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Ethanol-Induced Inhibition of Platelet Aggregation in Whole Blood from Healthy Donors

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Abstract

Ethanol is known to inhibit platelet aggregation. In order to examine effects of ethanol on platelet aggregation, isolated platelet-rich plasma and platelet suspension are often used. However, it remains to be clarified whether and how ethanol affects platelet aggregation in whole blood. In this concise study, we examined effects of ethanol on platelet aggregation induced by different stimulants in whole blood by using the screen filtration pressure method. Thapsigargin and 1-oleoyl-2-acetyl-sn-glycerol (OAG), which cause Ca^{2+} entry into platelets through the store-operated Ca^{2+} channels and the diacylglycerol-dependent Ca^{2+} channels, respectively, were used for inducing platelet aggregation. Thapsigargin-induced platelet aggregation was markedly inhibited by ethanol at 0.25% or higher, and OAG-induced platelet aggregation was significantly inhibited by ethanol at 1% or higher. These results indicate that inhibitory effect of ethanol was more prominent on thapsigargin-induced aggregation than on OAG-induced aggregation. Thus, ethanol inhibits thapsigargin- and OAG-induced platelet aggregation in whole blood, and the effect of ethanol on platelet aggregation through the store-operated Ca^{2+} channels is thought to be stronger than that on aggregation through the diacylglycerol-dependent Ca^{2+} channels.

Keywords: Ca^{2+} channels; Ethanol; Platelet aggregation; Whole blood

Introduction

Alcohol shows diverse effects on cardiovascular health. Although light-to-moderate amount of alcohol drinking is preventive for coronary heart disease [1], excessive alcohol drinking has been shown to increase the risk of hemorrhagic types of stroke such as cerebral hemorrhage and subarachnoid hemorrhage [2]. One reason for this harmful effect of alcohol is alcohol-induced hypertension [3]. Increase in bleeding tendency due to inhibition of platelet aggregation by alcohol is also proposed as a mechanism for the increased risk of stroke by drinking [4]. Moreover, alcohol has been reported to affect blood coagulation factors. Blood fibrinogen level has been shown to be inversely associated with alcohol consumption in previous epidemiological studies [5,6]. In experimental studies, protein production and mRNA expression of fibrinogen in lined hepatoma cells have been reported to be diminished by exposure of the cells to ethanol [7]. In addition, levels of von Willlebrand factor and factor VII were reportedly lower in moderate drinkers than in nondrinkers [8].

Ethanol is known to inhibit platelet aggregation [9,10], which is dependent on transmembraneous Ca^{2+} entry into platelets [11]. There are two main Ca^{2+} entry pathways, store-operated Ca^{2+} channels and diacylglycerol (DG)-dependent Ca^{2+} channels, in non-excitable cells including platelets [12,13]. In our previous study using isolated platelet suspension, ethanol showed diverse effects on the store-operated and DG-dependent Ca^{2+} channels [14]. However, it remains to be determined whether and how platelet aggregation mediated by the above different types of Ca^{2+} channels is influenced by ethanol in whole blood, a more physiological condition compared with other conditions, such as in washed platelet suspension and in platelet-rich plasma, which are generally used for experiments of platelet aggregation. Although in vitro platelet aggregation using platelet-rich plasma have been reported in other animal species [15,16], reports on platelet aggregation using whole blood have been rare in other animal species as well as in humans. The purpose of this study was therefore to clarify the effects of ethanol on platelet aggregation in whole blood in response to stimulation with thapsigargin and 1-oleoyl-2-acetyl-sn-glycerol (OAG), which induce Ca^{2+} entry into platelets through the store-operated and DG-dependent Ca^{2+} channels, respectively.

Materials and Methods

Preparation of whole blood

Venous blood was obtained from healthy male and female donors aged from 39 to 47 years who had not been administered any drugs for at least 10 days before the experiments, and the platelet counts in whole blood of the donors were $(20 \pm 5) \times 10^4/\mu l$. The blood from each donor (18 ml) was rapidly transferred to a plastic tube containing 2 ml of 3.2% sodium citrate, and then mixed. Thus, the final concentration of sodium citrate used as an anticoagulant was 0.32%. Since stabilization of platelets for about 1 hour after blood collection is needed in order to elude the effects of prostacyclin included in whole blood before experiments, the experiments using whole blood were done from 1 to 2 hours after blood collection.

Measurement of platelet aggregation by the screen filtration pressure method

Whole-blood aggregation was measured with a whole-blood aggregometer using the screen filtration pressure method (WBA-Neo, ISK, Tokyo, Japan) as reported previously [17,18]. While constantly stirring at 37°C, the reaction was started by an addition of 20 µl of a

solution, containing each stimulant, to 180 µl of whole blood. At 5 min after stimulation, the absorbing pressure of aggregated whole blood was measured through a micro sieve with 30 × 30 µm windows, and negative pressures of -130 mmHg and -6 mmHg were defined as 100% aggregation and 0% aggregation, respectively, the latter deviation from 0 mmHg being designated because of the viscosity of unstimulated whole blood.

Drugs

Thapsigargin and OAG (Sigma, St Louis, Missouri, USA) were dissolved in dimethylsulfoxide to make stock solutions of 1 mM and 50 mM, respectively, and stored at -80°C. Ethanol (Wako Pure Chemical Co., Osaka, Japan) was diluted with distilled water to obtain each required concentration just before use. Whole blood was stabilized by incubation in a cuvette at 37°C for 3 min. Then ethanol was added to the cuvette, followed by an addition of each stimulant 10 sec later and an addition of $CaCl_2$ (0.5 mM) further 10 sec later.

Statistical analysis

The data are presented as means ± standard deviations. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Scheffé's F-test. P values less than 0.05 were regarded as significant.

Results and Discussion

Figure 1 shows platelet aggregation in whole blood induced by different concentrations of thapsigargin (0.25 µM–2 µM) and OAG (25 µM–200 µM). Maximum level of platelet aggregation was induced by stimulation with thapsigargin at concentrations of 1 µM or higher or by stimulation with OAG at concentrations of 50 µM or higher. Then, thapsigargin at 1 µM and OAG at 50 µM were used to induce comparable levels of platelet aggregation, on which effects of ethanol were tested.

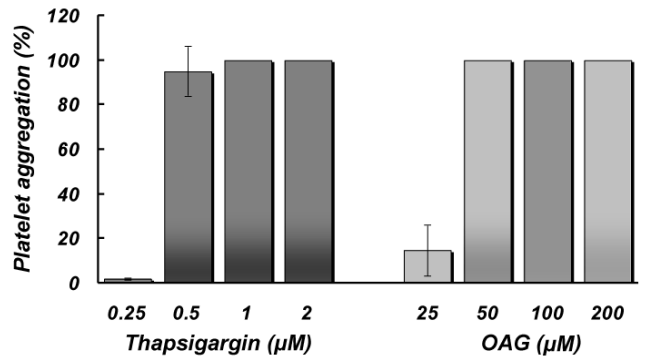

Figure 1: Platelet aggregation in whole blood induced by different concentrations of thapsigargin (0.25 µM–2µM) or OAG (25 µM–200 µM) n=5.

Figure 2 shows effects of different concentrations of ethanol on platelet aggregation induced by thapsigargin at 1 µM or OAG at 50 µM. Thapsigargin-induced aggregation was markedly inhibited by ethanol at concentrations of 0.25% or higher, and OAG-induced aggregation was significantly inhibited by ethanol at concentrations of 1% or higher. Thus, both thapsigargin- and OAG-induced platelet aggregation in whole blood was inhibited by ethanol, and the inhibitory effect of ethanol was more prominent on thapsigargin-induced aggregation than on OAG-induced aggregation.

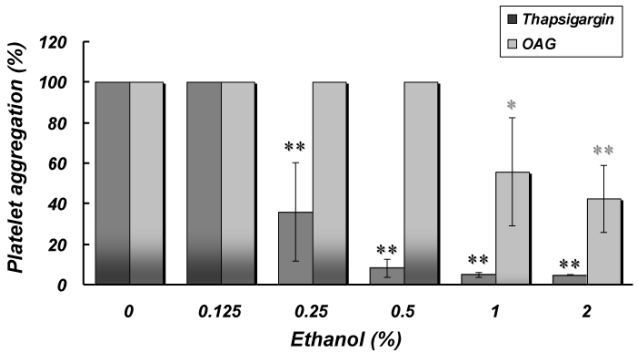

Figure 2: Effects of pretreatment with different concentrations of ethanol on platelet aggregation induced by thapsigargin (1 µM) or OAG (50 µM). Asterisks denote significant differences ($^{*}p<0.05$; $^{**}p<0.01$) from the control pretreated with a vehicle of ethanol. n=4–6.

This is, to the best of our knowledge, the first study showing effects of ethanol on platelet aggregation in whole blood induced by different stimulants, thapsigargin and OAG. The inhibitory effect of ethanol was much stronger on thapsigargin-induced aggregation than on OAG-induced aggregation. This difference may be due to diverse effects of ethanol on transmembraneous Ca^{2+} entry into platelets induced by thapsigargin and OAG: The former Ca^{2+} entry is inhibited by ethanol, whereas the latter Ca^{2+} entry is augmented by ethanol [14]. Regardless of augmenting action of ethanol on OAG-induced Ca^{2+} entry into platelets, ethanol significantly inhibited OAG-induced aggregation of platelets both in platelet suspension [14] and in whole blood as shown in the present study. Thus, ethanol has inhibitory action on a Ca^{2+} entry-independent pathway(s) of platelet aggregation. This action of ethanol is thought to be, at least partly, explained by its inhibitory action on phospholipase A_2 [10,19], which is a key enzyme for producing arachidonate metabolites including thromboxane A_2, a potent agonist for platelet aggregation. OAG, a mimic of DG, is a potent activator for protein kinase C, which causes an increase in Ca^{2+} sensitivity of actomyosin in platelet [20]. Thus, there is a possibility that ethanol inhibits the protein kinase C-mediated pathway of signal transduction in platelets, and further studies are needed to examine this hypothesis.

Thpsigargin and OAG activate different Ca^{2+} channels such as store-operated Ca^{2+} channels and DG-dependent Ca^{2+} channels, respectively. Thrombin, a physiological agonist for platelets, is known to activate both of the above types of Ca^{2+} channels following hydrolysis of phosphoinositides [21]. Interestingly, thrombin-induced Ca^{2+} entry was reportedly not affected by ethanol [14], suggesting a cancellation of the diverse effects of ethanol on store-operated and DG-dependent Ca^{2+} channels. In our previous study, thrombin-induced platelet aggregation was inhibited both in platelet suspension and in whole blood by ethanol at concentrations of 0.125% (about 21 mmol/l) or higher [22]. This threshold concentration of ethanol is clinically attainable [23]. Therefore, the inhibitory effect of ethanol on platelet aggregation is clinically significant and may, at least in part, explain acute blood coagulation disorder due to excessive drinking.

In conclusion, ethanol has inhibitory action on platelet aggregation in whole blood, and this action was stronger on thapsigargin-induced aggregation than on OAG-induced aggregation, suggesting that sensitivity to ethanol of the store-operated Ca^{2+} channels is higher than that of the diacylglycerol-dependent Ca^{2+} channels.

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