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**ACUTE EFFECTS OF RED WINE ON CYTOCHROME P450 EICOSANOIDS AND
BLOOD PRESSURE IN MEN**

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ABSTRACT

Objective: The vasodilation accompanying acute alcohol ingestion is hard to reconcile with the strong evidence linking chronic alcohol consumption with hypertension. Cytochrome P450 eicosanoids derived from arachidonic acid include vasodilator epoxyeicosatrienoic acids (EETs) and the vasoconstrictor 20-hydroxyeicosatrienoic acid (20-HETE). This study aimed to examine the relationship between CYP450 eicosanoids and blood pressure, and compared the effect of single session of drinking red wine (RW) with de-alcoholised red wine (DRW) or water (W) over 24 hours.

Methods: 25 normotensive men were randomly assigned to drink either 375ml of RW (41g of alcohol) or the equivalent volume of DRW or W, with a light meal on 3 separate days. Ambulatory BP and heart rate were measured over 24hr. Blood samples were obtained before and 2, 4 and 24hr after beverage consumption.

Results: BP fell in the first 4hr after RW ($p=0.001$) but was significantly higher after 20hrs ($p=0.037$). Plasma 20-HETE fell in the 2hrs after consumption of all beverages but over the 24hr period was relatively higher after RW ($p=0.025$). The largest difference in 20-HETE was 2hr after consuming RW and coincided with the highest blood alcohol level. There were no significant effects of RW on plasma EETs.

Conclusion: Acute consumption of alcohol as RW results in a relative increase in plasma levels of the vasoconstrictor 20-HETE over 24hr without affecting EETs and may contribute to the BP elevation that associates with a binge drinking pattern or be a homeostatic response to the acute fall in BP induced by alcohol.

KEY WORDS: red wine, de-alcoholized red wine, epoxyeicosatrienoic acids, 20-hydroxyeicosatrienoic acid, F₂-isoprostanes, endothelin-1, binge drinking pattern

BACKGROUND

Chronic moderate to high alcohol consumption is recognised to be a major contributor to elevated blood pressure (1-4). However, the mechanism of this hypertensive action of chronic alcohol consumption has been difficult to reconcile with the equally well recognised acute vasodilator effect of consuming alcohol. The role of cytochrome P450 (CYP450) enzymes in the metabolism of alcohol is well established. Isoenzymes including CYP2E1, 1A2, and 3A4 are known to contribute to ethanol metabolism (5). There is accumulating evidence that the cytochrome P450 metabolites of arachidonic acid play critical roles in the regulation of vascular, renal cardiac and pulmonary function in animal models and humans. The main product of the CYP450 ω -hydroxylase enzyme is 20-hydroxy eicosatrienoic acid (20-HETE). 20-HETE is a vasoconstrictor but also has natriuretic effects inhibiting sodium re-absorption in the proximal and distal tubules of the kidney. . 20-HETE is an important mediator of cerebral blood flow and in models of ischaemic stroke (6) and subarachnoid haemorrhage (7), its inhibition protects against brain damage. 20-HETE is a second messenger mediating vasoconstriction of agents such as angiotensin II (8) , endothelin-1 (9) and noradrenalin (10). The epoxygenase products of arachidonic acid are known as epoxyeicosatrienoic acids (EETs). The 4 regioisomers, 5,6-EET, 8,9-EET, 11,12-EET and 13,14 EET, all have vasodilator actions as well as inhibiting sodium re-absorption in the proximal tubule of the kidney (11). The vasodilator action of the EETs is dependent on their rate of metabolism by the enzyme soluble epoxide hydrolase to their dihydroxyeicosatetraenoic acid metabolites (DHETs) that are thought to be less vasoactive (11, 12) (Figure 1).

There are relatively few studies measuring CYP450 eicosanoids in humans because sensitive gas chromatography mass spectrometry is required for their measurement. However, we have shown that plasma and urinary 20-HETE associate with increased oxidative stress (13,

14), which may also contribute to development of hypertension. In a randomised controlled trial, we also showed that that urinary 20-HETE excretion was elevated after 4 weeks of increased alcohol consumption (15). This increase in 20-HETE was associated with an increase in plasma F₂-isoprostanes, an in vivo marker of oxidative stress (15). In another study, we showed that increased alcohol consumption for 4 weeks resulted in higher urinary endothelin-1 levels suggesting that endothelin-1 may in part contribute to the effects of alcohol on blood pressure (16). Although acute alcohol consumption has a vasodilator effect studies have shown that consumption of large quantities of alcohol in a single session ‘binge drinking’ associates with higher blood pressure (17) and an increased risk of stroke (18). Understanding the mechanisms that underly this association is of great importance given world-wide the prevalence of binge drinking particularly amongst healthy adolescents (19). We therefore sought to examine the involvement of CYP450 eicosanoids in the acute responses to a single session of alcohol consumption designed to mirror the effects of a binge pattern of drinking.

We hypothesised that altered CYP450 metabolites would contribute to the blood pressure changes observed after acute consumption. This study aimed to examine the acute effect of alcohol consumption on EETs and 20-HETE and oxidative stress (F₂-isoprostanes) in a randomised controlled trial in men.

METHODS

Recruitment and study design.

The study protocol was passed by the University of Western Australia Human Research Ethics Committee. The trial was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR), ACTRN12608000467336. Healthy male drinkers aged 20–65 years were sought by advertisement. Volunteers were required to be drinking between 40-110g alcohol/day. Women were excluded from study because the protocol required that a relatively high dose of

alcohol be consumed in a single session. Other exclusion criteria were smoking within the last 6 months, body mass index (BMI) $> 30 \text{ kg/m}^2$, chronic liver disease, cardiovascular disease, diabetes mellitus, blood pressure $>160/90 \text{ mmHg}$ or treatment with anti-hypertensive agents, total cholesterol $> 7.5 \text{ mmol/L}$ or use of lipid lowering agents, aspirin or NSAID's. At a screening visit blood pressure was measured using a Dinamap and an ECG recording obtained. Blood was collected for measurement of glucose, lipids and liver function. A medical examination by a physician (IBP) determined each volunteer's suitability for the study.

Twenty five men were recruited for the study. We estimated that studying 20-25 men would give us at least 80% power to detect a 20% difference in plasma 20-HETE and a 5 mmHg difference in systolic blood pressure at a significance level of $p < 0.05$. The men were asked to maintain their usual drinking, dietary and exercise habits during the study but to abstain from drinking alcohol for 48hr prior to each study visit and from taking any dietary supplements for 2 weeks prior to and during the study. They attended the study unit in the morning in a fasted state and were studied over 6 weeks on three separate days each two weeks apart. They were randomized using permuted block randomization by a statistician who was not involved with the study to drink a different beverage each day. On the study days volunteers drank either 375ml of red wine (RW) (41g of alcohol) or the equivalent volume of de-alcoholised red wine (DRW) or water (W), with a light meal that consisted of a bagel with crème cheese. Each beverage was consumed at room temperature over a thirty minute period. An ambulatory blood pressure monitor (Spacelabs 90207, Spacelabs Healthcare, Issaquah, WA, USA Spacelabs) was fitted by a trained researcher prior to consuming the beverage. Blood pressure and heart rate were monitored over 24h at 20 minute intervals while awake and at 30 minute intervals while asleep. Blood samples were obtained before and 2, 4 and 24hr after beverage consumption for measurement of plasma 20-HETE, EETs, endothelin-1 and F_2 -isoprostanes. Blood alcohol was monitored prior to drinking the beverage and 2, 4 and 24 hours after ingestion of the beverage.

Volunteers were transported home by taxi, 4-6 hours after consuming the beverage. They were instructed not to consume any other alcohol during the evening and to eat their normal evening meal and breakfast before their 24h visit to the research unit. The red wine and dealcoholized red wine were supplied by Orlando Wines, South Australia and had an alcohol content of ~14% and 0.6%, respectively.

Measurement of Plasma 20-HETE

Plasma free 20-HETE was measured in 125 μ l of plasma. After the addition of 1ng of d₆-20-HETE internal standard the plasma was acidified with 0.1M sodium acetate buffer pH 4.6 and applied under vacuum to Bond-Elut Certify II columns (200mg) (Varian, Australia), that were pre-washed with methanol and 0.1M sodium acetate pH 7.0 with 5% MeOH. The column was then washed with methanol/water (50:50) and dried under vacuum (6mmHg for 2 mins) prior to elution of 20-HETE with hexane/ethyl acetate/acetic acid (75:25:1). The sample was dried and derivatised as previously described for urine (20).

Measurement of Plasma EETs and DHETs

The four epoxyeicosatrienoic acid regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET) are not chromatographically resolved using gas chromatography mass spectrometry (GCMS). Therefore we measured plasma total EETs and DHETs in 0.5ml plasma. After addition of 5ng each of d₁₁14,15-EET and d₁₁14,15-DHET internal standard (Cayman Chemical Co.), the EETs and DHETs were hydrolysed under nitrogen with 1M KOH in methanol at 40°C for 30 min. Sodium Acetate 0.1M, pH 4.6 was added and the pH was adjusted to 4.6 with 1M HCl. The sample was applied to Bond-Elut Certify II columns (200mg) (Varian, Australia), pre-washed with methanol and 0.1M sodium acetate pH 7.0 with 5% MeOH. The column was then washed with methanol/water (50:50) and dried under vacuum (6mmHg for 2 mins) prior to elution of EETs and DHETs with hexane/ethyl acetate/acetic acid (75:25:1). The sample was dried under

vacuum and reconstitute in 50µl of MeOH prior to HPLC as previously described (20). Fractions corresponding to EETs and DHETs were collected dried under vacuum. EETs were derivatised with 40µl 10% PFBBr and 20µl 10% DIPEA, for 30 minutes at room temperature. The sample was dried under N₂ and analysed by GCMS monitoring ions *m/z* 319 and 330, as previously described (21). DHETs were derivatised with 40µl 10% PFBBr and 20µl 10% DIPEA, dried and derivatised with 20µl of BSTFA and 10µl Pyridine at 45°C for 20 minutes. The DHETs were dried under N₂ and analysed by GCMS monitoring ions *m/z* 481 and 492 as previously described (21).

Measurement of F₂-Isoprostanes and endothelin-1

Plasma F₂-Isoprostanes were measured by GCMS using methods previously described (14). Plasma endothelin1 was measured by enzyme immune assay (Assay Designs, Ann Arbor, MI).

Biochemistry

Blood alcohol levels were measured in the Pathwest laboratories at Royal Perth Hospital.

STATISTICAL ANALYSIS

The normality of the dependent variables was tested using the Shapiro-Wilk test (22). Plasma 20-HETE, EET, DHET and plasma F₂-isoprostanes were all found to follow a lognormal distribution and analysis proceeded using the natural logarithm for these variables. SBP, DBP and heart rate showed only minor departures from normally and these variables were not transformed.

Changes in SBP, DBP and heart rate; plasma cytochrome P450 arachidonic acid metabolites, F₂-isoprostanes and endothelin 1 levels after consumption of the three beverages were compared over time using linear mixed models with restricted maximum likelihood estimation (23). The intervention group was treated as a fixed effect and time was treated as a random

effect. This approach to the analysis was chosen because of its flexibility with respect to variable distributions and power. The linearity of continuous independent variables was examined using reduced cubic splines (24, 25). Models incorporating the baseline value for the dependent variable were found to be most powerful and for these baseline-corrected models time zero was omitted from the analysis. Random intercept models were used to estimate the arithmetic or geometric mean for the dependent variables over time as appropriate. A covariate was entered into each of the regression analyses to test for the effects of the randomisation order on each of the outcome variables.

A corrected *p* value of less than 0.05 was taken to indicate a statistically significant result.

Analysis used the Stata package (Version 11.2, StatCorp LP, College Station, Tx)

The characteristics of the men and plasma alcohol levels are reported as mean \pm SD.

RESULTS

Twenty four men completed the study; one withdrew because of work commitments. They were middle aged, slightly overweight but otherwise healthy with blood pressure, lipids and glucose within the normal range for adults (Table 1).

Blood alcohol levels

Plasma ethanol levels were not detectable over the 24 hours after consuming water or DRW. After consumption of RW blood ethanol levels were $0.06 \pm 0.01\%$ at 2 hours, $0.03 \pm 0.01\%$ at 4 hours and undetectable at 24 hours.

Blood Pressure and Heart Rate

After drinking RW blood pressure fell over the 24 hour period compared with DRW and W (SBP- 2.1 ± 0.4 mmHg, $p=0.0001$ and DBP- 1.4 ± 0.3 mmHg, $p=0.0001$ (Figure 2). The overall fall in blood pressure after RW was mainly due to a significant fall in the first 4 hours SBP (-

4.7±0.6 mmHg, p=0.001) and DBP (-3.9±0.5 mmHg, p=0.001) (Figure 2). Heart Rate was significantly increased over the 24h after RW (+1.8±0.3beats/min, p=0.001) with the largest differences in the first 4hrs (+2.4±0.4 beats/min, p=0.001) (Figure 2). SBP and DBP followed the normal diurnal variation after consumption of the 3 beverages but in the last 4 hours of the 24 hr period SBP in was significantly elevated after RW (+1.8±0.8 mmHg, p=0.037) compared with DAW or W.

Plasma 20-HETE

Plasma 20-HETE was similar between the groups at baseline, fell in all groups 2 hrs after consuming each beverage and returned to baseline levels by 24h (Figure 3a). However, there was a marked attenuation of the fall in plasma 20-HETE in the first 4 hours after consuming RW (p=0.001) that resulted in significantly (p=0.025) higher levels of 20-HETE over the 24h period compared with DRW.

Plasma EETS and DHETs

Plasma EETs were not significantly affected by consumption of RW over the 24 hours of study and in contrast to plasma 20-HETE were not different 2 hours after consumption of RW (Figure 3b). Plasma DHETs were also not significantly affected by consumption of red wine with levels not different at any of the time points (Figure 2c).

Plasma F₂-Isoprostanes and Endothelin-1

Plasma F₂-Isoprostanes and plasma endothelin-1 were not significantly affected by consumption of RW over the 24 hours of the study (Figure 4).

There were no significant effects of treatment order on any of the above outcome variables.

DISCUSSION

To our knowledge this is the first study to examine the effects of consuming ~4 glasses of red wine in a single session of on cytochrome P450 eicosanoids and blood pressure over 24hrs in healthy men. This is an important study because it investigates a possible mechanism connecting a binge pattern of drinking with subsequent elevations of blood pressure. The study is broadly applicable with many countries reporting considerable percentage of adults consuming a large quantity of alcohol in a single session (26). Our study was confined to healthy normotensive men who were moderate to heavy drinkers so that effects of a similar quantity of alcohol on blood pressure and cytochrome P450 eicosanoids could be more profound if consumed in hypertensive volunteers or women who are less tolerant to alcohol.

The present study demonstrated that the fall in plasma 20-HETE observed when drinking 375 ml of dealcoholized red wine or water was attenuated in the 24 hour period after acute consumption of the same volume of red wine. The effect of red wine on plasma 20-HETE accompanied a significant reduction in systolic and diastolic blood pressure and an increased heart rate over the same period. Within the 24 hour period after consumption of red wine, biphasic responses were observed for both blood pressure and plasma 20-HETE. The largest fall in blood pressure was observed between 2 and 4 hours after which systolic and diastolic BP followed a similar diurnal pattern to that seen with consumption of the other beverages. During the last 4 hours of the 24 hour period a significant increase in systolic BP was observed after red wine relative to the other beverages. Plasma 20-HETE which fell in the first 2 hours after consumption of all beverages returned to baseline levels at 24hr. After drinking red wine the fall in plasma 20-HETE was significantly attenuated with the most notable difference at 2 hours when the blood alcohol levels were highest.

The fall in blood pressure after acute alcohol consumption is well described (27-30) and is

accompanied by an increase in heart rate and reduced heart rate variability (31). We had hypothesised that the fall in blood pressure after acute alcohol consumption would be accompanied by reduced levels of the vasoconstrictor 20-HETE or increased levels of vasodilator EETs. The lack of effect of red wine on the epoxygenase products EETs and their metabolites DHETs over the 24 hours suggests they are unlikely to play a major role in acute vascular responses to red wine. The attenuation of the fall in 20-HETE after drinking red wine occurred at a time when blood alcohol levels were highest, and may be an important mediator preceding the subsequent increase in BP observed after 20hrs, or alternatively a homeostatic response to restore blood pressure. Further studies are required to investigate this relationship. We have previously shown that moderate chronic alcohol consumption associates with increased levels of urinary endothelin-1 and markers of oxidative stress (F₂-isoprostanes) and both have been associated with increased synthesis of 20-HETE. Although stimulation of either of these pathways could have contributed to the attenuated 20-HETE responses after red wine, we found no evidence to support this.

There is a reciprocal relationship between nitric oxide and 20-HETE such that nitric oxide donors have been shown to inhibit the formation of 20-HETE in renal and cerebral arterioles (32-35). Although we did not measure nitric oxide in our study, nitric oxide metabolites have been reported to increase in the first two hours after eating (36) and the fall we observed in 20-HETE in all groups over the first 2 hours may be associated with nitric oxide release after eating. Interestingly the same authors reported that the increase in nitric oxide metabolites was attenuated when food was consumed with alcohol (36), raising the possibility that a similar interaction between nitric oxide and 20-HETE after eating and drinking red wine in our study. The attenuation of the fall in 20-HETE after red wine could also result from stimulation of the

sympathetic nervous system and an increase in noradrenalin, that has been reported to occur within the first two hours of consuming alcohol (37-40). In this regard, a centrally mediated increase sympathetic nerve activity and mean arterial blood pressure has been demonstrated up to 2 hours after intravenous infusion of alcohol (39) raising the possibility that the increase in plasma 20-HETE may be due to CNS stimulation of the sympathetic system. Another mechanism that might account for the attenuation of the fall in 20-HETE relates to its catabolism. 20-HETE is a substrate for alcohol dehydrogenase-4 (ADH4) which converts it to 20-COOH-arachidonic acid. It has been suggested that as ethanol is a good substrate for ADH4 and it is possible that ADH4 could act to decrease catabolism of 20-HETE leading to higher 20-HETE levels that may affect vascular function (41). Other possible mechanisms that may have contributed to the attenuation in plasma 20-HETE after red wine are altered electrolyte balance that has been reported after acute alcohol consumption

The de-alcoholised red wine was not significantly different to water with respect to effects on systolic or diastolic blood pressure, heart rate or plasma 20-HETE, EETs or DHET suggesting that the effects of red wine on blood pressure, heart rate and 20-HETE were due to the alcohol content rather than the polyphenols contained in red wine. This finding agrees with previous studies that showed no effect of consumption of a similar quantity of de-alcoholized red wine polyphenols for 4 weeks on blood pressure and heart rate (42, 43), but not with the study of Chiva-Blanch et al. who suggested de-alcoholized red wine reduced blood pressure and increased nitric oxide levels (44). Differences in the findings of these studies may be due to the patient populations, the type of blood pressure monitoring and the statistical analysis of the data.

The increased systolic blood pressure observed after 20hrs after drinking red wine is consistent

with reports of a biphasic effect of alcohol on blood pressure using ambulatory blood pressure monitoring (28). This effect occurs within the timeframe of blood pressure elevation after a binge drinking session and is supported by studies showing that withdrawal of alcohol after weekend drinking associates with a higher blood pressure on Mondays compared with alcohol consumption spread more evenly across the week (45, 46). These findings are important as binge drinking associates with an increased risk for stroke even after adjusting for alcohol consumption (18). Chronic alcohol consumption has been associated with elevated urinary 20-HETE, F₂-isoprostanes and endothelin-1 (15, 16). Only 20-HETE was elevated over the whole 24hr period after drinking red wine suggesting that a combination of different mechanisms may be responsible for the relative increase in 20-HETE during acute and chronic intake of alcohol.

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Figure 1. Enzymatic pathways of arachidonic acid metabolism. AA is metabolised by cyclooxygenase (COX) to prostaglandins, (e.g. prostacyclin and thromboxane); by lipoxygenase to leukotrienes and stereoselective hydroxyeicosatetraenoic acids (HETEs). Metabolism of AA by cytochrome P450 (CYP450) ω -hydroxylase yields C-terminal HETEs (e.g. 20-HETE) and epoxygenases produce 4 possible epoxides vasodilator EETs (5,6-EET; 8,9-EET; 11,12-EET & 14,15-EET) that are metabolised to the less active dihydroxyeicosatrienoic acids (DHETs) by the enzyme soluble epoxide hydrolase (sEH)

Figure 2. Ambulatory systolic blood pressure (SBP) (a) diastolic blood pressure (DBP) (b) and heart rate (c) over 24 hours after consumption of 375 ml of water (open squares), de-alcoholised red wine (open triangles) or red wine (closed circles). Red wine significantly reduced SBP and DBP $p=0.0001$ and increased heart rate $p=0.0001$ over the 24 hour period compared with de-alcoholized red wine or water. SBP was significantly increased from 20-24 hours after red wine $p=0.037$.

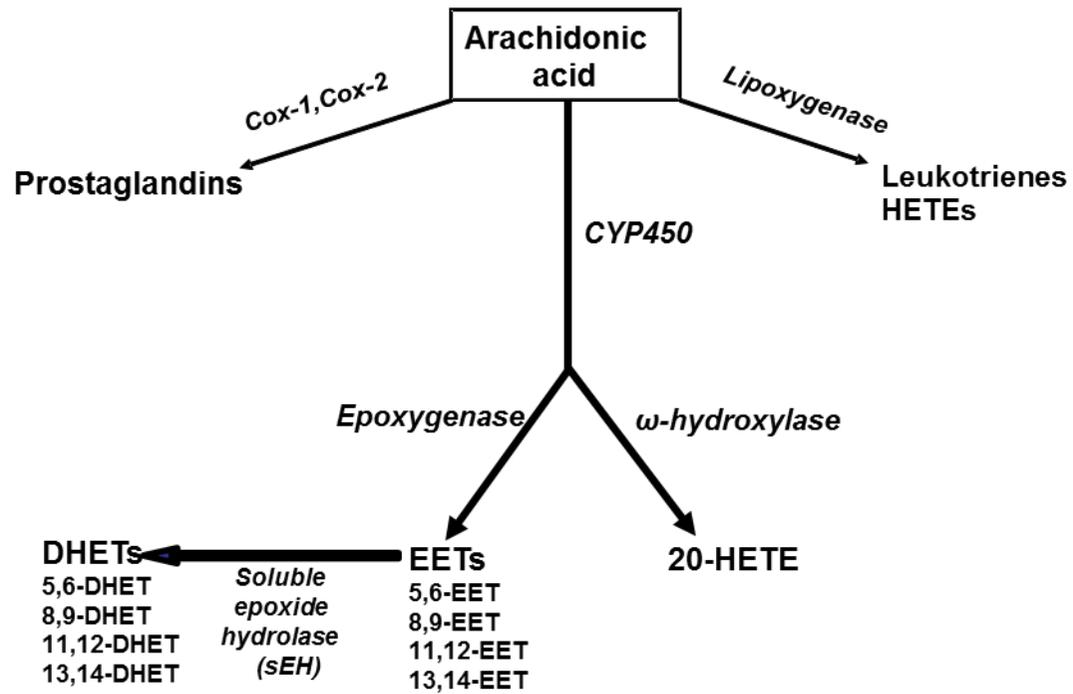
Figure 3. Plasma 20-HETE (a), EETs (b) and DHETs (c) at 0 hours and at 2, 4 and 24hr after consuming 375 ml of water (open squares), de-alcoholised red wine (open triangles) or red wine (closed circles). Plasma 20-HETE was significantly higher after red wine compared with de-alcoholized red wine or water over 24hr and 4hr, $p=0.025$ and $P=0.0001$, respectively.

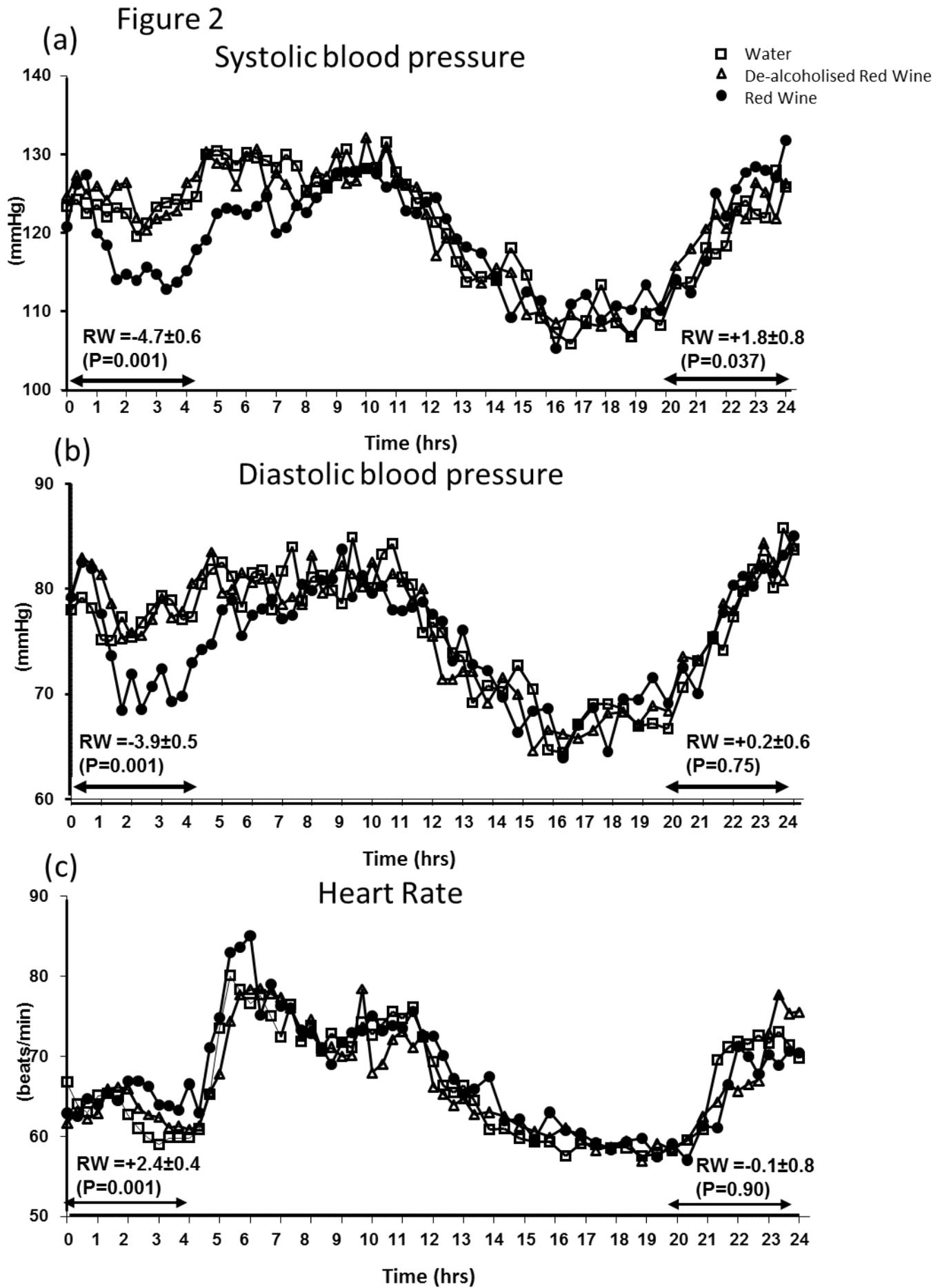
Figure 4. Plasma F₂-isoprostanes (a), endothelin-1 (b) at 0 hours and at 2, 4 and 24hr after consuming 375 ml of water (open squares), de-alcoholised red wine (open triangles) or red wine (closed circles). No significant differences were observed between beverages across time.

TABLE 1.**Characteristics of the men studied**

Characteristic	Mean \pm SD
Age (yrs)	56.0 \pm 5.6
Average Alcohol intake (g/day)	57 \pm 14
BMI (Kg/m ²)	26.7 \pm 2.5
SBP (mmHg)	115 \pm 11
DBP (mmHg)	72 \pm 6
Cholesterol (mmol/L)	5.4 \pm 1.0
Triglycerides (mmol/L)	1.18 \pm 0.79
LDL-cholesterol (mmol/L)	3.46 \pm 0.80
HDL-cholesterol (mmol/L)	1.36 \pm 0.26
Glucose (mmol/L)	4.8 \pm 0.6
Creatinine (μ mol/L)	84 \pm 10
Gamma-glutamyl transferase γ GT (U/L)	32 \pm 20
Alanine transaminase (U/L)	31 \pm 13
Aspartate transaminase (U/L)	29 \pm 7

Figure 1





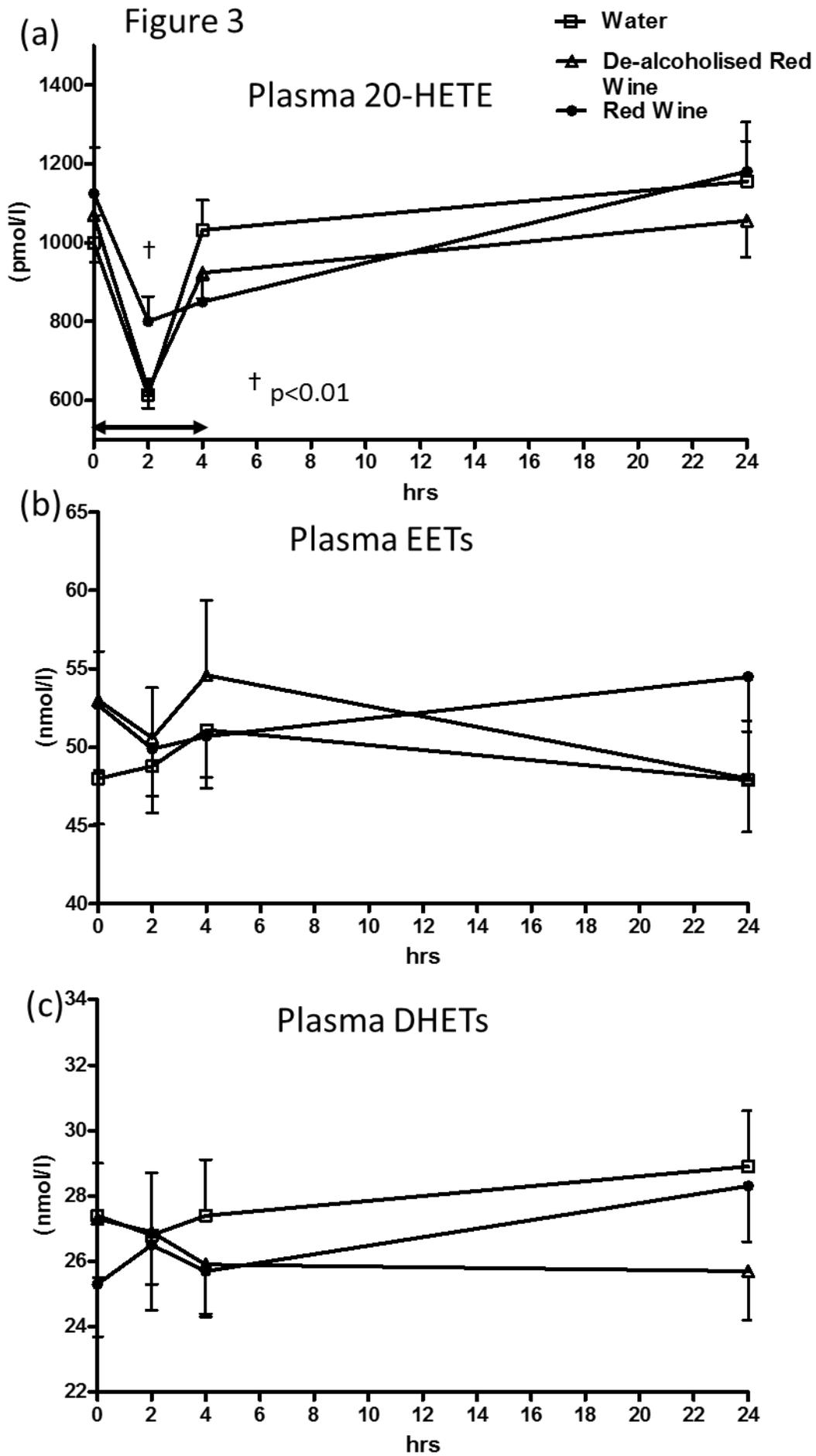


Figure 4

