Effects of Ethanol Administration at a High-Dose Level on the Stimulatory Action by Bradykinin in Vascular Permeability

Yoshiko TANAKA¹ and Yoshiaki YAMASHITA²

¹ Department of Food Science and Nutrition, School of Human Environmental Sciences, Mukogawa Women’s University, 6–46 Ikibinaki, Nishinomiya, Hyogo 663–8558, Japan
² Department of Life Science, Kinran College, 5–25–1 Fujishirodai, Suita, Osaka 565–0873, Japan

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Summary The effects of ethanol (EtOH) administration at a high-dose level on the stimulatory action by bradykinin in vascular permeability were examined in rats, as compared with the effects of histamine and hyaluronidase. Oral administration (7.5 g/kg) and intraperitoneal injection (5 g/kg) of EtOH markedly potentiated the vascular permeability accelerated by bradykinin, but they suppressed in reverse such effects induced by histamine and hyaluronidase. EtOH did not affect the stimulatory action of bradykinin on the vascular permeability when intracutaneous injection was done under the coexistence with bradykinin. The blood pressure was found to descend 30 min later, though there was a transient rise immediately after the oral administration of EtOH (7.5 g/kg). The oral administration of EtOH (7.5 g/kg) caused no change in both enzyme activities of aspartic acid aminotransferase and alanine aminotransferase in blood for 3 h. The intraperitoneal injection of EtOH (5 g/kg) lowered the blood bradykinin level and increased the blood hyaluronidase activity. In vitro, EtOH elicited a concentration-dependent increase in the kallikrein activity, trypsin activity, and bradykinin-decomposed activity in plasma. These results strongly suggest that vascular permeability results from elevation in the bradykinin level, direct action of EtOH on inflamed skin site, and actions of EtOH or its metabolites on bradykinin-regulator, which involves bradykinin receptor and NO and endothelin productions.

Key Words ethanol, bradykinin, vascular permeability, histamine, hyaluronidase

Ethanol (EtOH) has many pharmacological actions such as central inhibitory effects, angioteletasia action, and effects on the endocrine and on the metabolic system in the liver. EtOH is metabolized by the alcohol dehydrogenase to acetaldehyde and becomes acetic acid and acetyl CoA by aldehyde dehydrogenase. It has been well documented that EtOH has been closely related to the causes of human sickness because of habitual drinking, which results in neuropathy and organ failure such as heart, kidneys, and liver (1–3). EtOH induces microcirculatory disturbances in many organs such as heart and brain (4, 5). In the liver, the infusion of EtOH into the portal vein elicits vasoconstriction in the hepatic microvasculature, leading to hepatic tissue hypoxia and eventually to hepatocellular necrosis (6, 7).

Kinsics and amines including bradykinin (8), histamine, and serotonin are known to induce the extension of vascular endothelial cells, which are concerned with the initial stage of inflammation as a chemical mediator. It is also known that hyaluronidase (EC 3.2.1.35) is profoundly associated with the causes of inflammation such as the decrease of viscosity of the interstititial tissue, material transition into and out of the organs/cells, and vascular permeability accentuation induced by hydrolase of the mucopolysaccharide, which is also a constituent of various organs and cells. Hyaluronidase is also known to play an important role in the segregation, and histamine of the mast cell plays roles in the occurrence of the type 1 allergy (9). Although stimulatory effects in vascular permeability by these factors concerning the induction of the inflammation are also reported on the involvement of the autonomic nervous system (2), besides the influences on metabolic functions involved in the actions of the amine system and the kinin system, many important points on the causes of increase of vascular permeability remain to be elucidated.

Therefore the possible mechanisms involved in the stimulatory effect of EtOH on bradykinin-accelerated vascular permeability were examined by administrating EtOH to rats in a high dose level, compared with those of histamine and hyaluronidase.

MATERIALS AND METHODS

Experimental animals. Male rats of the Wistar strain (CLEA Japan, Tokyo), weighing 200 to 250 g, were maintained on an Oriental yeast solid feed-NMF (Tokyo) ad libitum, and were usually allowed free access to drinking water. The animals were bred at a room temperature of 23±1°C and at humidity of 55±5% in animal-rearing facilities under 24-h ventilation. One week later, the animals with no abnormalities in general condition and with no weight gain were selected. The handling for the animals was based on the Guidelines for Animal Experiments at the Mukogawa Women’s University, Hyogo, Japan.
Chemical application. Plasma kallikrein (EC 3.4.21.34, derived from human plasma, with 0.5 unit) and tissue kallikrein (EC 3.4.21.35, from pig pancreas derivation with 50 units) were purchased from Sigma Co. (USA); Markit M Bradykinin from Dainippon Pharmaceutical (Osaka); Fuji Drychem Slide ALT-P and Fuji Drychem Slide AST-P were from Fuji Photo Film (Kanagawa, Japan), and the other reagents were from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of solutions. Bradykinin, histamine, and hyaluronidase derived from bovine ovaries were respectively adjusted to give a concentration of 140 μg/mL, 1 mg/mL, and 3,000 units/mL by using the physiological saline, as well as each solution of 0.5% Evans blue and 50% EtOH were also prepared with the same solution.

Measurement of the vascular permeability action and EtOH administration. One-tenth milliliters of bradykinin (140 μg/mL), histamine (1 mg/mL) or hyaluronidase (3,000 units/mL) was intradermally injected in each of 3 sites on the right side of the median line in the rat’s shaven back, at 5 min after injection of 0.5% Evans blue into the tail vein. The shaving of rats selected for the experiment was carried out on the previous day. Physiological saline was similarly injected on the left side as a control. The effect of vascular permeability was determined by area produced by the leakage of the dye. This area was calculated by substituting for the following equation after each diameter was measured with slide calipers.

Area (cm²) = \[ \frac{\text{Diameter (cm) × Long Diameter (cm)}}{2} \times 3.14 \]

The EtOH group received administrations orally (1.5 mL/100 g body weight) or intraperitoneally (1 mL/100 g body weight), at 30 min before each injection of bradykinin, histamine, or hyaluronidase solutions.

Measurement of blood pressure. The blood pressure of the rat was measured by use of an unobserved style automatic sphygmomanometer MK-1000-S for laboratory animals (Muromachi Kikai, Tokyo, Japan).

Measurement of kallikrein activity. A mixture of substrate (50 μL), water (150 μL), 3 mM 1,10-phenanthroline (100 μL) and physiological saline (100 μL) was kept for 5 min at 30°C. Sequentially, after adding 0.01 unit plasma kallikrein or 1 unit tissue kallikrein 100 μL was added, the mixture was kept at 30°C for 30 min. The bradykinin formed was then measured using Markit M Bradykinin for the bradykinin measurement based on ELISA, as described above.

Measurement of tissue kallikrein activity. A mixture of substrate (50 μL), water (150 μL), 3 mM 1,10-phenanthroline (100 μL), and physiological saline (100 μL) was kept for 5 min at 30°C. Sequentially, after adding 0.01 unit plasma kallikrein or 1 unit tissue kallikrein 100 μL was added, the mixture was kept at 30°C for 30 min. The bradykinin formed was then measured using Markit M Bradykinin for the bradykinin measurement based on ELISA, as described above.
Fig. 1. Effects of ethanol (EtOH) on dye leakage produced by bradykinin, histamine, and hyaluronidase into dorsal skin. Each solution (0.1 mL/site) of bradykinin (40 μg) (A), histamine (1 mg) (B), or hyaluronidase (3,000 units) (C) was intracutaneously injected at 3 sites on the left side and on the right side on dorsal skin after intravenous injection (1 mL/animal) of 0.5% Evans blue solution. A solution of 50% EtOH was administered orally to give a dose of 7.5 g/kg (n=5; upper panels) (●) or intraperitoneally to give a dose of 5 g/kg (n=5; bottom panels) (◆) 30 min before an injection of each solution of bradykinin, histamine or hyaluronidase as indicated above. Physiological saline (n=5) was given as control (○). Ellipsoidal dye-leaked areas were then evaluated. Ordinate and abscissa represent ellipsoidal dye-leaked area (dimension, cm²) and time (min) after an injection of bradykinin, histamine, or hyaluronidase. Each point and vertical bar represents the mean ± SE. EtOH raised the stimulatory action by oral administration and intraperitoneal injection of bradykinin, but lowered that of histamine and hyaluronidase in spite of the route of ingestions (p<0.05 by ANOVA). A single asterisk indicates a significant difference from control (without EtOH) at p<0.05.

RESULTS
Effect of EtOH on the vascular permeability accelerated by treatments with hyaluronidase, histamine, and bradykinin

As shown in Fig. 1, the vascular permeability was enhanced remarkably by injecting each solution of hyaluronidase, histamine and bradykinin in the absence of EtOH into the rat’s back intraderma (○). The manner of stimulatory actions by hyaluronidase, histamine, and bradykinin on vascular permeability was observed as follows: Bradykinin maintained a constant level on vascular permeability from 15 to 150 min (Fig. 1A), and the effects of histamine becomes more markedly after 30 min in comparison with 15 min after its injection (Fig. 1B), whereas that of hyaluronidase elicited a time-dependent stimulation (Fig. 1C). The effects of EtOH on each of the stimulatory actions of hyaluronidase, histamine, and bradykinin in vascular permeability were examined by oral administration (upper panel) or intraperitoneal injection (lower panel). EtOH raised the stimulatory action by both oral and intraperitoneal administrations of bradykinin, but lowered those of histamine and hyaluronidase as shown in the case of ingestions (●).
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Fig. 2. Effects of the simultaneous administration of EtOH on dye leakage into dorsal skin produced by bradykinin. Each mixture of bradykinin (40 μg) and EtOH of 0%, (control □, n=26, 0 mg/kg), 0.25% (■, n=10, 3.75 mg/kg), or 2.5% (△, n=10, 37.5 mg/kg) was intracutaneously injected 0.1 mL at each site on dorsal skin, totaling 3 sites. Control received physiological saline instead of EtOH. An injection of Evans blue solution was carried out as described in the legend of Fig. 1. Each column and vertical bar represents the mean value±SE.

blood volume of the animal is calculated to be 15 mL/animal. EtOH was present at final concentrations of 0.25%, 2.5% and 5% in bradykinin solution, and when the solutions of the mixture or bradykinin alone were administered in the rat’s back intraderma, the effects of EtOH administration on stimulatory action by bradykinin were examined. As shown in Fig. 2, there were no statistically significant differences between each mixture of varying concentrations of EtOH in the presence of bradykinin and bradykinin alone in the degrees of vascular permeability.

Effect of EtOH on blood pressure

Figure 3 shows the influences of EtOH on rat blood pressure. Although there was a slight rise of blood pressure within 10 min after the administration of EtOH, it reversely fell 30 min later, and this tendency became remarkable over time after administration.

Effect of EtOH on serum AST and ALT activities

The effect of oral administration of EtOH (7.5 g/kg) on the serum AST activity and ALT activity was investigated. The AST activity (unit/L) at 60 min after EtOH administration was found to be 79.0±6.7, and in the control receiving the water instead of EtOH 79.0±9.0, both being of almost equal value. The value of 88.0±8.7 and 79.0±9.0 was respectively shown at 180 min after the administration of EtOH, and the statistical significance was not found in the EtOH-administrated group on in serum AST activity of the nonadministration group (p<0.05). On the other hand, the value of ALT activity (unit/L) at 60 min after EtOH administration was 30.0±3.0, and that of the control group was 28.0±4.0; neither had statistical significance (p<0.05). The ALT activity after 180 min was also most equal, such as 30.0±3.0 and 28.0±4.0. From these results, it is judged that serum ALT activity was not affected by EtOH because there is no statistical significance between the EtOH-administrated group and the nonadministration group.

Effect of EtOH on bradykinin in blood

The influences of EtOH administration on the bradykinin level in rat blood were examined. As illustrated in Fig. 4, the bradykinin content was obviously lowered by EtOH administration.

Effect of EtOH on serum hyaluronidase activity

The effect of oral administration (7.5 g/kg) and intraperitoneal injection of EtOH (5.0 g/kg) to the rat on serum hyaluronidase was tested in preliminary experiments. Hyaluronidase activities at 60 min after the oral administration of EtOH or water (control) were obtained: Control was 3.6±0.8, and the EtOH-administrated group 8.0±0.6 as N-acetylglucosamine quantity (μg/mL/min). EtOH-elicited accentuation was statistically significant (p<0.05). EtOH enhanced from the hyaluronidase activity of 3.5±0.6; control to 14.2±1.4 at 180 min after the administration, this effect being approximately 2.6-fold stimulation and statistically significant (p<0.05). On the other hand, an intraperitoneal injection of EtOH also elicited stimulatory effects in hyaluronidase activity, making 32.5±5.7 from 4.2±0.4, control group after 60 min and being also statistically significant (p<0.05) in comparison with control. Hyaluronidase activity was increased significantly even 180 min following intraperitoneal injection.
Fig. 4. Changes in blood bradykinin concentration after an intraperitoneal injection of EtOH. Bradykinin concentrations in serum were measured after an intraperitoneal injection of EtOH (5 g/kg), as described in the text. Control received physiological saline instead of EtOH. Each column and vertical bar represents the mean value ± SE of 5 animals. A single asterisk indicates significant difference from control (without EtOH) at p<0.05.

Fig. 5. Effects of EtOH on kallikrein activity. We carried out the enzyme assay as described in the text, using the kallikrein preparations from human plasma and plasma separated from rat blood as substrate. Each column and vertical bar represents the mean value ± SE of five experiments. A single asterisk indicates significant difference from control (without EtOH) at p<0.05.

Fig. 6. Effects of EtOH on trypsin activities in rat plasma. We performed the enzyme assay as described in the text, with N-α-benzoyl-д-arginine-p-nitroanilide as substrate. Each column and vertical bar represents the mean value ± SE of five experiments. A single asterisk indicates significant difference from control (without EtOH) at p<0.05.

Fig. 7. Effect of EtOH on bradykinin-decomposed activity. We carried out the assay as described in the text, with bradykinin as substrate and rat plasma as enzyme preparation. We assayed the bradykinin amount with kinin measuring reagent, Markit M Bradykinin based on ELISA. Each column and vertical bar represents the mean value ± SE of five experiments. A single asterisk indicates significant difference from control (without EtOH) at p<0.05.

of EtOH, as follows: The activities with EtOH administration and without EtOH were 20.3±4.1 and 4.0±0.4, respectively.

**Effect of EtOH on the kallikrein activity in plasma**

Figure 5 shows the effect of EtOH on the plasma kallikrein activity in the in vitro experiment. Varying concentrations of EtOH from 0.2% to 1.0% seldom affected the kallikrein activity, but such high concentrations of EtOH as 6%, 12%, and 20% significantly accelerated the activity. When the tissue kallikrein was used, the activity was remarkably weak at 0.5 μg/min in comparison with plasma kallikrein; furthermore it was not affected by EtOH (data not shown).

**Effect of EtOH on the trypsin activity**

The effects of EtOH concentrations at a range of 0.6% to 20% on the trypsin activity were examined in vitro. As demonstrated in Fig. 6, there was an increase in the trypsin activity EtOH-concentration dependently.

**Effect of EtOH on the bradykinin-decomposed activity**

The effects of EtOH concentrations of 0.02%, 0.2%, 0.6%, 6%, 12%, and 20% against the activities of bradykinin decomposition by rat plasma in vitro are shown in Fig. 7. EtOH from 0.02% to 20% caused a
concentration-dependent promotion in the bradykinin decomposition.

**DISCUSSION**

Although oral administration and intraperitoneal injection of EtOH potentiated bradykinin-enhanced vascular permeability, these stimulations were suppressed in reverse by the actions of histamine and hyaluronidase with a property similar to bradykinin as a chemical mediator. The effects of EtOH on blood pressure and serum AST- and ALT-activities were examined to clarify a mechanism for the actions of EtOH on a stimulation of the vascular permeability by bradykinin. As reported previously, the rat’s blood pressure rose immediately after EtOH administration, followed by a quick return to normal. It was shown that there is no direct relevance on the transient hypertensive response to EtOH, because a potentiation of EtOH on the accelerated action of bradykinin in vascular permeability appeared 30 min after the EtOH administration. It was supported that the potentiation on the enhancement of bradykinin in vascular permeability by EtOH is not caused by the pharmacological action of EtOH on blood pressure, since EtOH did not demonstrate similar actions in the cases of histamine and hyaluronidase.

It is generally known that acute heart failure and acute liver disease (11) by viral infection and the administration of certain types of drug increases AST- and ALT-activities in blood (12, 13). The potentiating effect of EtOH is not due to an induction of acute inflammation in heart and liver, because the EtOH dose used in this experiment did not induce the changes of AST- and ALT-activities in blood, as described in the text. Harada et al. (14) reported that EtOH concentration in blood at 30 min after administration became 200 mg/dL by oral administration (6 g/kg body weight) of EtOH to the rat, and similar levels were maintained at 180 min after. EtOH concentration in blood at 30 min after administration was found to be retained throughout 2 h following intraperitoneal injection (5 g/kg body weight) of EtOH to the mouse (15), and it lowers only about 2/3 of the blood EtOH concentration following 4 h. This seems to show that EtOH itself sufficiently exists in blood for expressing various pharmacological effects in vivo (Fig. 1).

EtOH did lower the blood bradykinin level (Fig. 4), but was reversely enhanced the hyaluronidase activity in the blood (in the text). The increase in the concentration of chemical mediator in blood became not always the potentiation by EtOH, although the result in the case of histamine had not been obtained. Therefore the potentiating effects of EtOH on the action of bradykinin in vascular permeability is not concerned with the raised bradykinin in the blood level; also it does not depend on the direct action of EtOH in circulation (Fig. 2).

EtOH not only increased the plasma kallikrein activity to stimulate bradykinin formation from kininogen (Fig. 5), but it also accentuated the bradykinin-decomposed activity, whereas the oral administration of EtOH into rat lowered the blood bradykinin level (Fig. 7). Although EtOH affected the degrading and the producing systems in the metabolism of bradykinin, it is shown that EtOH did more markedly affect the degrading activity than it did the production system. Moreover, the activation of trypsin by EtOH (Fig. 6), which may be involved in the bradykinin metabolic system, seems interesting and should be clarified in future studies. The expression of the tissue kallikrein activity was weak; this seems to be because the rat kininogen does not receive the action of tissue kallikrein from human (16).

It is reported that the rise of the blood kallikrein activity and the activation of the tissue kallikrein are observed just as the blood acetalddehyde reaches its peak 1 h after the ingestion of alcohol (17). When this report is considered, the result shown in Fig. 2, namely, EtOH administered in the rat back intraderma did not potentiate a bradykinin-stimulation effect, indicates that the difference in note of reduction in blood acetalddehyde level compared with that in oral administration and intraperitoneal injection may result from the difference in the rise of activity.

Both bradykinin and histamine have stimulatory actions on vascular permeability and vasodilator action. These results cause the contraction of endothelial cells in the venule and the gap forming between endothelial cells. The action is known to be generated through an endothelium-derived relaxing factor in the vascular endothelial cells in which nitric oxide (NO) is mainly concerned (18–31), as well as leukotriene (LTC₄, LTD₄, and LTE₄) (32, 33).

The infusion of EtOH into the portal vein elicits vasoconstriction in the hepatic microvasculature, leading to hepatic tissue hypoxia and eventually hepatocellular necrosis (6, 7). An inhibition of the action of endogenous NO was associated with an increase in hepatic vasoconstriction and hepatocellular damage. Endogenous NO acts as a vasodilator that reduces EtOH-induced vasoconstriction, improving microcirculation and leading to decreased hepatic damage (34). Endothelin-1 enhances the EtOH-induced hepatic vasoconstriction and disturbs hepatic microcirculation, resulting in hepatic hypoxia and reduction of mitochondrial respiratory cytochromes (6). Two endothelium-derived vasoactive factors, NO and endothelin-1, regulate hepatic vascular tone in the presence of EtOH (35, 36) and gastric mucosal microcirculation (37). Furthermore, Suematsu et al. (38) reported that carbon monoxide produced by heme oxygenase could function as an endogenous modulator of vascular perfusion in the liver. Therefore it seems that stimulatory actions of EtOH involving epinephrine (2) segregation action from the adrenal and NO and endothelin productions (36, 37) with arteriole endothelial cell-derivative relaxant effect were related to its potentiating action on the vascular permeability of bradykinin, though the difference in the actions of EtOH on the stimulated permeabilities by bradykinin and histamine remains unknown. Ehringer et al. (19) reported that histamine stimulates the vascular permeability in a calcium-dependent manner, but the stimulatory effect of bradykinin does not.
depend on it. Bradykinin inhibits the DNA synthesis and stimulates the phospholipid hydrolysis and arachidonic acid release through the \( B_1 \) and \( B_2 \) receptors in human breast stromal cells (23). Therefore the stimulatory effect was obtained with bradykinin as a chemical mediator but not with other mediators as illustrated in Fig. 1, might show the result from the difference in the mode of stimulatory action on vascular permeability. It is assumed that the potentiation of EtOH on the enhancement of bradykinin in vascular permeability may be due to the elevations of bradykinin level and its action in the inflamed skin site by EtOH or its metabolites. Furthermore, it is assumed to involve the effects on the binding mechanism between bradykinin and its receptor and on the actions of NO and endothelin.

To clarify the further action of EtOH on the vascular permeability enhanced by bradykinin, we are now attempting to analyse the influences of EtOH and acetaldehyde on the actions of bradykinin receptor in its regulation by using the agonist and antagonist of receptor, in comparison with that of histamine.

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