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

Mikio Marumo* and Ichiro Wakabayashi

Department of Environmental and Preventive Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan

*Corresponding author: Marumo M, Department of Environmental and Preventive Medicine, Hyogo College of Medicine, Mukogawa-cho 1-1, Nishinomiya, Hyogo 663-8501, Japan. Tel: +81-798-45-6562; Fax: +81-798-45-6563; E-mail: m-mail@hyo-med.ac.jp

Received date: Mar 04, 2014, Accepted date: Mar 23, 2015, Published date: Mar 27, 2015

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 Ca²⁺ entry into platelets through the store-operated Ca²⁺ channels and the diacylglycerol-dependent Ca²⁺ 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 Ca²⁺ channels is thought to be stronger than that on aggregation through the diacylglycerol-dependent Ca²⁺ channels.

Keywords: Ca²⁺ 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 Willebrand 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 transmembrane Ca²⁺ entry into platelets [11]. There are two main Ca²⁺ entry pathways, store-operated Ca²⁺ channels and diacylglycerol (DG)-dependent Ca²⁺ channels, in non-excitatory cells including platelets [12,13]. In our previous study using isolated platelet suspension, ethanol showed diverse effects on the store-operated and DG-dependent Ca²⁺ channels [14]. However, it remains to be determined whether and how platelet aggregation mediated by the above different types of Ca²⁺ 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 Ca²⁺ entry into platelets through the store-operated and DG-dependent Ca²⁺ 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 ± 5) × 10⁴/µl. 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 CaCl₂ (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.

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 transmembrane Ca²⁺ entry into platelets induced by thapsigargin and OAG: The former Ca²⁺ entry is inhibited by ethanol, whereas the latter Ca²⁺ entry is augmented by ethanol [14]. Regardless of augmenting action of ethanol on OAG-induced Ca²⁺ 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 Ca²⁺ 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 A₂ [10,19], which is a key enzyme for producing arachidonate metabolites including thromboxane A₂, a potent agonist for platelet aggregation. OAG, a mimic of DG, is a potent activator for protein kinase C, which causes an increase in Ca²⁺ 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.

Thapsigargin and OAG activate different Ca²⁺ channels such as store-operated Ca²⁺ channels and DG-dependent Ca²⁺ channels, respectively. Thrombin, a physiological agonist for platelets, is known to activate both of the above types of Ca²⁺ channels following hydrolysis of phosphoinositides [21]. Interestingly, thrombin-induced Ca²⁺ entry was reportedly not affected by ethanol [14], suggesting a cancellation of the diverse effects of ethanol on store-operated and DG-dependent Ca²⁺ 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\textsuperscript{2+} channels is higher than that of the diacylglycerol-dependent Ca\textsuperscript{2+} channels.

Acknowledgement

This work was supported by a Grant-in-Aid for researchers at Hyogo College of Medicine (2012).

References

1. Marmot MG (2001) Alcohol and coronary heart disease. Int J Epidemiol 30: 724-729.
2. Reynolds K, Lewis B, Nolen JD, Kinney GL, Sathya B, et al. (2003) Alcohol consumption and risk of stroke: a meta-analysis. JAMA 289: 579-588.
3. Klatsky AL (1996) Alcohol and hypertension. Clin Chim Acta 246: 91-105.
4. Hillbom ME (1987) What supports the role of alcohol as a risk factor for stroke? Acta Med Scand Suppl 717: 93-106.
5. Meade TW, Imeson J, Stirling Y (1987) Effects of changes in smoking and other characteristics on clotting factors and the risk of ischaemic heart disease. Lancet 2: 986-988.
6. Brien SE, Ronksley PE, Turner BJ, Mukamal KJ, Ghali WA (2011) Effect of alcohol consumption on biological markers associated with risk of coronary heart disease: systematic review and meta-analysis of interventional studies. BMJ 342: 636.
7. Wang Z, Barker TH, Fuller GM (1999) Alcohol at moderate levels decreases fibrinogen expres-sion in vivo and in vitro. Alcohol Clin Exp Res 23: 1927-1932.
8. Mukamal KJ, Jadhav PP, D’Agostino RB, Massaro JM, Mittleman MA, et al (2001) Alcohol consumption and hemostatic factors: analysis of the Framingham Offspring cohort. Circulation 104: 1367-1373.
9. Renaud SC, Ruf JC (1996) Effects of alcohol on platelet functions. Clin Chim Acta 246: 77-89.
10. Rubin R (1999) Effect of ethanol on platelet function. Alcohol Clin Exp Res 23: 1114-1118.
11. Rink TJ, Sage SO (1990) Calcium signaling in human platelets. Annu Rev Physiol 52: 431-449.
12. Parekh AB, Putney JW (2005) Store-operated calcium channels. Physiol Rev 85: 757-810.
13. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, et al (1999) Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397: 259-263.
14. Marumo M, Wakabayashi I (2010) Diverse effects of ethanol on Ca\textsuperscript{2+} entry and subsequent aggregation of platelets. Alcohol 44: 343-350.
15. Casella S, Giudice E, Giannetto C, Marafioti S, Piccione G (2011) Effects of hydrocortisone and aminophylline on the aggregation of equine platelets in vitro. J Vet Sci 12: 215-219.
16. Casella S, Giannetto C, Giudice E, Marafioti S, Fazio F, et al. (2013) ADP-induced platelet aggregation after addition of tramadol in vitro in fed and fasted horses plasma. Res Vet Sci 94: 325-330.
17. Ozeki Y, Sudo T, Toga K, Nagamura Y, Ito H, et al. (2001) Characterization of whole blood aggregation with a new type of aggregometer by a screen filtration pressure method. Thromb Res 101: 65-72.
18. Tabuchi A, Taniguchi R, Takahashi K, Kondo H, Kawato M, et al. (2008) Action of aspirin on whole blood-aggregation evaluated by the screen filtration pressure method. Circ J 72: 420-426.
19. Toivanen J, Ylikorkala O, Viinikka L (1984) Ethanol inhibits platelet thromboxane A2 production but has no effect on lung prostacyclin synthesis in humans. Thromb Res 33: 1-8.
20. Yamami Y, Higashihara M, Kurokawa K, Ozaki Y, Kume S (1994) Effects of the prior activation of protein kinase C on human platelet activation induced by thrombin. Int J Hematol 59: 201-209.
21. Münzer P, Tolios A, Peliz L, Schmid E, Schmidt EM, et al. (2013) Thrombin-sensitive expression of the store operated Ca\textsuperscript{2+} channel Orai1 in platelets. Biochem Biophys Res Commun 436: 25-30.
22. Marumo M, Wakabayashi I (2009) Sensitivity of thrombin-induced platelet aggregation to inhibition by ethanol. Clin Chim Acta 402: 156-159.
23. Kupari M, Heikilä J, Ylikahri R (1983) Acute effects of alcohol on left ventricular dynamics during isometric exercise in normal subjects. Clin Cardiol 6: 103-108.