

## Effect of ethanol on capsaicin-induced nerve-mediated vasorelaxation in rat arteries

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**Abstract:** Capsaicin, a pungent component of red chili peppers, is a potent vasorelaxant. Although the mechanism of capsaicin-induced relaxation seems to involve binding of capsaicin to the transient receptor potential vanilloid type 1 (TRPV1) cation channel and release of calcitonin gene-related peptide (CGRP) from the sensory nerve terminals, there is little evidence to suggest that capsaicin-induced relaxation is affected by ethanol. Therefore, in this study, we examined the effects of ethanol and several pharmacological antagonists on capsaicin- or CGRP-induced relaxation in rat arteries. Experiments were performed on hepatic and superior mesenteric arteries of male Wistar rats. We measured the isometric tension in the rings of these arteries with their endothelium removed to exclude the influence of the endothelial cells. Then, the arteries precontracted with phenylephrine were exposed to cumulative concentrations of capsaicin or CGRP in the absence or presence of ethanol and several antagonists. Capsaicin and CGRP caused dose-dependent relaxation in both arteries precontracted with phenylephrine. Capsaicin-induced relaxation was significantly inhibited by TRPV1 antagonists capsazepine, ruthenium red and CGRP receptor antagonist CGRP 8-37. Ethanol also significantly inhibited capsaicin-induced relaxation but did not affect CGRP-induced relaxation. Capsaicin-induced relaxation is mediated by the release of CGRP from the sensory nerve terminals via activation of the TRPV1, and ethanol did not inhibit relaxation at the smooth muscle level but inhibited CGRP release from the sensory nerve terminals.

**Key Words:** ethanol, relaxation, capsaicin, rat hepatic artery, rat superior mesenteric artery.

### INTRODUCTION

Vasorelaxation is mainly regulated by endothelium-dependent relaxation or nerve-mediated relaxation [1–2]. It is well known that nerve-mediated relaxation is regulated not only by sympathetic adrenergic nerves [3], but also by nonadrenergic noncholinergic (NANC) vasodilator innervation [4–5]. The transient receptor potential cation channel subfamily V member 1 (TRPV1), also known as the capsaicin receptor and the vanilloid receptor 1 (VR1), exists in sensory nerves, which are a type of NANC nerve. The vascular effects of capsaicin have been shown to be due to excitation of TRPV1 in the primary sensory nerves in the vessel wall and consequent

release of vasodilator neuropeptides such as calcitonin gene-related peptide (CGRP) [6–7]. On the other hand, endothelium-dependent relaxation is induced by nitric oxide (NO) [1, 8] or endothelium-derived hyperpolarizing factor (EDHF) [9–10], however, the mechanism is unknown.

Ethanol has been reported to decrease both coronary and hepatic blood flow, possibly via its depressive effect on endothelium-dependent relaxation [11]. However, whether ethanol affects nerve-mediated vasorelaxation has not yet been investigated. The present study was designed to investigate the effects of ethanol on nerve-mediated relaxation induced by capsaicin in rat hepatic and superior mesenteric arteries.

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## MATERIALS AND METHODS

### *Tissue preparation*

All procedures were approved by the committee on ethics in animal experiments of Nara Medical University and were conducted in conformity with institutional guidelines.

Male Wistar rats (10–12 weeks old and weighing 330–350 g) were anaesthetized by injection of pentobarbital (40 mg/kg) into the abdominal cavity and killed by blood loss. The common hepatic artery (HA) and second-order branches of the superior mesenteric artery (SMA) were then removed from the rats. The arteries were cut into 1-mm wide ring segments and mounted horizontally on 50- $\mu$ m diameter tension hooks in 4 mL tissue baths that contained Krebs-Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 2.5 mM CaCl<sub>2</sub>, and 10 mM D-glucose). The solution was maintained at 37 °C (pH 7.4), and aerated with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The endothelium was removed from all the rings to exclude the influence of the endothelial cells by rubbing the intimal surface with a stainless steel wire. Successful removal of the endothelial cells was confirmed by the inability of 10<sup>-6</sup> M acetylcholine to induce relaxation [12].

### *Drugs*

Capsaicin, capsazepine and ethanol were obtained from Wako Pure Chemical (Tokyo, Japan). NG-nitro-L-arginine (L-NNA) and ruthenium red were obtained from Sigma-Aldrich (USA). CGRP 8-37 was obtained from Tocris Bioscience (UK). Capsaicin, capsazepine and CGRP 8-37 were dissolved in dimethyl sulfoxide at a final concentration of 0.1%, since this concentration of dimethyl sulfoxide did not affect phenylephrine-induced vascular contraction (data not shown). All other drugs were dissolved in distilled water. Drug concentrations are reported as the final molar concentration in a Krebs-Ringer solution bath.

### *Measurement of tension*

Isometric tension, monitored with a force-displacement transducer (Primetech Co., Tokyo, Japan) to which one side of the tension hooks were connected, was recorded with a pen recorder (Nihon Kohden Kohgyo Co., Tokyo, Japan). The rings were allowed to equilibrate for approximately 1 h, during which time the medium was replaced every 15 min and the rings were adjusted for a resting tension of 0.2 g. Relaxant responses were studied in preparations that had been pre-contracted with phenylephrine (1–3  $\times$  10<sup>-6</sup> M). When stable contractions were obtained, capsaicin (10<sup>-9</sup> to 10<sup>-6</sup> M) or CGRP (10<sup>-10</sup> to 5  $\times$  10<sup>-8</sup> M) was added to the bath to determine the relationship between drug concentration and relaxation response. Capsazepine (3  $\times$  10<sup>-6</sup> M), CGRP 8-37 (10<sup>-6</sup> M) or L-NNA (10<sup>-4</sup> M) was added to the tissue bath 20 min

before the addition of phenylephrine. Ruthenium red (5  $\times$  10<sup>-5</sup> M) or ethanol (50 mM or 100 mM) was added to the bath 5 min before the addition of phenylephrine. The incubation period of 5 min with ethanol was selected to minimize evaporation during incubation in the tissue bath at 37 °C.

### *Measurement of alcohol concentration*

Alcohol concentration in the organ chamber was measured 30 min after the addition of alcohol using gas chromatography. Ethanol added to the tissue bath at a final concentration of 50 or 100 mM resulted in concentrations of approximately 30 mM and 70 mM, respectively, after approximately 30 min from the addition of ethanol, due to evaporation.

### *Calculation and statistical analysis*

Relaxation was expressed as a percentage of the contraction in response to 1–3  $\times$  10<sup>-6</sup> M phenylephrine. Data are presented as mean  $\pm$  standard error of mean (SEM). Statistical analysis was performed using Dunnett's test, and the results were considered statistically significant when P < 0.05.

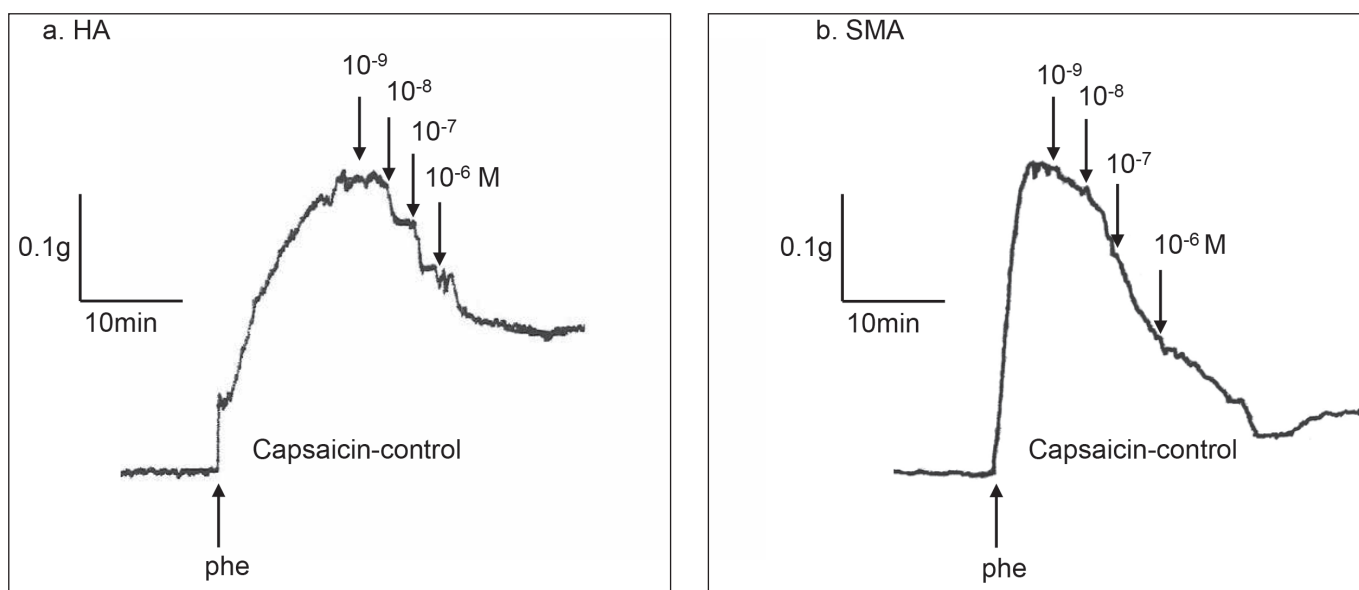
## RESULTS

### *Effect of ethanol on phenylephrine-induced contraction*

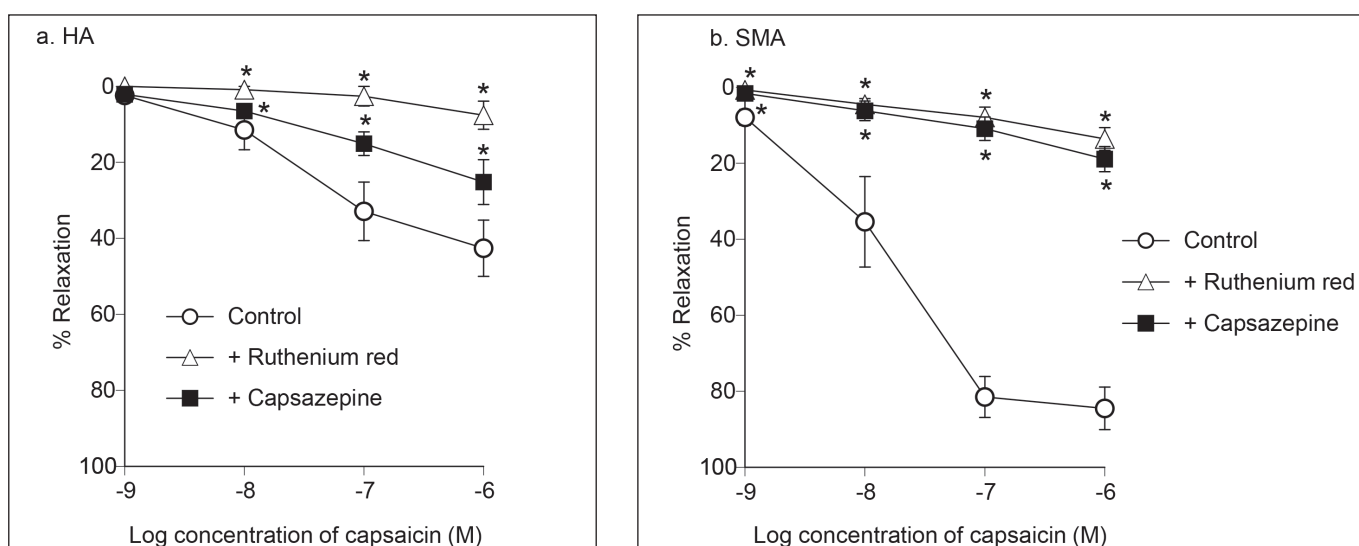
In both arteries, the initial levels of contractions induced by phenylephrine (1–3  $\times$  10<sup>-6</sup> M) were stable, long-lasting and not affected by the presence of 50 mM or 100 mM ethanol. The initial tension levels induced by phenylephrine in the presence of 0, 50, and 100 mM ethanol were 236  $\pm$  27 mg (n = 7), 216  $\pm$  20 mg (n = 5), and 222  $\pm$  23 mg (n = 8), respectively, in the HA, and 246  $\pm$  20 mg (n = 7), 223  $\pm$  32 mg (n = 5) and 279  $\pm$  23 mg (n = 8) respectively in the SMA (data not shown).

### *Effect of various inhibitors or ethanol on capsaicin-induced relaxation in the HA and SMA*

Capsaicin caused concentration-dependent relaxation in rat HA and SMA (Figs 1a and 1b), and the relaxation response was greater in the SMA than in the HA. In both arteries, capsaicin-induced relaxation was significantly inhibited by capsazepine and ruthenium red (Figs 2a and 2b). CGRP 8-37 also almost completely inhibited the relaxation induced by capsaicin in both arteries, while L-NNA did not inhibit capsaicin-induced relaxation (Figs 3a and 3b). In both arteries, capsaicin-induced relaxation was significantly inhibited by ethanol (50 mM or 100 mM) (Figs 4a and 4b). As shown in Table 1, in both arteries, pretreatment with ethanol significantly decreased the maximum relaxation (E<sub>max</sub>) induced by capsaicin. Also, in the SMA, the pD<sub>2</sub> values were significantly decreased by pretreatment with ethanol (Table 1).



**Figure 1.** Concentration-response curves for capsaicin-induced relaxation of the hepatic artery (HA) and superior mesenteric artery (SMA) in rats. Data are represented as mean  $\pm$  SEM, n = 5.



**Figure 2.** Effect of capsazepine ( $3 \times 10^{-6}$  M) and ruthenium red ( $5 \times 10^{-5}$  M) on the vasorelaxant response to capsaicin in the hepatic artery (HA) (a) and superior mesenteric artery (SMA) (b) in rats. Data are represented as mean  $\pm$  SEM, n = 5–6. \*P < 0.05 indicates significant differences from control values.

**Table 1.** pD<sub>2</sub> and E<sub>max</sub> values for capsaicin –induced relaxation in arteries

Artery/Treatment	pD <sub>2</sub>	E <sub>max</sub> (%)
<b>HA</b>		
No treatment	7.38 $\pm$ 0.47	42.6 $\pm$ 7.4
50 mM EtOH	7.04 $\pm$ 0.25	21.1 $\pm$ 4.5*
100 mM EtOH	7.01 $\pm$ 0.27	5.4 $\pm$ 2.9*
<b>SMA</b>		
No treatment	7.84 $\pm$ 0.36	84.5 $\pm$ 5.6
50 mM EtOH	7.33 $\pm$ 0.33*	31.4 $\pm$ 9.9*
100 mM EtOH	7.37 $\pm$ 0.05*	25.2 $\pm$ 5.8*

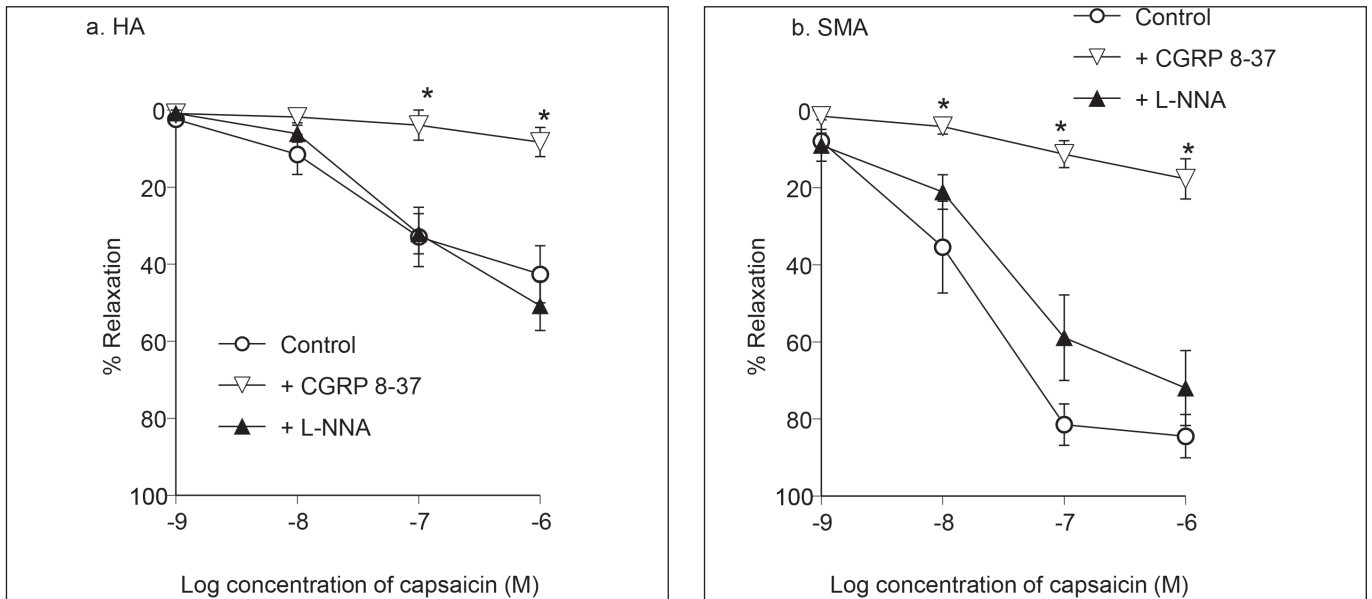
Data are represented as mean  $\pm$  SEM, n = 5–6. E<sub>max</sub>, maximal capsaicin-induced relaxation expressed as % of precontraction. pD<sub>2</sub>, negative logarithm of the molar concentration of capsaicin producing 50% of the maximal response. \*P < 0.05 indicates significant differences from control values.

**Effect of ethanol on CGRP-induced relaxation**

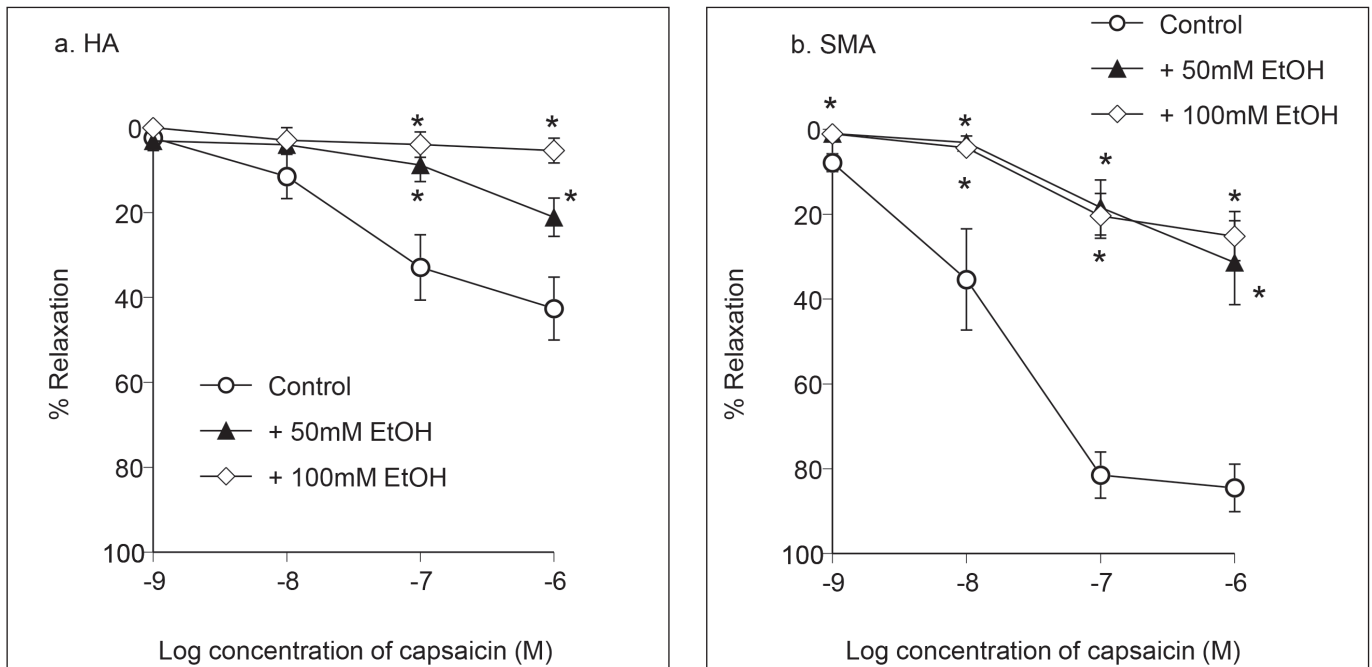
CGRP induced concentration-dependent relaxation in the HA and SMA (Figs 5a and 5b), and ethanol had no effect on the relaxation induced by CGRP in either artery (Figs 6a and 6b).

**DISCUSSION**

In the present study, capsaicin induced arterial relaxation in rat HA and SMA, and the level of relaxation was greater in the SMA than in the HA. The difference in the relaxation response between the 2 arteries may be due to differences in the sensitivity of, or the number of, TRPV1. TRPV1 antagonists capsazepine, ruthenium red and CGRP1 receptor antagonist CGRP 8-37 inhibited capsaicin-induced relaxation in the 2 arteries. These



**Figure 3.** Effect of CGRP 8-37 ( $10^{-6}$  M) and nitro- L-arginine (L-NNA) ( $10^{-4}$  M) on vasorelaxant responses to capsaicin in the hepatic artery (HA) (a) and superior mesenteric artery (SMA) (b) in rats. Data are represented as mean  $\pm$  SEM,  $n = 5-7$ . \* $P < 0.05$  indicates significant differences from control values.

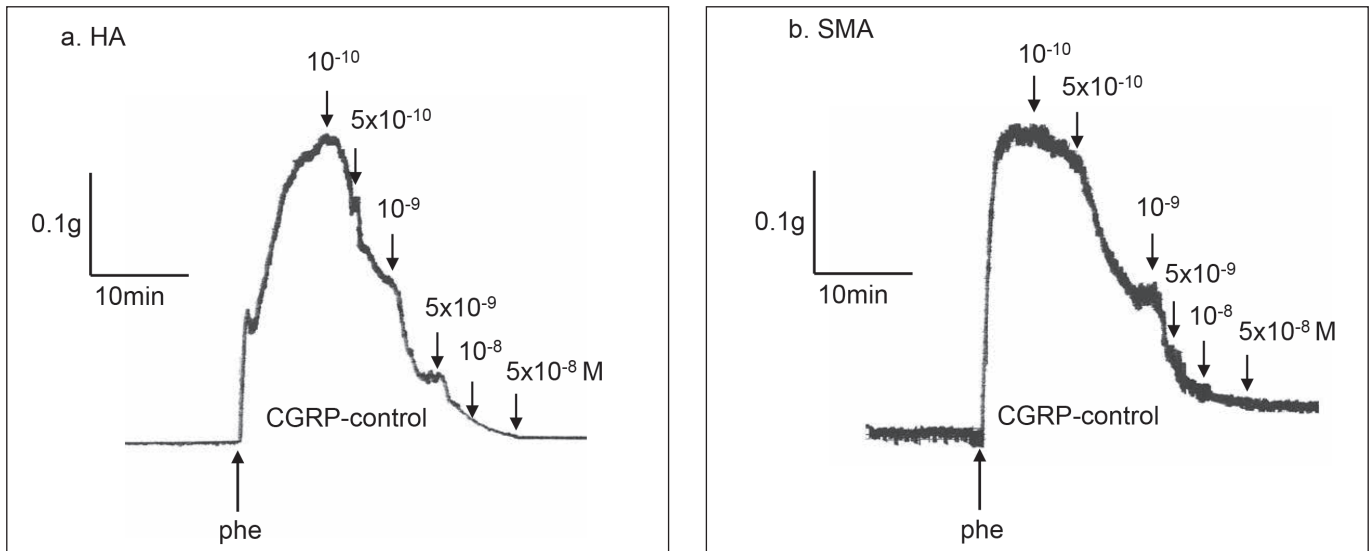


**Figure 4.** Effect of ethanol on vasorelaxant responses to capsaicin in the hepatic artery (HA) (a) and superior mesenteric artery (SMA) (b) in rats. Data are represented as mean  $\pm$  SEM,  $n = 5-6$ . \* $P < 0.05$  indicates significant differences from control values.

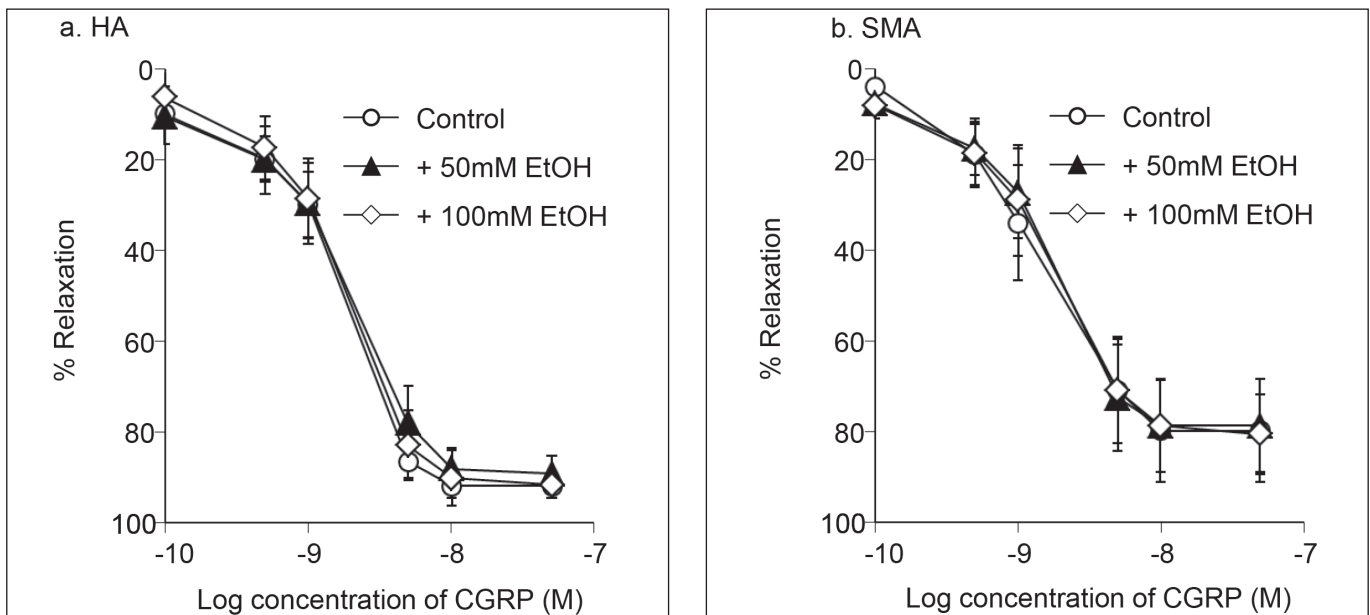
results suggest that capsaicin-induced relaxation is mediated by CGRP release from sensory nerve terminals via TRPV1 on the surface membrane of nerves, and the inhibitory action of these antagonists is consistent with previous reports [13]. However, the relaxation was not inhibited by L-NNA, the NO synthase inhibitor. This result suggests that neuronal nitric oxide synthase (nNOS) is not involved in capsaicin-induced relaxation. CGRP has been previously reported to cause vascular relaxation via an NO-dependent mechanism of endothelial activation [14]. Furthermore, endothelium-derived NO has been reported to facilitate the release of CGRP from nerve terminals and to be a secondary vasorelaxation

messenger of CGRP [15]. Therefore, in the present study, we used denuded arteries to exclude the involvement of endothelium-derived NO and investigated whether nerve-derived NO is involved in capsaicin-induced relaxation. As L-NNA did not inhibit capsaicin-induced relaxation in either artery, our results suggest that nerve-derived NO is not involved in the relaxation mechanism. It has been reported that NO is partially involved in the relaxation of electrically stimulated HA [16], so the differences observed here may be due to the fact that the present study used chemical stimulation.

Ethanol significantly reduced  $E_{max}$  and  $pD_2$  values for capsaicin-induced relaxation in the 2 arteries. Further,



**Figure 5.** Concentration-response curves for CGRP-induced relaxation of the endothelium-denuded isolated hepatic (HA) and superior mesenteric artery (SMA) in rats. Data are represented as mean  $\pm$  SEM, n = 6–8.



**Figure 6.** Effect of ethanol on vasorelaxant responses to CGRP in the hepatic artery (HA) (a) and superior mesenteric artery (SMA) (b) in rats. Data are represented as mean  $\pm$  SEM, n = 6–8.

ethanol did not inhibit relaxation induced by the CGRP in the 2 arteries. Therefore, our data suggest that ethanol did not inhibit the relaxation at the smooth muscle level but rather inhibited CGRP release from sensory nerve terminals. Ethanol may inhibit CGRP release from sensory nerve termini in the following two ways. First, as ethanol is a membrane-fluidizing agent [17, 18], its action may alter the properties and functions of the membrane receptor system including TRPV1. This may result in either a decrease in efficient receptor coupling or a disturbance in the receptor-mediated trans-membrane process, which then affects nerve-mediated CGRP release. Second, the receptor-evoked release of CGRP is dependent upon the presence of extracellular calcium [19, 20]. Therefore, ethanol may exert its inhibitory action on the release of CGRP by affecting calcium which bind to TRPV1 of the nerve membrane.

Ethanol has been previously reported to reduce the contraction induced by contractile agents, and the degree to which a vascular strip relaxes depends on the level of contraction prior to the administration of the relaxing agent [21]. However, in the present study, the initial contraction levels induced by  $3 \times 10^{-6}$  M phenylephrine did not show a significant reduction in the presence of 50 mM or 100 mM ethanol. Thus, inhibition of capsaicin-induced relaxation by ethanol is not affected by the contraction level before ethanol administration. Furthermore, the depressant effect of ethanol may be due to damage of smooth muscles by high doses of ethanol. However, the level of contraction induced by phenylephrine in the presence of 100 mM ethanol was not different from the level of the contraction induced by phenylephrine in its absence, after ethanol was washed away. Thus, the vessel after exposure to 100 mM ethanol

showed a reversal of the contractile force and damage of smooth muscle, therefore, is not likely to be due to the depressant effect of ethanol.

On the other hand, ethanol has been reported to mediate relaxation through the release of CGRP via the activation of TRPV1 in porcine coronary arteries and, after treatment of these arteries with capsaicin, ethanol did not induce relaxation [22]. However, in our preliminary study, 100 mM ethanol caused no relaxation in either rat HA or SMA. In these arteries, ethanol caused relaxation at a high concentration (200 mM) which non-specific effects might start, but the relaxation was not inhibited by CGRP 8-37 or pretreatment (desensitization) with capsaicin (data not shown). These findings suggest that ethanol-induced relaxation is not mediated by CGRP in rat HA or SMA. Differences in these results seem due to the differences in species and vascular bed.

In general, the physiological ethanol concentration is about 20 mM during alcohol intake. The concentration used in the present study was high (50 mM and 100 mM) but the ethanol concentrations added to the organ bath resulted in lower final concentrations (approximately 30 mM and 60 mM) after the end of the *in vitro* experiment due to evaporation. Thus, ethanol appears to depress capsaicin-induced relaxation at lower concentrations. Furthermore, it is likely that the inhibitory effect of ethanol can be evoked at lower concentrations *in vivo* than *in vitro* because, as the exposure time to ethanol becomes longer, the effect of ethanol is observed at lower concentrations [23]. Thus, ethanol at physiological concentrations may have inhibitory effects on the

relaxation responses *in vivo*.

CGRP shows vasodilatory action in the hepatic vascular bed, and nerves containing CGRP in such vessels may play a role in maintaining blood flow to the liver [24–27]. Furthermore, in the right and left ventricle walls and in the interventricular septum, CGRP-containing nerve fibers are observed to run parallel to the coronary arteries and their collaterals [28–31]. Our results suggest, therefore, that ethanol might cause hemodynamic dysfunction of the hepatic microcirculation and vasculature by inhibiting CGRP-mediated relaxation, resulting in hepatic hypoxia and alcoholic liver damage. In addition, ethanol may be involved in vasospasm or decreased blood flow in the coronary artery by inhibiting CGRP-mediated relaxation, leading to alcohol-induced sudden death.

## CONCLUSION

We demonstrated that capsaicin-induced relaxation was mediated by the release of CGRP from the sensory nerve terminals via activation of the TRPV1. Ethanol reduced capsaicin-induced relaxation by inhibiting CGRP release from sensory nerve terminals. Further investigations are required for the elucidation of the detailed restraint mechanism of ethanol on this pathway.

**Conflict of interest.** The authors declare that they have no conflict of interest concerning this article.

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