Korean J Physiol Pharmacol
Vol 5: 47–53, February, 2001

Pulse Exposure to Ethanol Augments Vascular Contractility Through Stress Response

Eun-Kyoung Yang[1] and InKyeom Kim[2]

[1]Departments of Physiology and [2]Pharmacology, Kyungpook National University School of Medicine, Taegu 700–422, Korea

Drinking excessive alcohol has been recognized as a risk factor for hypertension. However, the mechanism by which alcohol intake causes hypertension still remains elusive. We tested the hypothesis that ethanol itself acts as a stress factor on vasculature and indirectly modulates vascular contractility. After end of exposure to 1, 2.5 and 5% ethanol for 45 min, rat aortic strips were subjected to contractile responses, immunoblot for Hsp70 and the measurement of levels of myosin light chain phosphorylation. Exposure to 5% ethanol not only augmented contractions to KCl or phenylephrine, but also increased expression of Hsp70 and the levels of myosin light chain phosphorylation. There were no significant differences in contractions produced by 1 μmol/L phorbol 12,13-dibutyrate, a protein kinase C activator, whether the tissues were exposed to 5% ethanol or not. This is the first report to show that even short exposure to ethanol has a delayed effect to increase vascular smooth muscle contractility through a modulation of thick filament regulation. It may be a mechanism by which ingestion of alcohol induces hypertension.

Key Words: Ethanol, Hsp 70 family, Hypertension, Myosin Light Chains, Phosphorylation, Contraction

Abbreviations: ANOVA, analysis of variance; DTT, dithiothreitol; Grp, glucose-regulated protein; HSP, heat shock family of stress proteins; Hsp, heat shock protein; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; RLC, regulatory myosin light chain.

INTRODUCTION

Drinking excessive alcohol has been identified as a risk factor for hypertension. Although epidemiological studies demonstrated a positive correlation between blood pressure and alcohol consumption (Klatsky et al, 1977; Dyer et al, 1981), the mechanism by which alcohol intake causes hypertension still remains elusive. Stress has been commonly implicated in cardiovascular pathological conditions such as hypertension (Chan et al, 1985; Blake et al, 1995). Recently we reported that vascular stress such as heat shock potentiated vascular contractility in isolated aortic strips of rats (Kim et al, 1999). Since ethanol has been known to induce a family of heat shock proteins (HSPs) in mammalian cells such as Hsp70 and Hsp28 (Hahn et al, 1991), Hsc70 (Miles et al, 1991), and Grp78 and Grp94 (Miles et al, 1994), there is a possibility that ethanol itself acts as a stress factor on vasculature and indirectly modulates contractile responses to vasoactive agents.

The acute effect of alcohol drinking on blood pressure has been reported to be diverse from hypotensive to hypertensive actions depending on the amount of ethanol consumption as well as the timing of BP measurement (Kawano et al, 1992; Seppa, 1999; Narkiewicz et al, 2000). Moderate alcohol intake (ethanol, 1 ml/kg) has biphasic effects on 24-hour blood pressure in hypertensive patients; a depressor effect that lasts for several hours after drinking followed by a pressor effect (Abe et al, 1994). Even though reduction or cessation of alcohol drinking lowered blood pressure during the next three

Corresponding to: InKyeom Kim, Department of Pharmacology, Kyungpook National University School of Medicine, 101 Dongin-2-Ga, Taegu 700-422, Korea. (Tel) +82-53-420-6939, (Fax) +82-53-426-7345, (E-mail) inkim@knu.ac.kr

weeks or one month (Ueshima et al, 1993; Aguilera et al, 1999), prior chronic alcohol exposure had yet residual pressor effects even after a mean of 35 days of abstinence in detoxified alcoholics (York & Hirsch, 1996). These results suggest that the increased blood pressure by alcohol drinking is not caused by ethanol itself, but by secondary biological changes. Although there have been many studies to show direct effects of ethanol on vascular contraction (Werber et al, 1997; Jover et al, 1999), there is no study to demonstrate residual effects of alcohol on vascular reactivity regarding stress response.

In view of the suggested important role of ethanol in the development of hypertension, we tested the hypothesis that a stress response induced by ethanol, but not ethanol itself, may modulate vascular smooth muscle contractility. To our knowledge, this is the first report to demonstrate an indirect and delayed effect of ethanol to increase vascular contractility following exposure to ethanol in isolated vascular strips.

METHODS

Organ bath study

Male Sprague Dawley rats weighing 300~330 g were used in this study. Under anesthesia with sodium pentobarbital (50 mg kg^{-1} i.p.), thoracic aorta was excised immediately and immersed in ice-cold modified Krebs-bicarbonate solution composed of (in mM) NaCl, 115.0; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and dextrose, 10.0. All adherent connective tissues of the aorta were cleaned off on moistened filter paper and it was cut into four ring segments (4 mm in length). The endothelium was denuded from some of the rings by rubbing internal surface with a forcep. Two stainless steel triangles were inserted through each vessel ring. Care was taken to avoid rubbing the endothelial surface of vessels. Each aortic ring was suspended in a water-jacketed organ bath (20 ml) maintained at 37°C and aerated with a gas mixture of 95% O_2 and 5% CO_2 (pH 7.4). One end was anchored to a stationary support and the other end was connected to an isometric force transducer (Grass FT03C, Quincy, MA, USA). Aortic rings were stretched passively by imposing the optimal resting tension, 2.0 g, which was maintained throughout the experiment. The isometric contractions were recorded on an ink-writing polygraph (Grass model 7, Quincy, MA, USA).

Pulse exposure to ethanol

Aortic ring preparations were exposed to 1, 2.5, and 5% ethanol for 45 min followed by further incubation in physiological salt solution, whereas control tissues were left on the physiological salt solution. All the rings were subjected to contractions by KCl in one and five hours followed by phenylephrine.

Western blot

After functional study, some muscle strips were quickly frozen by immersion in acetone containing 10 mM dithiothreitol (DTT) precooled with dry ice. Muscles were stored at −80°C overnight, brought back to room temperature and dried up by putting them on filter paper next day. Samples were homogenized in a buffer (pH 7.0) containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, the protease inhibitor leupeptin, trypsin inhibitor (10 μg/ml each), aprotinin (2 μg/ml), and PMSF (100 μg/ml). Protein matched samples (Bradford assay) were electrophoresed on SDS-PAGE (7.5%), transferred to nylon membranes, and subjected to immunoblot with a specific Hsp70 antibody (1 : 1,000, Sigma, St. Louis, MO, USA). Anti-mouse IgG (goat) conjugated with horse-radish peroxidase was used as a secondary antibody (1 : 2,000, Sigma, St. Louis, MO, USA). The bands containing Hsp70 were detected with enhanced chemiluminescence (ECL) visualized on films, acquired by Adobe Photoshop™, and then analyzed by NIH image™.

RLC phosphorylation

At 3 minutes after administration of 0.1 or 1.0 μmol/L phenylephrine, muscle strips were quickly frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) precooled in dry ice as described (Kim et al, 2000). Muscles were washed 3 times with acetone containing 5 mM DTT for 15 min each to remove TCA and soaked in 100 μl of sample buffer containing 20 mmol/L Tris base, 23 mmol/L glycine (pH 8.6), 8.0 mol/L urea, 10 mmol/L DTT, 10% glycerol, and 0.04% bromphenol blue. The urea-extracted samples (20 μl) were electrophoresed at 400 V for 2.5

hours and transferred to nitrocellulose membranes, and subjected to immunoblot with a specific myosin light chain antibody (1 : 1,000, Sigma, St. Louis, MO, USA). The bands containing myosin light chains were visualized with enhanced chemiluminescence (ECL) on films, acquired from Adobe PhotoShop™, and then analyzed by NIH image™.

Statistical analysis

The data were expressed as mean±S.E.M and were analyzed by ANOVA followed by Bonferroni's method for contraction and by Student's t-test for myosin light chain phosphorylation. $P<0.05$ were regarded as statistically significant.

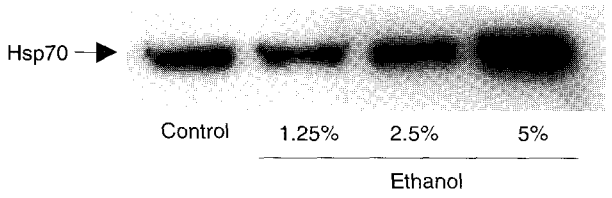

Fig. 1. Western blot for Hsp70 in control, 1, 2.5 and 5% ethanol-exposed rat aortic rings. Pulse exposure to 5% ethanol for 45 min markedly increased expression of Hsp70 in rat aorta 5 hours later as compared to control. Three independent experiments showed similar results.

RESULTS

After functional study, some muscle strips were subjected to immunoblot for Hsp70. Pulse exposure to 5% ethanol for 45 min markedly increased expression of Hsp70 in rat aorta as compared to control (Fig. 1).

One and five hours after the end of exposure to ethanol, aortic rings were challenged with 55 mM KCl. Exposure of tissues to 5% ethanol augmented the tension five hours later compared with control, whereas exposure to ethanol at any concentration did not affect vascular contractility one hour after the end of exposure (Fig. 2. *Left*). Concentration-response curves for the contractile responses to KCl were obtained from control and ethanol-exposed rat aortic rings five hours after the end of the exposure (Fig. 2. *Right*).

In order to obtain concentration-response relationships to phenylephrine, an α_1-adrenergic agonist, it was added to the organ bath in a cumulative fashion. Phenylephrine induced contractions in concentration-

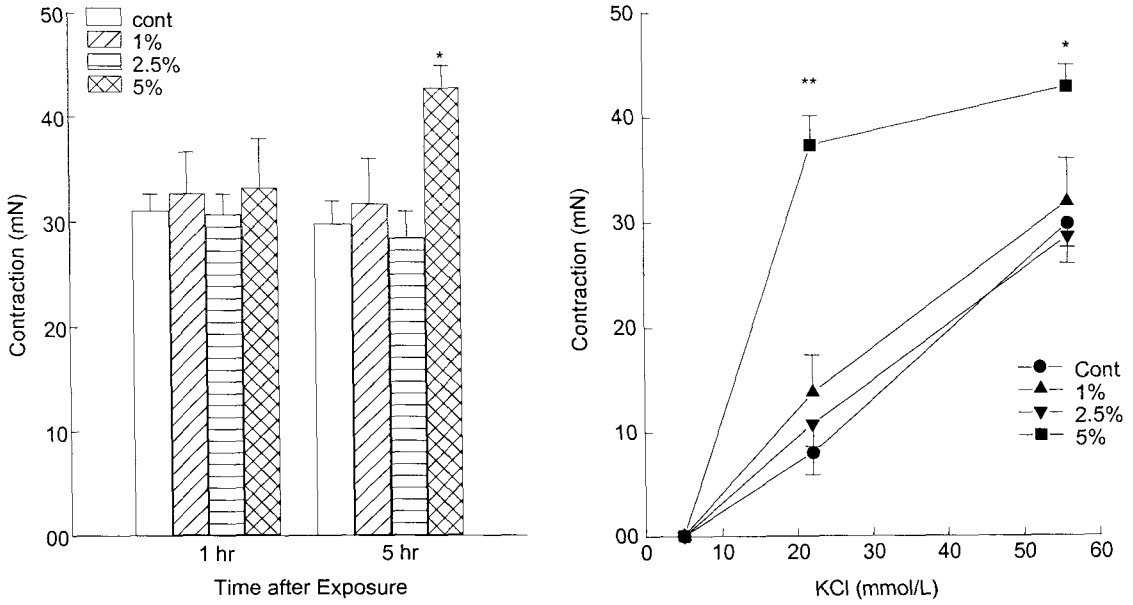

Fig. 2. Contractile responses to KCl in control and ethanol-exposed rat aortic rings. *Left:* Exposure to 5% ethanol for 45 min enhanced vascular contractility to 55 mmol/L KCl five hours but not one hour after the end of the exposure. *Right:* Contractile responses to KCl five hours after the end of the exposure in control and ethanol-exposed rat aortic rings. Tension is expressed in mN. The data are expressed as means of five experiments with vertical bars showing S.E.M. *$P<0.05$, **$P<0.01$ with control.

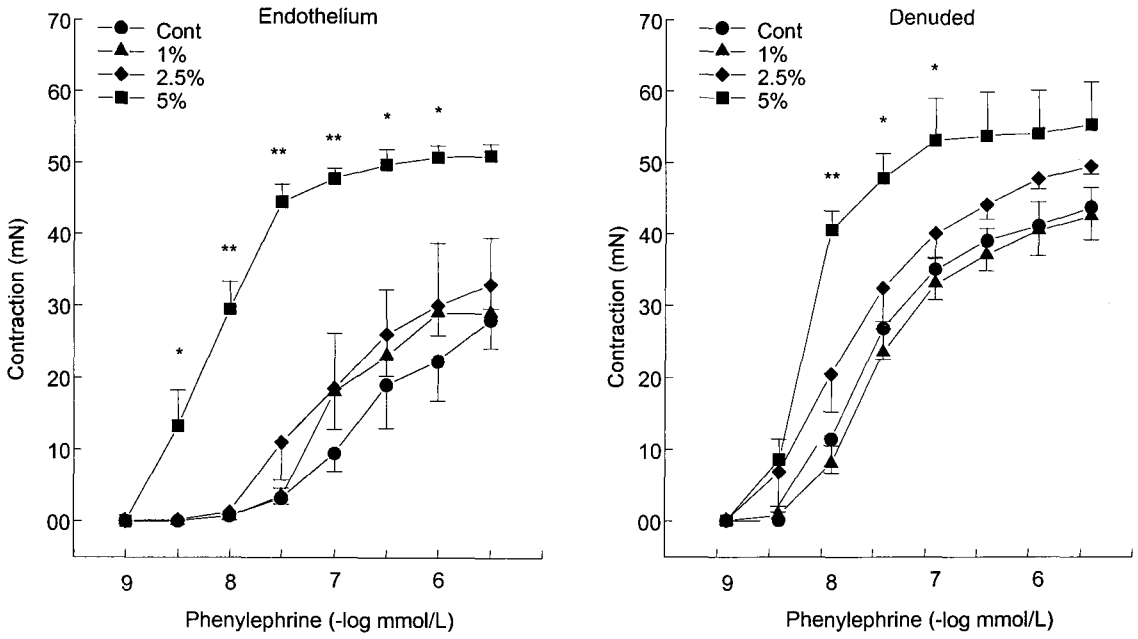

Fig. 3. Cumulative log concentration-response curves for the contractile responses to phenylephrine in control and ethanol-exposed rat aortic rings with (left) or without (right) endothelium. Exposure to 5% ethanol for 45 min statistically significantly enhanced vascular contractility to phenylephrine five hours after the end of the exposure compared with control whether endothelium was present or denuded. Tension is expressed in mN. The data are expressed as means of five experiments with vertical bars showing S.E.M. *P<0.05, **P<0.01 with control.

dependent manner in isolated rat aortas from a concentration of 1.0 nmol/L to that of 3 μmol/L. Three μmol/L caused a maximal effect. Phenylephrine-induced vascular contraction was statistically significantly enhanced in 5% ethanol-treated rat aortic rings compared with control whether endothelium was present or denuded (Fig. 3). However, the difference in contractility between 5% ethanol-treated rat aortic rings and the others were less marked after denudation of endothelium (Fig. 3. *Right*).

In parallel, we measured levels of regulatory myosin light chain (RLC) phosphorylation as a biochemical marker for calcium-dependent contraction. Urea-extracted samples were electrophoresed in a glycerol/urea gel where phosphorylated RLC (pRLC) moves down faster than unphosphorylated RLC because the former has higher anionic charges (Fig. 4. *Upper*). The levels of RLC phosphorylation in 5% ethanol-exposed tissues were statistically significantly increased compared with respective controls (Fig. 4. *Lower*).

In order to investigate whether pulse exposure to ethanol affect calcium-independent contraction, we tried phorbol 12,13-dibutyrate (1 μmol/L), a PKC ac-

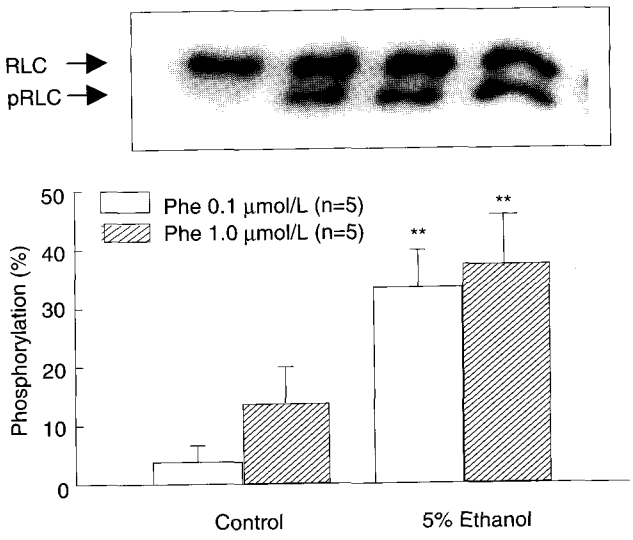

Fig. 4. *Upper:* A representative of regulatory myosin light chain (RLC) phosphorylation (pRLC) to 0.1 or 1.0 μmol/L phenylephrine in control and 5% ethanol-exposed rat aortic rings. Description of each lane is same as below. *Lower:* Exposure to 5% ethanol for 45 min statistically significantly enhanced the levels of RLC phosphorylation compared with their respective controls. The data are expressed as means of five experiments with vertical bars showing S.E.M. **P<0.01.

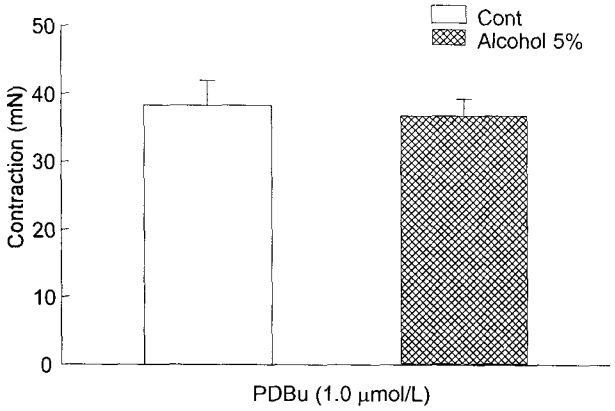

Fig. 5. Contractile responses to 1.0 μmol/L phorbol 12, 13-dibutyrate (PDBu) administrated in a Ca^{2+}-free solution with 2 mmol/L EDTA in control and 5% ethanol-exposed rat aortic rings. Exposure to 5% ethanol for 45 min did not affect vascular contractility induced by 1.0 μmol/L PDBu. Tension is expressed in mN. The data are expressed in means of five experiments with vertical bars showing S.E.M.

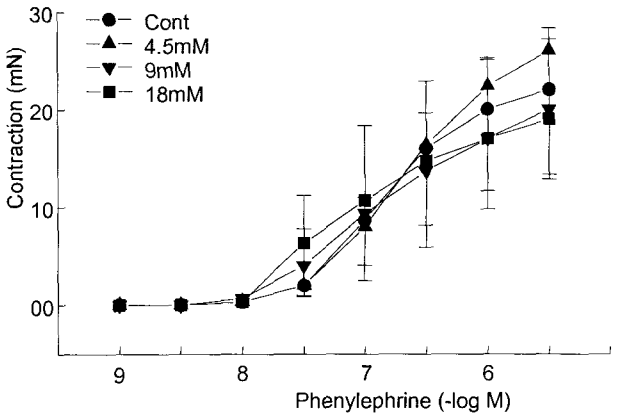

Fig. 6. Cumulative log concentration-response curves for the contractile responses to phenylephrine in control and acetaldehyde-exposed rat aortic rings. Exposure to acetaldehyde at any concentration for 45 min did not affect vascular contractility to phenylephrine five hours after the end of exposure as compared with control. Tension is expressed in mN. The data are expressed as means of five experiments with vertical bars showing S.E.M.

tivator, in a calcium-free solution containing 2 mmol/L EDTA. There was no significant difference in contraction between control and 5% ethanol-exposed groups (Fig. 5).

Because acetaldehyde is a major metabolite of ethanol, we questioned whether pulse exposure to acetaldehyde would also augment vascular contractility. Exposure to acetaldehyde did not affect phenylephrine-induced vascular contraction whether the strips were treated in 4.5, 9.0 and 18 mM acetaldehyde or not (Fig. 6).

DISCUSSION

Pulse exposure to 5% ethanol for 45 min not only augmented vascular contractility to KCl as well as phenylephrine five hours later but also increased expression of Hsp70. In a previous study, direct heat shock of isolated rat aortic rings also increased vascular contractility eight hours later in accordance with increased Hsp70 expression (Kim et al, 1999). The results of these two studies have a common feature in that vascular stresses, whether physical or chemical, have a delayed effect of augmenting vascular contractility. It is clear that ethanol did not directly affect the contractility since ethanol was washed off several times after its exposure. This notion is also supported by an observation that augmentation of vascular contraction was seen five hours after the end of exposure, but not as early as one hour after the end of exposure (Fig. 2). It is conceivable that the augmentation of contractility was caused by secondary biological change but not by a direct chemical action of ethanol itself. Presumably, several hours are required for proteins to be synthesized and to have an effect. Heat shock also augmented vascular contractility three hours, but not as early as 1 hour after heat shock (unpublished data). From these results we speculate that a biological reaction occurred in vascular smooth muscle cells in response to the stressor. Because so many proteins are known to be induced by ethanol (Hahn et al, 1991; Miles et al, 1991; 1994), it is not yet concluded which protein is responsible for the increased vascular contractility after exposure to ethanol.

From the results of the present study, we speculate that a certain inducible protein or its complex may modulate vascular contractility by a mechanism to increase RLC phosphorylation (Fig. 4). The level of myosin phosphorylation is controlled by a balance of the activities of two key enzymes, myosin light chain kinase and myosin light chain phosphatase (Horowitz et al, 1996). With further study it will be clear which mechanism is responsible for increased contractility of vascular smooth muscles after a stress response. However, it is less likely that a calcium-independent contractile pathway plays a role in the potentiating

action of ethanol on vascular contraction because the contractions induced by phorbol 12,13-dibutyrate in a calcium-free solution showed no significant difference between the control and ethanol-exposed groups (Fig. 5).

There were many suggestions for a pressor effect of ethanol; interference of central inhibitory pathways, increased sympathetic activity, impairment of baroreceptor reflex, activation of renin-angiotensin system, excessive corticosteroid and mineralocorticoid production, increase of calcium in the vascular smooth muscle cells, and suppression of endothelium-dependent relaxation (Abdel-Rahman & Wooles, 1987; MacMahon, 1987; Criscione et al, 1989). Abe et al (1994) did not hesitate mentioning a possibility that there also may be other slow pressor mechanisms since the 24-hour blood pressure pattern on day 7 of the alcohol phase seemed to be elevated in parallel compared with that on day 1. This delayed effect of ethanol on vasculature may be a mechanism to explain a slow pressor effect following a depressor effect after the last drink in hypertensive patients with repeated alcohol intake (Abe et al, 1994). The pressor effect of alcohol remained even after a mean of 35 days of abstinence in detoxified alcoholics (York & Hirsch, 1996), implying that the pressor effect might be mediated by a delayed alcohol-induced biological change but not by a direct pharmacological action of alcohol or its metabolites.

A depressor effect is considered to be caused by the direct action of alcohol or its metabolite following a moderate amount (1.0 g/kg body weight) of alcohol intake (Kawano et al, 1992; Narkiewicz et al, 2000). Since ethanol shifted the pressor response curve of phenylephrine rightward, it was found to have an adrenergic α-receptor blocking action (Abdel-Rahman & Wooles, 1987). In Orientals, post-drinking facial flush was associated with elevated blood acetaldehyde, marked BP reduction, and tachycardia (Kupari et al, 1983). This study showed that acetaldehyde had no residual effect on vascular contraction even at a sublethal dose (Fig. 6).

Ethanol has been reported to have diverse actions on endothelial nitric oxide synthase activity depending on the duration of ethanol exposure as well as the concentration of ethanol (Criscione et al, 1989; Davda et al, 1993). The fact that augmentation of contraction by exposure to 5% ethanol was sustained after denudation of endothelium indicates that the effect was at least in part directly on the smooth muscle cells themselves (Fig. 4).

The results of the present study may be criticized in 3 respects. First, the concentration of ethanol to create an effect was far higher than that usually observed physiologically (~500 mg/dl) (El-Mas & Abdel-Rahman, 2000). Due to limitation of time in in-vitro experiment, we tried high concnetrations of ethanol for a brief incubation time to test the hypothesis. Moreover, rats are about 20 times as tolerable to ethanol in the development of hypertension as human beings. Sprague-Dawley rats maintained on ethanol (10 g/kg/day) did not become hypertensive until 4 weeks but showed a modest increase in mean arterial pressure after 12 weeks (Abdel-Rahman & Wooles, 1987), whereas three drinks or more per day (1 drink is an equivalent of 8~10 g of ethanol) has been reported to be sufficient to elevate blood pressure in humans (MacMahon, 1987). In this study, 5% ethanol was chosen since 9% of ethanol was reported to be a comparable cellular stress to 43°C for 60 min in U937 and PEER human lymphoid tumor cells (Gabai et al, 1997). In a previous study, heat shock of 45 min, but not 15 min, at 42°C was suitable to increase vascular contractility in isolated rat aortas (Kim et al, 1999). Second, 5% ethanol might be enough to make high osmolarity in solution, 856 mM. Because ethanol is water-miscible and diffuse easily, it does not produce osmolality in an aqueous solution. Third, the aorta used in this study is just a conduit vessel, not a resistance vessel that plays an important role in regulating blood pressure. In this study, we intended to test a hypothesis that stress response induced by ethanol, but not ethanol itself, may modulate vascular smooth muscle contractility. Resistance vessels such as the mesenteric artery might be stressed by physiological concentrations of ethanol, especially in hypertensive-prone subjects.

Thus, some questions remain unanswered by our findings. Nevertheless, to the best of our knowledge, this is the first report to demonstrate an indirect and delayed effect of ethanol on the augmentation of vascular smooth muscle contractility following pulse exposure to ethanol. The relevance of a stress response to regulation of vascular smooth muscle contractility is being studied regarding pathogenesis of essential hypertension.

ACKNOWLEDGMENTS

This work was supported by research fund from Hangok Medical Foundation 2000. The authors are

extremely grateful to Dr. Michael J. Taggart (Manchester Royal Infirmary, UK) for his critical reading of this manuscript.

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