EFFECTS OF ALCOHOL AND THE EVENING MEAL ON SHEAR-INDUCED PLATELET AGGREGATION AND URINARY EXCRETION OF PROSTANOIDS

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Abstract — Moderate regular alcohol intake has been found to be associated with a decreased risk for coronary heart disease and stroke. We investigated the effects of acute intake of red wine (60 g ethanol) and a standard dinner under controlled conditions on haemostatic factors. Shear-induced platelet aggregation (SIPA) decreased after the intake of alcohol irrespective of whether the subjects were fasting or not, and also after the intake of food. The intake of alcohol inhibited the postprandial increase of von Willebrand factor multimers. Plasma levels of plasminogen activator inhibitor 1 activity (PAI-1) and serum triglycerides were increased by alcohol. Excretion of the platelet thromboxane A2 metabolites 11-dehydrothromboxane B1 and 2,3-dinor-thromboxane B2, as well as the endothelial prostacyclin metabolite 2,3-dinor-6-ketoprostaglandin F1α into urine was not influenced by either alcohol or food. We conclude that eating a dinner together with red wine has no untoward effect on SIPA and that the decrease of SIPA is not specific for alcohol.

INTRODUCTION

Moderate regular alcohol intake is associated with a decreased risk for coronary heart disease (Langer et al., 1992; Gaziano et al., 1993; Hein et al., 1996; Rimm et al., 1996) and ischaemic stroke of atherothrombotic origin (Palomäki and Kaste, 1993; Palomäki et al., 1993). These associations could be due to the protective effects of either ethanol itself or the non-alcoholic components of the beverages. It has been suggested that the effects of ethanol on blood lipids (Langer et al., 1992; Renaud and De Lorgeril, 1992; Gaziano et al., 1993) could explain half of the protective effect. The effects of ethanol on factors of primary haemostasis (Mikhailidis et al., 1983; Landolfi and Steiner, 1984; Renaud et al., 1992) and fibrinolytic activity (Hendriks et al., 1994; Ridker et al., 1994b) have also been suggested to contribute, but their role is unclear.

Several previous experimental studies on humans have addressed the effects of moderate drinking of alcohol on the haemostatic (Renaud and Ruf, 1996) and fibrinolytic (Reeder et al., 1996) systems. Two studies have separately investigated the effect of a small dose of alcohol in the course of a standard dinner. In these two studies, fibrinolytic activity seemed to be inhibited immediately after the ingestion of alcohol, whereas a rebound increase was observed shortly after the alcohol had been eliminated (Veenstra et al., 1990; Hendriks et al., 1994). One hour after the ingestion of alcohol, collagen- and ADP-induced platelet aggregation was increased, whereas a rebound decrease of platelet aggregation was observed on the next morning after an overnight fast (Veenstra et al., 1990).

Shear-induced platelet aggregation (SIPA) and urinary excretion of thromboxane and prostacyclin metabolites have not been studied as yet after acute ingestion of a moderate dose of alcohol. We investigated these parameters, plasminogen activator inhibitor, von Willebrand factor antigen and multimers together with several other laboratory parameters.

Healthy human volunteers, who served as their own controls, were randomly assigned to ingest a moderate dose of red wine (or water) with or without an evening meal. The aim was to find out the possible beneficial effects of alcohol on SIPA and other parameters of the haemostatic and fibrinolytic systems in healthy human volunteers during fasting and after the ingestion of a standard evening meal.

METHODS

Subjects and study design

Twelve healthy men aged 33–59 years volunteered for the trial. After a full explanation of the purpose, nature and risks of the study, written informed consent was obtained from the subjects. The study protocol was approved by the Ethical Committee of the University Hospital of Oulu.

The volunteers were infrequent light-to-moderate drinkers, and all but one were non-smokers. They had abstained from alcoholic beverages for two weeks before the trial, and they denied having taken any drugs within one week before the trial. None had arterial hypertension, heart disease, haematological disorders or any other serious disease in their history, nor were any on regular medication. The descriptive characteristics and baseline laboratory values of the subjects are shown in Table 1.

Each subject participated in four 12-h sessions on consecutive days: (1) alcohol with food; (2) mineral water with food; (3) alcohol without food; and (4) mineral water without food (fasting). The sequences of the four sessions were randomized for each individual. Two of the sessions were assigned to include 633 ml of Hungarian red wine (60 g ethanol, Egri Bigaver), whereas two others did not include alcohol, but included the same volume of mineral water. Thus, each participant underwent four tests, two of which were postprandial tests, one a post-alcohol test without a meal and one a fasting test. The sessions always began at 18.00 (with or without a
between the sessions they had breakfast (07.00) and lunch were kept supine and allowed to sleep. During the daytime (12.00) and were allowed to move freely on our metabolic rates of eating and drinking were similar for all the subjects.

The period day (10.00–18.00) preceding the session and during the night dinner) and ended at 06.00 the next morning. The period allowed for eating the dinner and drinking was 3 h, and the rates of eating and drinking were similar for all the subjects. During the following night from 22.00 until 06.00 the subjects were kept supine and allowed to sleep. During the daytime between the sessions they had breakfast (07.00) and lunch (12.00) and were allowed to move freely on our metabolic ward. During the whole study week the subjects were served standardized meals: breakfast, lunch and dinner. Each meal contained the same relative amount of carbohydrates (55%), proteins (15%), and fats (30%). The total amount of energy per day was kept constant, either 14.0 or 22.0 kcal/kg, depending on whether only breakfast and lunch or also dinner were served, respectively.

Blood and urine sampling

Blood samples were taken four times at 4-h intervals, starting just before each session. Urine was collected after voiding the bladder from 10.00 till 18.00 and from 22.00 till 06.00.

Shear-induced platelet aggregation

SIPA was measured at 4-h intervals between 18.00 and 06.00. Blood was collected with minimal stasis via a plastic cannula into tubes containing 1/10 volume of 3.8% trisodium citrate. To obtain platelet-rich (PRP) and platelet-poor (PPP) plasma, part of the blood samples were centrifuged at 330 g for 10 min and at 1500 g for 5 min. Aggregation was induced by applying a high shear force, 108 dynes/cm², for 5 min by means of a turbidimetric technique that uses a thermostated cone-plate streaming chamber. The method has been described in detail elsewhere (Fukuyama et al., 1989; Uchiyama et al., 1994). SIPA was expressed as the maximum percentage change in light transmittance during the 5 min period.

SIPA in PRP was also assessed after addition of ethanol to the test tube. First, pure ethanol was added to homologous PPP to yield PPP with different concentrations of ethanol. A constant volume of PPP, with or without ethanol, was always added into the sample of PRP to be tested 10 min before starting the aggregation assay. The final concentrations of ethanol in PRP were 0, 4, 8, 16, 33, 65 and 130 mmol/l.

Determination of prostanoids

Urine was collected for the determination of 2,3-dinor-6-ketoprostaglandin F₁α (2,3-dinor-PGF₁α), 11-dehydrothromboxane B₂ (11-dehydro-TXB₂) and 2,3-dinorthromboxane B₂ (2,3-dinor-TXB₂). Samples of the urine excreted during the day (10.00–18.00) preceding the session and during the night following the session (22.00–06.00) were frozen immediately after collection and stored at −70°C until extraction. The urine excreted during the first 4 h of each session was discarded, because the subjects did not succeed in collecting their urine between 18.00 and 10.00 during the alcohol sessions.

11-Dehydro-TXB₂ and 2,3-dinor-PGF₁α were determined by radioimmunoassay as described previously (Riutta et al., 1992, 1994). One-step and two-step solid-phase extraction methods were applied to 11-dehydro-TXB₂ and 2,3-dinor-PGF₁α respectively. The urinary excretion rates were expressed as picograms per micromole of creatinine.

For the analysis of 2,3-dinor-TXB₂, 2,3-dinor-[³H]TXB₂ was prepared from [³H]TXB₂ (Amersham International, Bucks, UK) by beta-oxidation in vitro using rat hepatocytes as previously described for 2,3-dinor-PGF₁α (Riutta et al., 1994). The labelled 2,3-dinor-TXB₂ was separated from [³H]TXB₂ by HPLC on a Spheri-5 RP-C₁₈ column (Pierce, Breda, The Netherlands) using a water:acetonitrile:acetic acid (72:28:0.1, by volume) mobile phase. After thawing, the urine samples were centrifuged and 2,3-dinor-[³H]TXB₂ was added (10 000 d.p.m. to 1 ml of urine). The urine was diluted with water and applied to a C₁₈ silica cartridge (Varian, Walnut Creek, CA, USA). 2,3-Dinor-TXB₂ was eluted with 5 ml of ethyl acetate from the cartridge and, after evaporation, the dry residue was dissolved into radioimmunoassay (RIA) buffer. RIA was carried out in duplicate in 50 mM phosphate buffer containing 0.1% gelatin and 0.01% thiomersal using [¹²⁵I]thromboxane-tyrosine methyl ester (Institute of Isotopes, Budapest, Hungary) as a labelled radioligand and non-labelled 2,3-dinor-TXB₂ (Cayman Chemical Co., Ann Arbor, MI, USA) for the calibration of the standard curve. The antibody raised against TXB₂ (Institute of Isotopes) showed a 100% cross-reaction with 2,3-dinor-TXB₂ across the whole range of the standard curve. After incubation at 4°C overnight, dextran-coated charcoal (Sigma, St Louis, MO, USA) was used to separate the bound fraction from the free.

Determination of von Willebrand factor antigen and multimers

Von Willebrand factor antigen (vWF:Ag) was measured with an enzyme-linked immunosorbent assay technique using TMB (3,3,5,5-tetramethylbenzidine; Sigma) as substrate (Cejka, 1982). Antibodies to the rabbit anti-human von Willebrand factor and to the peroxidase-conjugated rabbit anti-human von Willebrand factor were from Dako A/S (Glostrup, Denmark). A normal plasma pool calibrated with the international standard (Factor VIII and von Willebrand factor in plasma, WHO International Institute for Biological Standards and Controls, UK) was used as a working standard.

For the assessment of von Willebrand factor (vWF) multimers, citrated plasma samples were diluted with Tris buffer containing urea and sodium dodecyl sulphate (SDS) (10 mmol/l Tris, 2% SDS, 8 mol/l urea and 0.005% bromphenol blue, pH 8.0) and run in 1% SDS–1.3% agarose (Sea Kem, Rockland, ME, USA) in Tris buffer (0.05 mol/l Tris, pH 8.35, containing 0.384 mol/l glycine and 0.1% SDS). Proteins were transferred to nitrocellulose and the vWF multimers were visualized (Blake et al., 1994) with rabbit immunoglobulins to human vWF and alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (Dako A/S). vWF:Ag was expressed as units. Coded plasma samples were used to study the size of vWF multimers

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<th>Table 1. Characteristics of the study subjects</th>
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HDL, high-density lipoprotein.
and the number of bands on the immunoblots was evaluated independently by one of us (R.K.).

**Determination of plasminogen activator inhibitor activity**

Citrated plasma was used to analyse PAI-1 activity (U/ml), which was measured as duplicate determinations with a colorimetric assay (Stachrom PAI, Diagnostica Stago, Paris, France) in the kinetic mode using an automatic coagulometer (Thrombolyzer, Behnk Elektronik GMBH & Co., Norderstedt, Germany) equipped with a chromogenic channel.

**Determination of other laboratory parameters**

Plasma fibrinogen, serum triglycerides, cholesterol, high-density-lipoprotein cholesterol, gamma-glutamyl transferase, blood ethanol, glucose and urinary creatinine were measured with routine clinical laboratory methods. The mean blood-ethanol concentrations at 22.00 (i.e. 4 h after starting to drink) were 18.1 mmol/l (95% CI 16.4–19.8) and 12.9 mmol/l (95% CI 11.4–14.3) after the intake of wine without and with an evening meal, respectively.

**Statistical methods**

The demographic results are expressed as mean ± SD. For all other means, 95% confidence intervals (CI) were calculated. The comparisons between the baseline values and those obtained at 4-h intervals during the session were performed by paired t-test. The time-dependent changes were also compared by repeated-measures analysis of variance (ANOVA), either as a one-way analysis for a single session or as a two-way analysis between the sessions. All analyses were calculated by Statview II or SuperAnova (Abacus Concepts, USA).

**RESULTS**

Ingestion of alcohol produced a transient decrease in SIPA (Table 2). The effect was small but was the same irrespective of whether a standard evening meal was taken together with the alcohol or not. The time needed to eliminate the dose of alcohol from the body was ~10 h. Accordingly, the effect on SIPA seemed to last until the alcohol was eliminated from the body. No alcohol was present in the blood of the volunteers 12 h after the baseline, i.e. at 06.00. SIPA was not influenced by mineral water, but food ingested together with mineral water also decreased it, though not significantly. Thus, one-way ANOVA indicated statistically significant decreases in SIPA during the wine-drinking sessions (wine with meal, \( F_{3,11} = 4.67, P < 0.01 \); wine without meal, \( F_{3,10} = 3.34, P < 0.05 \), but not during the water-drinking sessions. However, the differences between the alcohol and water sessions were not statistically significant when compared by two-way ANOVA.

We then examined the effect of ethanol on SIPA in vitro. A slight and insignificant decrease was observed at a very low concentration (4 mmol/l of ethanol). SIPA remained unchanged at a concentration of 8 mmol/l, but was significantly increased at concentrations of 33, 65 and 130 mmol/l (Table 2). One-way ANOVA showed the increase to be statistically highly significant (\( F_{6,5} = 29.8, P < 0.001 \)).

PAI-1 activity was significantly increased by the intake of alcohol (Fig. 1). An increase was observed irrespective of whether the alcohol was ingested with \( (F_{3,11} = 28.0, P < 0.001) \) or without \( (F_{3,10} = 10.7, P < 0.001) \) a meal. We also observed a slight increase during the control sessions. However, the time course was different. During the alcohol sessions PAI-1 increased to high levels relatively rapidly after drinking, whereas during the control sessions it increased modestly and slowly. The difference between the alcohol + meal and the water + meal sessions was statistically significant (two-way ANOVA, \( F_{4,11} = 19.7, P < 0.002 \)).

Serum triglycerides increased upon the ingestion of alcohol (Fig. 2). The effect peaked at 8 h after the baseline (wine without meal: \( F_{3,10} = 22.2, P < 0.001 \); wine with meal: \( F_{3,11} = 22.2, P < 0.001 \)). Eating a meal without alcohol also caused a
slight increase in triglycerides (water with meal: $F_{1.10} = 12.9$, $P < 0.001$), but the effect was weaker and occurred earlier. Serum triglycerides were not increased during the session without alcohol and meal. Two-way ANOVA showed a significant difference between the dinner sessions ($F_{1.10} = 9.4$, $P = 0.012$), but not between the fasting sessions ($F_{1.10} = 2.5$, $P = 0.14$). PAI-1 values correlated with those of serum triglycerides (slope 3.56, 95% CI 1.69–5.44, $t = 3.75$, $P < 0.001$, $r^2 = 0.07$).

Neither the drinking of alcohol nor consumption of an evening meal influenced plasma vWF:Ag or fibrinogen (Figs 3 and 4 respectively). By contrast, an evening meal increased the number of vWF multimers, so that multimers of highest size were detected, but the effect was inhibited by simultaneous drinking of alcohol (Fig. 3). Two-way ANOVA revealed a significant difference between the meal with alcohol and the meal without alcohol sessions ($F_{1.11} = 43.0$, $P < 0.001$). SIPA showed no statistically significant correlations with vWF multimers, triglycerides or fibrinogen (data not shown).

The urinary excretions of 11-dehydro-TXB$_2$, 2,3-dinor-TXB$_2$ and 2,3-dinor-PGF$_{1a}$ were similar during the day and the night. We did not observe any significant differences between the alcohol and water sessions with or without food (Table 3).

**DISCUSSION**

We found that the ingestion of a moderate dose of alcohol significantly decreased SIPA (peak blood ethanol 18.1 mmoll/l). In contrast, pure ethanol added to a test tube increased SIPA at concentrations ≥33 mmoll/l, whereas small concentrations showed no significant effects. The effect of alcohol on SIPA has not been reported before. In the present study, the intake of a meal together with mineral water also reduced SIPA, which indicates that the effect was not specific for alcohol. However, the effect of alcohol on SIPA was more pronounced than that of the postprandial state. The effect could be speculated to be due to postprandial hyperlipaemia (Nimpf et al., 1989; Tholstrup et al., 1996), but we did not observe any significant correlation between SIPA and serum triglycerides.

Previous studies *in vitro* have shown that platelet aggregation stimulated by various agonists is usually inhibited after the addition of ethanol (Rubin and Rand, 1994; Renaud and Ruf, 1996). Our finding of an ethanol-induced increase in SIPA *in vitro* contrasts with previous findings. Because the SIPA level depends on the platelet membrane glycoprotein receptors (Ruggeri, 1997), we speculate that ethanol may influence these receptors or vWF multimers. A change in the
The affinity of the receptors mainly to vWF could be a consequence of the observed increase of SIPA in our in vitro experiment. However, the duration of such an effect remains unclear. A transient effect followed by a rebound could explain the apparent contrast between our observations in vitro and ex vivo.

SIPA is an attractive method for measuring platelet aggregation (Fukuyama et al., 1989; Ikeda et al., 1991), because it probably reflects the situation encountered by platelets in stenosed arteries or arterioles undergoing vasoconstriction more closely than the more conventional techniques.
It may also clarify the impacts of alcohol, because arterial blood flow is increased and even vasospasm may be provoked during alcoholic intoxication (Altura et al., 1995). Severe alcoholic intoxication could lead to increased shear stress and platelet activation in stenosed arteries, where thrombus formation is often triggered by plaque rupture. We applied a high shear force, because this is believed to correspond to conditions of pathophysiological relevance (Ikeda et al., 1991).

We have previously demonstrated that acute intake of a large dose of alcohol significantly increases the urinary excretion of 2,3-dinor-TXB₂ (Numminen et al., 2000). In the present study, the ingestion of neither alcohol nor an evening meal influenced the excretion of thromboxane metabolites into urine. The intake of a moderate dose of alcohol was not accompanied by any significant increase or decrease in the excretion of 11-dehydro-TXB₂ and 2,3-dinor-TXB₂. Accordingly, no platelet activation occurred in vivo.

SIPA may not require thromboxane A₂ synthesis, because SIPA is not inhibited by cyclooxygenase inhibitors, such as aspirin and indomethacin, or the thromboxane A₂ synthetase inhibitor CV-4151 (Uchiyama et al., 1993). Accordingly, SIPA could be inhibited by the drinking of alcohol without any inhibition of thromboxane synthesis, as previously reported for ticlopidine (Uchiyama et al., 1989).

The ingestion of alcohol inhibited the postprandial increase of vWF multimers. It remains possible that this effect could contribute to the observed decrease in SIPA, but no significant correlation emerged between vWF multimers and SIPA. SIPA may behave independently of the changes in vWF multimers, although in some subtypes of ischaemic stroke the amount of larger vWF multimers seems to correlate positively with SIPA (Uchiyama et al., 1994).

The most prominent finding was the increase of PAI-1 activity, which suggests inhibition of fibrinolysis. The effect was observable throughout the period for which alcohol was present in the blood, but disappeared directly after alcohol had been eliminated from the body. Eating a meal did not significantly influence PAI-1 activity, although triglycerides are included among the many regulators of PAI-1 (Salomaa et al., 1993). Acute alcohol intake has also been shown to increase tissue plasminogen activator antigen and, simultaneously, to decrease tissue type plasminogen activator activity (Veenstra et al., 1990; Hendriks et al., 1994), the effects being similar irrespective of whether wine, beer or spirits are ingested (Hendriks et al., 1994). On the whole, acute drinking of alcohol seems to decrease fibrinolytic activity in subjects who are not daily consumers of alcohol, whereas in regular and habitual drinkers it may lead to an increase (Ridker et al., 1994a,b; Urano et al., 1993).

Do our observations support the hypothesis of a beneficial effect of alcohol consumption on thrombogenesis? High plasma levels of PAI-1 (Dawson and Henney, 1992), PA antigen (Ridker et al., 1994a) and vWF (Koster et al., 1995) are risk factors for thrombotic diseases. Elevated SIPA seems to be an indicator of risk for ischaemic stroke of atherothrombotic origin (Uchiyama et al., 1994; Konstantopoulos et al., 1995). Increased excretion of 11-dehydro-TXB₂ into urine is frequently encountered after the onset of symptoms of acute ischaemic stroke, suggesting platelet activation (van Kooten et al., 1994).

We did not observe any increase in the urinary excretion of thromboxane metabolites after acute ingestion of a moderate dose of alcohol. Nor did we observe elevations in SIPA or elevated vWF:Ag and vWF multimers, but we did observe elevated PAI-1 activity. It is quite clear that, in our healthy volunteers, thrombogenesis was not activated by the drinking of alcohol, but it remains unresolved whether the transient elevation of PAI-1 could promote venous thrombosis in individuals prone to develop thrombosis. However, the initial decrease of fibrinolytic activity may be rapidly followed by a rebound effect (Hendriks et al., 1994). The transient postprandial and post-alcohol decreases in SIPA suggest a beneficial effect.

We conclude that acute ingestion of a moderate dose of red wine with or without a standard dinner causes a decrease in SIPA. Both the alcohol and the dinner transiently decrease SIPA. The decrease of SIPA by alcohol was specific to neither ethanol nor red wine. Further studies are needed to demonstrate whether a decrease in SIPA will be seen during habitual and heavy drinking of alcohol, and whether soluble tannins or some other non-alcoholic constituents of wine could influence SIPA.

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REFERENCES


*Clinica Chimica Acta* **246**, 77–89.


*Atherosclerosis* **103**, 1–11.


*Thrombosis and Haemostasis* **63**, 345–348.