intragastric application of ethanol. Briefly, 99.5% ethanol (1.5 ml) was orally administered to 24–26 h fasted rats (about 250 g) by means of a metal orogastric tube. One hour later, the animals were sacrificed by cervical dislocation under ether anesthesia, and the stomach was dissected out and inflated by injection of 10 ml 1.5% formalin to fix the inner and outer layers of the gastric walls. Subsequently, the stomach was incised along the greater curvature and the lengths of the necrotizing lesions were examined at $10\times$ magnification by two or three observers unaware of the treatment. The lesions were scored with arbitrary scales in which the severity rating and number of lesions were considered according to a modification of the scoring system of Martin et al. (1994): (0) no lesion; (1) less than five slight lesions < 5 mm in length and < 2 mm in width; (2) more than five slight lesions < 5 mm in length and < 2 mm in width; (3) less than five medium lesions > 5 mm in length and < 2 mm in width; (4) more than five medium lesions > 5 mm in length and < 2 mm in width; (5) from one to three hemorrhagic bands of moderate lesions in length < 5 mm and width > 2 mm; (6) more than four hemorrhagic bands of moderate lesions in length < 5 mm and width > 2 mm; (7) from one to three hemorrhagic bands of severe lesions in length > 5 mm and width > 2 mm; (8) from four to six hemorrhagic bands of severe lesions in length > 5 mm and width > 2 mm; (9) more than six hemorrhagic bands of severe lesions in length > 5 mm and width > 2 mm. Mean scores for each group were calculated. The samples were given orally 1 h prior to the application of ethanol. Omeprazole was used as a reference drug in this experiment.

2.4. Gastric secretion in pylorus-ligated rats

According to the method of Shay et al. (1945), rats (about 250 g, b.w.) were anesthetized with ether, then the abdomen was incised and the pylorus was ligated. Three hours later, the animals were sacrificed, and the cardia was ligated. The stomach was removed and opened along the greater curvature over a glass funnel for collection of gastric juice in a graduated centrifuge tube. After centrifugation, the volume and pH of the gastric juice were measured. Total acidity was titrated with 0.01 N NaOH and acid output (μEq/h) was calculated. The pepsin activity was determined by Anson's method (Anson, 1938) using bovine serum albumin as a substrate, and then output of pepsin (mg tyrosine/h) was calculated. The samples were given orally 1 h prior to the operation. Omeprazole was used as a reference drug in this experiment.

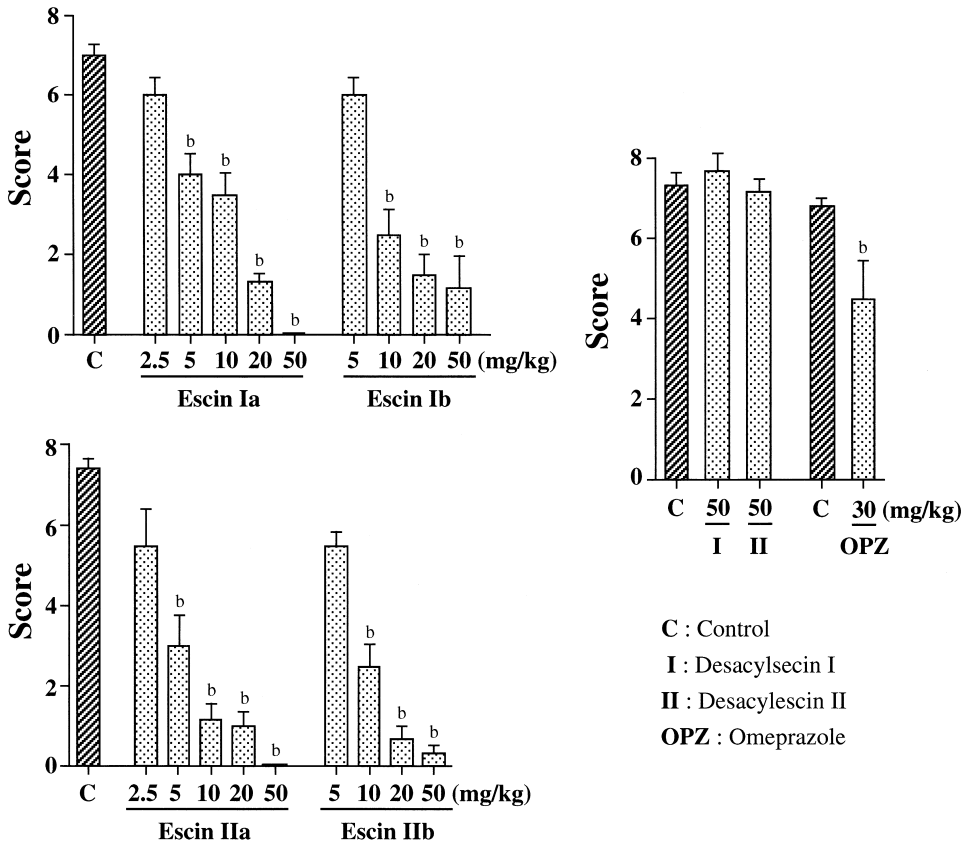


Fig. 2. Effects of escins Ia, Ib, IIa, and IIb, desacylescins I and II, and omeprazole on gastric lesions induced by ethanol in rats. The scores of gastric lesions were deteremined 1 h after administration of ethanol. Each test compound was given orally 1 h before administration of ethanol. Bars represent the means with S.E.M. (control group: $n = 9$, other groups: $n = 6$). Significantly different from the control group, $^{b}P < 0.01$.

2.5. Ethanol-induced gastric mucosal lesions in capsaicin-pretreated rats

The gastric mucosal lesions were induced, measured and scored as above method. To investigate the involvement of capsaicin-sensitive afferent neurons in the protective effects of escins Ia–IIb, capsaicin solution was prepared in a solution containing 99.5% ethanol, Tween 80, and saline (92:1:7, v/v/v). Rats (180–200 g) were injected with sodium pentobarbital (30 mg/kg in 1 ml saline, i.p., 15 min before), and treated with increasing doses of capsaicin for 2 consecutive days (25, 50 and

Table 1
Effects of escins Ia, Ib, IIa, and IIb on gastric secretion in pylorus-ligated rats

Treatment	Dose (mg/kg, p.o.)	*n*	Volume (ml/3 h)	pH value	Acid output (μEq/h)	Pepsin output (mg tyrosine/h)
Control	–	8	3.4 ± 0.3	1.36 ± 0.03	66.3 ± 10.0	21.1 ± 1.4
Escin Ia	10	6	4.5 ± 0.4	1.42 ± 0.02	107.7 ± 25.7	27.4 ± 5.2
	20	6	5.1 ± 0.2	1.22 ± 0.03	134.4 ± 5.9	34.9 ± 2.3[a]
Escin Ib	10	6	4.0 ± 0.6	1.27 ± 0.04	107.9 ± 26.5	27.9 ± 5.0
	20	6	5.2 ± 0.6	1.30 ± 0.05	131.5 ± 21.5	31.4 ± 4.3
Escin IIa	10	6	3.7 ± 0.4	1.30 ± 0.04	91.7 ± 18.0	23.5 ± 3.0
	20	6	4.8 ± 0.5	1.33 ± 0.04	105.2 ± 12.9	29.5 ± 3.1
Escin IIb	10	6	3.5 ± 0.4	1.33 ± 0.03	76.8 ± 11.6	24.3 ± 2.9
	20	6	5.1 ± 0.6	1.34 ± 0.02	119.1 ± 27.0	30.6 ± 2.6
Control	–	6	3.5 ± 0.5	1.45 ± 0.05	70.5 ± 11.3	22.7 ± 2.1
Omeprazole	20	6	2.2 ± 0.1[b]	7.12 ± 0.32[b]	1.5 ± 1.1[b]	10.3 ± 0.6[b]

The pylorus was ligated under ether anesthesia. Three hours later, the animals were sacrificed, and the gastric juice was collected and centrifuged. The volume and pH of the gastric juice were measured. Total acidity was titrated with 0.01 N NaOH and acid output (μEq/h) was calculated. The pepsin activity was determined by Anson's method using bovine serum albumin as a substrate and output of pepsin (mg tyrosine/h) was calculated. Each value represents the mean ± S.E.M. Significantly different from the control group, $^{a}P < 0.05$, $^{b}P < 0.01$.

50 mg/kg at 12-h intervals, s.c.) as a modification of the method described previously (Lambrecht et al., 1993; Barrachina et al., 1997). To counteract any respiratory impairment associated with administration of capsaicin, the rats were pretreated with aminophylline (10 mg/kg, dissolved in 1 ml saline, i.m.) before capsaicin injection. After 14 days, the efficiency of capsaicin pretreatment was verified by the corneal chemosensory test which consists of monitoring the wiping reflex in response to ocular instillation of a drop of 0.1% NH_4OH solution. None of the capsaicin-pretreated rats showed a wiping response, indicating effective ablation of primary sensory afferent neurons, whereas the wiping reflex was present in vehicle-pretreated rats.

2.6. Ethanol-induced gastric mucosal lesions in indomethacin-pretreated rats

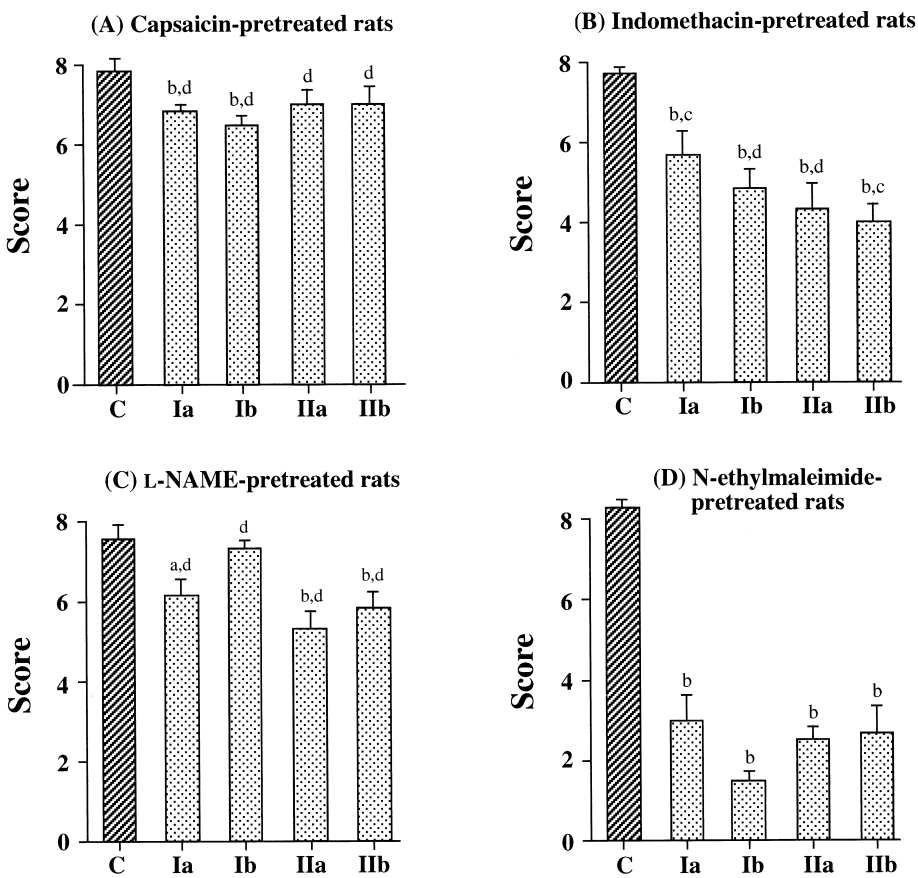
The gastric mucosal lesions were induced, measured and scored as above method. To investigate the involvement of endogenous prostaglandins in the protective effects of escins Ia–IIb, indomethacin (10 mg/kg, dissolved in $NaHCO_3$, and diluted in distilled water) was subcutaneously injected 30 min before the administration of the samples.

2.7. Ethanol-induced gastric mucosal lesions in N^G-nitro-L-arginine methyl ester-pretreated rats

The gastric mucosal lesions were induced, measured and scored as above method. To investigate the involvement of endogenous NO in the protective effects of escins Ia–IIb, N^G-nitro-L-arginine methyl ester (L-NAME, 70 mg/kg, dissolved in 5 ml saline) was intraperitoneally injected 30 min before the administration of escins Ia–IIb.

2.8. Ethanol-induced gastric mucosal lesions in N-ethylmaleimide-pretreated rats

The gastric mucosal lesions were induced, measured and scored as above method. To investigate the involve-



C : Control, **Ia** : Escin Ia (10 mg/kg), **Ib** : Escin Ib (10 mg/kg), **IIa** : Escin IIa (10 mg/kg), **IIb** : Escin IIb (10 mg/kg).

Fig. 3. Effects of escins Ia, Ib, IIa, and IIb on gastric lesions induced by ethanol in rats pretreated with capsaicin, indomethacin, L-NAME, and *N*-ethylmaleimide. (A) Capsaicin (125 mg/kg in total, s.c.) was injected to rats 2 weeks before the administration of test samples. (B) Indomethacin (10 mg/kg, s.c.) was injected to rats 30 min before the administration of test samples. (C) L-NAME (70 mg/kg, i.p.) was injected to rats 30 min before the administration of test samples. (D) *N*-Ethylmaleimide (10 mg/kg, s.c.) was injected to rats 30 min before the administration of test samples. Bars represent the means with S.E.M. (control group: $n = 7$, other groups: $n = 6$). Significantly different from the control group, $^aP < 0.05$, $^bP < 0.01$, from the corresponding group in Fig. 1 $^cP < 0.05$, $^dP < 0.01$.

ment of endogenous sulfhydryls in the protective effects of escins Ia–IIb, *N*-ethylmaleimide (10 mg/kg, dissolved in 5 ml saline) was subcutaneously injected 30 min before the administration of escins Ia–IIb.

2.9. Ethanol-induced gastric mucosal lesions in streptozotocin-pretreated rats

The gastric mucosal lesions were induced, measured and scored as above method. To investigate the involvement of streptozotocin-induced diabetes in the protective effects of escins Ia–IIb, streptozotocin (60 mg/kg, dissolved in 5 ml citrate buffer, pH 4.2) was intravenously injected 10 days before the administration of the samples. The serum glucose levels were tested the day before the experiment at non-fasted condition under ether anesthesia.

2.10. Statistics

Values were expressed as means ± S.E.M. For statistical analysis, One-way analysis of variance following Dunnett's test for parametric data and Kruskal–Wallis statistics following Steel's test for non-parametric data were used. Probability (P) values less than 0.05 were considered significant.

3. Results

3.1. Effects on gastric lesions induced by ethanol in rats

Ethanol-induced gastric mucosal lesions and the effects of escins Ia, Ib, IIa, and IIb and desacylescins I and II in rats are shown in Fig. 2. When ethanol (1.5 ml/rat) was administered to control animals, marked gastric mucosal lesions were induced. These lesions were characterized by multiple hemorrhage red bands (patches) of different sizes lesions macroscopically, and vasocongestion of the mucosa and submucosal hemorrhage histologically, along the long axis of the glandular stomach. Oral administrations of escins Ia, Ib, IIa, and IIb (10–50 mg/kg) showed a potent protective effect against ethanol-induced gastric lesions in rats. They dose-dependently reduced the scores of lesions, and improved the pathogenic changes (data were not shown). Whereas desacylescins I and II (50 mg/kg) had no such effect. Reference drug omeprazole (30 mg/kg) also significantly inhibited the lesions.

3.2. Effects on gastric secretion in pylorus-ligated rats

As shown in Table 1, ligation of the pylorus for 3 h produced an accumulation of gastric juice (3.4 ml/rat). Reference drug omeprazole (20 mg/kg, p.o.) potently reduced the volume, acid and pepsin outputs, and increased the pH value to 7.12. In contrast, escins Ia–IIb (10 and 20 mg/kg) tended to increase the volume, acid and pepsin outputs, whereas these escins did not alter the pH value of the gastric juice.

3.3. Effects on gastric lesions induced by ethanol in capsaicin-, indomethacin-, L-NAME-, or N-ethylmaleimide-pretreated rats

As shown in Fig. 3(A), pretreatment with capsaicin (125 mg/kg in total, s.c.) markedly attenuated the gastroprotections of escins Ia–IIb (10 mg/kg).

Pretreatment with indomethacin (10 mg/kg, s.c.), L-NAME (70 mg/kg, i.p.), or *N*-ethylmaleimide (10 mg/kg, s.c.) tended to aggravate ethanol-induced mucosal lesions. They had tendency to increase the length and score. Pretreatment with indomethacin partly reduced the gastric mucosal protective effect by escins Ia–IIb (10 mg/kg) as shown in Fig. 3(B). Pretreatment with L-NAME partly reduced the gastric mucosal protective effects afforded by escins Ia–IIb (10 mg/kg) as shown in Fig. 3(C). Pretreatment with *N*-ethylmaleimide did not attenuate the gastroprotections of escins Ia–IIb (10 mg/kg) as shown in Fig. 3(D).

3.4. Effects on gastric lesions induced by ethanol in streptozotocin-pretreated rats

Streptozotocin (60 mg/kg, i.v.) induced hyperglycemia (above 400 mg glucose/dl in serum) in non-fasted condition. This pretreatment increased the width and score of the lesions, but decreased the length. As shown in Fig. 4, it

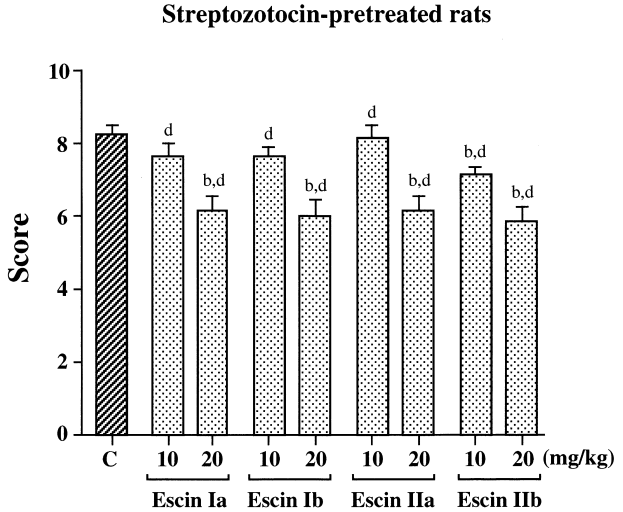


Fig. 4. Effects of escins Ia, Ib, IIa, and IIb on gastric lesions induced by ethanol in streptozotocin-induced diabetic rats. Streptozotocin (60 mg/kg, i.v.) was injected to rats 10 days before the administration of test samples. Bars represent the means with S.E.M. (C: control group, $n = 8$, other groups: $n = 6$). Significantly different from the control group, $^{b}P < 0.01$, from the corresponding group in Fig. 1, $^{d}P < 0.01$.

markedly attenuated the gastroprotections of escins Ia–IIb (10 and 20 mg/kg).

4. Discussion

Necrotizing agents such as ethanol, 0.6 N HCl, 0.2 N NaOH, etc. given intragastrically in the rat produce severe gastric hemorrhagic erosions (Robert et al., 1979; Szabo et al., 1981). In this study, comparing to reference drug omeprazole, escins Ia–IIb (10–50 mg/kg, p.o.) showed potent protective effects against ethanol-induced gastric mucosal lesions.

Gastric acid is not a primary cause but plays a permissive role in gastric mucosal damage induced by ethanol (Tarnawski et al., 1983). Previous studies have demonstrated that most of antiulcer agents, such as proton pump inhibitors (omeprazole, etc.) and histamine H_2 receptor antagonists (cimetidine, etc.), have the activity of antisecretion (Larsson et al., 1983; Yamamoto et al., 1984; Lindberg et al., 1990; Kinoshita et al., 1997). In the present study, omeprazole strongly inhibited gastric secretion by decreasing the volume of gastric juice, raising the pH value, and lowering the acid and pepsin outputs at the dosage, which inhibited the gastric mucosal lesions induced by ethanol. In contrast, escins Ia–IIb tended to increase gastric secretion by increasing the volume of gastric juice and the acid and pepsin outputs, but they did not alter the pH value of the gastric juice at the doses, which inhibited the gastric mucosal lesions. These results indicate that the protections by escins Ia–IIb are acid-independent.

Profound long-standing suppression of acid secretion causes bacterial overgrowth in the upper gut (Wingate, 1990; Larner and Lendrum, 1992) and hypergastrinemia (Carlsson, 1989). Life-long elevation of serum gastrin levels often stimulates the development of gastric enterochromaffin-like cell carcinoids in rodents (Poynter et al., 1985; Carlsson et al., 1986; Betton et al., 1988). In humans, hypergastrinemia induced by antisecretory agents causes hyperplastic change but not dysplasia in endocrine cells of the stomach (Lamberts et al., 1993). Therefore, a drug which have an effect of antiulcer without inhibiting gastric acid secretion or neutralizing intraluminal acid is desirable for treatment of peptic ulcer to avoid the possibility of microbial overgrowth, excessive hypergastrinemia, tolerance, and rebound acid hypersecretion. Interestingly, escins Ia–IIb at low dosages showed the gastroprotective activity without inhibiting the gastric secretion.

Capsaicin-sensitive afferent neurons are involved in gastric mucosal protection against ulcerogenic factors. This type of gastric defense is primarily due to a local mechanism initiated by sensory nerve ending in the gastric mucosa (Holzer and Sametz, 1986). Capsaicin is widely used to ablate sensory C fibers. It has been used systematically to ablate all capsaicin-sensitive C fibers. The protections of escins Ia–IIb on ethanol-induced gastric mucosal lesions were partly attenuated by the pretreatment with capsaicin. This result suggests that capsaicin-sensitive afferent neurons participate the protections of escins Ia–IIb on ethanol-induced gastric lesions.

Since vascular changes in gastric mucosa appear to be the most pronounced feature of absolute ethanol-induced injury, maintenance of mucosal vasculature and normal blood flow may be the major mechanism of cytoprotection. It has been demonstrated that the gastric mucosa could form endogenous NO derived from L-arginine (Whittle et al., 1990). NO participates in the gastric defense mechanisms by regulating the gastric mucosal blood flow and gastric mucus secretion (Brown et al., 1992, 1993; Whittle and Lopez-Belmonte, 1993). NO increases prostaglandins biosynthesis in physiological and pathophysiological conditions, and there is an up- or down-regulation of NO by prostaglandins (Salvemini et al., 1995; Sautebin et al., 1995; Di Rosa et al., 1996). Endogenous NO has a role acting in concern with prostacyclin and sensory neuropeptides, in the modulation of gastric mucosal integrity (Whittle et al., 1990). Previous studies (Peskar et al., 1991; Ali, 1995) and our present study demonstrated that pretreatment with L-NAME, an inhibitor of NO synthase, enhanced ethanol-induced gastric mucosal lesions. 'Escin' raised the vascular tonic and helped in the regulation of the microcirculation (Antoni et al., 1979). The inhibition of NO synthase by L-NAME attenuated the gastroprotection of escins Ia–IIb. The combination of this result with above result indicates that endogenous NO, possibly together with prostaglandins, take part in the gastric mucosal protection by escins Ia–IIb against ethanol-induced lesions.

Prostaglandins are involved in cytoprotection. Robert (1979) pointed out that exogenous prostaglandins were capable of protecting gastric mucosa against necrotizing agents. Chaudhury and Robert (1980) also demonstrated that mild irritants protected the gastric mucosa against damaging via release of endogenous prostaglandins as well. Pretreatment with indomethacin attenuated the protection afforded by escins Ia–IIb against ethanol-induced gastric mucosal lesions. Prostaglandins seem to participate in the protective effect of escins Ia–IIb.

Ethanol-induced gastric damage has been shown to be associated with depletion of endogenous sulfhydryls, and pretreatment with sulfhydryl-blockers prevented the gastroprotection of sulfhydryl-containing substances (Szabo et al., 1981; Szelenyi and Brune, 1986; Szabo and Brown, 1987). Pretreatment with *N*-ethylmaleimide, a sulfhydryl-blocker, did not reduced the protection afforded by escins Ia–IIb. It indicates that endogenous sulfhydryls may be not involved in the protection of escins Ia–IIb.

The sympathetic activation may enhance prostaglandin synthesis (Kuratani et al., 1994). On the other hand, pancreatic β-cells secrete and release insulin and amylin. Though it is unknown that whether insulin has the effect on ethanol-induced gastric mucosal lesions, amylin, a pep-

tide hormone co-secreted with insulin (Inoue et al., 1990), potentially protects the gastric mucosa from the damages induced by ulcerogenic agents, such as ethanol, indomethacin (Guidobono et al., 1997), reserpine, serotonin (Clementi et al., 1997). In the present study, the protections of escins Ia–IIb on ethanol-induced gastric mucosal lesions were attenuated in streptozotocin-pretreated rats, in which the activity of sympathetic nervous system was abnormal (Young et al., 1983), and the secretory activity of β-cells was disrupted. This result suggests that the protections of escins Ia–IIb on ethanol-induced gastric lesions may be relative to the sympathetic nervous system and/or the secretion of pancreatic β-cells.

5. Conclusion

These results suggest that capsaicin-sensitive afferent neurons, endogenous NO, and prostaglandins participate in the protections of escins Ia–IIb against ethanol-induced gastric mucosal lesions. The mechanisms of sympathetic nervous system and the secretion of pancreatic β-cells in the gastroprotection by escins Ia–IIb should be studied further.

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