Moderate Daily Intake of Red Wine Inhibits Mural Thrombosis and Monocyte Tissue Factor Expression in an Experimental Porcine Model

L. Casani, MS; E. Segales, MS; G. Vilahur, MS; A. Bayes de Luna, MD, PhD, FESC; L. Badimon, PhD, FESC

Background—Moderate consumption of red wine has been epidemiologically associated with a reduction in cardiovascular disease, but its mechanism of action is not fully understood. The objective was to study whether the protective effects of a daily intake of red wine (Tempranillo, 12.8% alcohol vol/vol) could be related to inhibition of thrombosis in an experimental model of diet-induced hyperlipemia.

Methods and Results—For 100 days, animals were fed a western-type proatherogenic diet containing 2% cholesterol and 20% saturated fat. Three doses of red wine were studied (20, 30, and 40 g wine-ethanol/d) and compared with placebo-control animals not taking any wine. Thrombosis under flow conditions was evaluated by radioisotopic quantification of deposited platelets on damaged arteries. Changes in RhoA translocation in platelets and monocyte tissue factor expression were also analyzed. Mural platelet deposition was significantly reduced in animals ingesting red wine with their food. Expression of RhoA in the platelet cytoplasm (inactive form) was increased in wine-fed animals. Tissue factor mRNA expression in lipopolysaccharide-stimulated monocytes was reduced in wine-fed animals. Total cholesterol levels were not significantly different among groups.

Conclusions—Moderate red wine intake significantly reduces platelet deposition triggered by damaged vessel wall, partially explained by inhibition of RhoA translocation to the platelet membrane. Hence, a daily moderate intake of wine seems to inhibit different pathways that converge in a reduced thrombotic risk on vessel wall injury. (Circulation. 2004;110:460-465.)

Key Words: wine ■ thrombosis ■ cardiovascular disease ■ tissue factor

Coronary heart disease (CHD) is the leading cause of death in the adult population in developed countries. The cardiovascular benefits of red wine consumption have been reported by different epidemiological studies. Moderate alcohol drinkers have lower CHD mortality than nondrinkers or binge drinkers. It has also been described that the French population has lower cardiovascular mortality than expected despite its high ingestion of saturated fats, which has been called the French paradox. The mechanisms to produce these beneficial effects are not clearly understood. A decrease in LDL-particle oxidation, an increase of HDL cholesterol, a favorable change in plasma lipid profile, a reduction of in vitro platelet aggregation, or an anti-inflammatory action have been proposed as candidate mechanisms.

Thrombosis on atherosclerotic lesions is the major cause of clinical presentation of acute coronary syndromes, and although ex vivo platelet aggregation has been used as a surrogate marker and indicates platelet activation, it does not properly indicate thrombosis because it uses Ca²⁺-depleted blood. The effects of wine on mural thrombosis triggered by a thrombogenic vessel under characterized flow conditions have not yet been analyzed. Therefore, the objective of our study was to investigate whether red wine intake with a controlled hyperlipemic western-type diet could reduce thrombotic risk in a standardized flow model, using the Badimon perfusion chamber and mimicking the thrombotic risk elicited by acute plaque rupture and exposure of prothrombotic surfaces to platelets. We have hypothesized that red wine may exert a cardioprotective effect by inhibiting thrombosis and platelet activity and found that moderate intake of red wine induces significant inhibitory effects on mural thrombosis triggered by damaged vessel wall.

Methods

Animal Model and Experimental Design

A total of 22 male, white commercial fixed-breed pigs (final weight, 97.83±0.88 kg) were used in this study. Animals were fed a western-type atherosclerotic diet, rich in saturated fatty acids (20%...
beef tallow) and cholesterol (2% cholesterol), for 100 days. Animals were housed individually, and water was provided ad libitum.

In a preliminary study, a group of animals (group A) received a daily moderate dose of wine (30 g wine-ethanol/d) and were compared with a control group (group B). After completion of this first study, results led to a second experimental design assaying 2 different doses of wine. Animals were divided into 3 groups: group HD, receiving a moderate dose of wine equivalent to 40 g wine-ethanol/d; group LD, a low dose, 20 g wine-ethanol/d; and group HL, the hyperlipemic placebo control.

Red Tempranillo wine was provided by the Foundation for Wine Research (Table 1). Red wine was administered once a day with the daily chow (2.5% body weight). Animals were weighed individually weekly, and daily chow and wine-ethanol dose were calculated. The last wine dose was administered 24 hours before the thrombosis assay.

A third and final study was performed to analyze the thrombotic response of acute ingestion of red wine in normolipemic animals. Two pigs (60 kg body weight) were fed 1 single dose of red wine (40 g wine-ethanol) with chow intake just before the perfusion experiment, and another 2 pigs were fed only commercial chow.

All procedures followed in the study were in accordance with the American Heart Association guidelines for experimental research and were approved by the Ethical Committee of the local government.

Perfusion Chamber
At the end of the experimental period, thrombotic risk was assessed by exposure of blood from studied animals to a thrombus-triggering damaged artery in the previously validated and standardized Badimon perfusion chamber. After an overnight fasting, animals were tranquillized (8 mg/kg Stressnil, Esteve) and anesthetized (5 mg/kg pentobarbital, Braun Medical), and a carotid artery–jugular vein shunt was established to place the Badimon perfusion chamber. Blood was perfused through the chamber for 3 and 5 minutes and at 2 different shear rates of 212 s⁻¹ and 1690 s⁻¹ typical of patent or mildly stenotic coronary arteries. Homologous porcine vessel wall with 2 types of damage, severe (ruptured vessel wall) and mild (eroded vessel wall), was used as substrate. Perfusions were performed both with native blood and after heparinization (50 IU/kg).

Ex Vivo Platelet Aggregation
Platelet aggregation was performed as previously reported, using ADP and collagen as agonists. Blood was obtained in sodium citrate (0.16 mol/L), and extent of aggregation was determined in platelet-rich plasma with an optical aggregometer (Aggrecorder-II) and in whole blood with an impedance aggregometer (Chrono-Log).

Rho-A Expression
Platelets were obtained from blood gently dropped into plastic tubes containing acid citrate dextrose. Platelet number was adjusted to 4×10⁸ platelets and mixed with 100 µL of lysis buffer (10 mmol/L Tris/HCl, pH 7.5, 0.15 mol/L KCl, 2.9 mmol/L PMSF, 0.1 mmol/L DTT, 10 µmol/L leupeptin, 1350 UI aprotinin, 1% Triton), then sonicated for 10 seconds and centrifuged at 9000 g at 4°C for 15 minutes. Platelet subfractionation was performed as described. Equal amounts of platelet cytoplasm and membrane protein were subjected to SDS/PAGE and transferred onto nitrocellulose mem-

### Table 1. Analysis of Red Wine Composition According to Manufacturers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Alcohol degree</td>
<td>12°87</td>
</tr>
<tr>
<td>Density</td>
<td>994</td>
</tr>
<tr>
<td>Tannins (including catechines), g/L</td>
<td>3.36</td>
</tr>
<tr>
<td>Anthocyanins, ppm</td>
<td>448</td>
</tr>
<tr>
<td>Resveratrol, ppm</td>
<td>1.75</td>
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</table>

Figure 1. Total platelet deposition (PLT×10⁶/cm²) of nonheparinized blood over severely damaged vessel wall at 2 different shear rates, 212 s⁻¹ (n=9, group A; n=9, group B) and 1696 s⁻¹ (n=9 per group). Black bars, hyperlipemic control animals; hatched bars, wine-treated animals. *Significantly different (P<0.05).

Blood and Plasma Determinations
Blood was withdrawn by venipuncture from the ear marginal vein in fasting animals at baseline and at days 15, 30, 50, 75, and 100 of the study.

Blood cell counting (System-9000, Serono-Baker Diagnostics) and coagulation parameters (ST4 coagulometer, Diagnostica-Stago) were followed up. Blood alcohol concentration was determined by use of an alcohol dehydrogenase assay kit (332-UV) according to the manufacturer’s instructions (Sigma Chemical). Liver enzymes, total cholesterol, HDL cholesterol, and triglycerides were determined enzymatically (Kodak Ektachem DT, Eastman Kodak Co).

Figure 2. Perfusions with nonheparinized blood. Perfusions were run for 3 minutes over mildly and severely damaged vessel wall, at 212 s⁻¹ (n=16 per group) and 1696 s⁻¹ (n=16 per group). Black bars, hyperlipemic control animals; hatched bars, hyperlipemic with 40 g wine-ethanol/d animals; open bars, hyperlipemic with 20 g wine-ethanol/d animals. *Significantly different (P<0.05), HD group vs HL group, and †significantly different (P<0.05), LD group vs HL group.
LDL Oxidation
Lipoprotein oxidation capacity was analyzed in EDTA-collected blood. Samples were obtained at the end of the perfusion experiment and in the control study at baseline and 30, 50, and 120 minutes after red wine administration. LDLs were isolated by sequential ultracentrifugation, with oxidation induced by copper as described. Lipoprotein oxidation was also analyzed by thiobarbituric acid–reactive substances.

Tissue Factor Expression in Induced Monocytes
Blood collected in sodium citrate from normolipemic animals fed with and without 40 g wine-ethanol was used to obtain monocytes by an elutriation centrifuge. Elutriated monocytes were harvested on RPMI 1640 medium with Glutamax-1 (Gibco) overnight and then adhered on 100-mm plastic dishes (Falcon) and induced with lipopolysaccharide (LPS) (10 μg/10^6 cells). Four hours later, supernatants were aspirated and centrifuged; dishes were scraped with Tripure (Boehringer Mannheim Corp) and processed to obtain RNA by conventional techniques. The centrifuged supernatants (700 g, 5 minutes) containing nonadherent cells were also dissolved with Tripure.

Tissue factor (TF) mRNA and a control GAPDH mRNA were amplified in semiquantitative reverse transcription–PCR analysis. RNA (0.5 μg) was reverse-transcribed in a final 15-μL reaction mixture, performed at 42°C for 1 hour. cDNA (1 μL) was amplified in a total 25-μL reaction. The specific oligonucleotides selected for TF amplification were as follows: sense, 5′-AGAGTTTCACACCTTACCTTGGA-3′ and antisense, 5′-AGTTTTCCTCTTATACCAT-3′; for GAPDH, sense, 5′-GTCACCAGGGCTGTCTTTTAA 3′ and antisense, 5′-ACCGAAGGGCCATGCCCCAGTG-3′. Amplification of TF was performed by 35 cycles of 95°C for 2 minutes, 55°C for 40 seconds, and 72°C for 1 minute, followed by a final extension of 72°C for 10 minutes. A ratio of TF/GAPDH was used to normalize TF values. PCR products were resolved by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Data Analysis
Multiple group means were compared by single-factor or multiple-factor ANOVA, followed by Fisher’s protected least significant difference and Scheffé’s F test. Results are expressed as mean±SEM, and a value of P<0.05 was considered significant.

Results
Thrombotic Response and Ex Vivo Platelet Aggregation
The thrombotic response to severely damaged vessel wall was significantly decreased in the group ingesting 30 g wine-ethanol/d of red wine at both high and low shear rates (Figure 1).

Platelet deposition also showed a statistically significant reduction with 20 and 40 g wine-ethanol/d in native blood perfusions over mildly and severely damaged vessel wall and at both shear rate conditions (Figure 2). When heparinized blood was used, the inhibitory effects of red wine on platelet deposition were clearly significant in severely damaged substrates (P<0.05) at high and low shear rates in perfusions of 3 and 5 minutes (Figure 3B). In eroded vessel wall, because of the low levels of platelet deposition (platelet adhesion), the reduction was significant (P<0.05) only at a low shear rate (Figure 3A), whereas results did not reach significance at high shear rate.
Red wine intake did not produce any significant difference in blood and plasma determinations. Western blotting of RhoA protein. Platelets obtained at end of perfusion experiment were subfractioned in cytoplasm (c) and membrane (m). Blotting membranes were exposed for 1 minute to evidence RhoA protein expression in platelet membranes. Overexposure of these blotting membranes, only platelet cytoplasm of high-dose animals was expressed (picture not shown). 1. Hyperlipemic control animals; 2. hyperlipemic with 40 g wine-ethanol/d animals; 3, hyperlipemic with 20 g wine-ethanol/d animals.

Normolipemic animals fed a unique dose of red wine showed only a significant decrease (P<0.05) of platelet deposition in perfusions over severely damaged vessel wall at low shear rate (Figure 3D) compared with normolipemic animals not taking any wine.

Ingestion of these light to moderate doses of red wine did not cause any significant reduction in either whole blood or platelet-rich plasma aggregation ex vivo induced by collagen or ADP (data not shown).

Rho-A Protein Translocation
The expression of RhoA protein in the platelet membrane (active form) showed a reduction in groups ingesting wine (Figure 4). RhoA protein in platelet cytoplasm (inactive form) was increased in groups ingesting the high dose of red wine (40 g wine-ethanol/d) compared with the hyperlipemic placebo group (data not shown).

Blood and Plasma Determinations
Red wine intake did not produce any significant difference in biochemical parameters, blood cell counts, or platelet counts.

### TABLE 2. Plasma Lipid Composition

<table>
<thead>
<tr>
<th></th>
<th>Group HD</th>
<th>Group LD</th>
<th>Group HL</th>
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<tbody>
<tr>
<td>Total Cholesterol, mg/dL</td>
<td>515.07±52.27</td>
<td>407.43±36.56</td>
<td>523.03±45.50</td>
</tr>
<tr>
<td>HDL Cholesterol, mg/dL</td>
<td>69.79±5.63</td>
<td>59.57±4.99</td>
<td>69.11±5.11</td>
</tr>
<tr>
<td>Non-HDL Cholesterol, mg/dL</td>
<td>167.91±51.70</td>
<td>233.67±45.50</td>
<td>196.32±49.40</td>
</tr>
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<table>
<thead>
<tr>
<th></th>
<th>Group LD</th>
<th>Group HL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max CD, nmol/mg protein</td>
<td>258.9±11.3</td>
<td>285.23±39.1</td>
</tr>
<tr>
<td>Lag time, min</td>
<td>36.2±2.6</td>
<td>52.85±12.01†</td>
</tr>
<tr>
<td>Vmax CD, nmol/min/g protein</td>
<td>8.13±0.68</td>
<td>3.85±0.51†</td>
</tr>
<tr>
<td>TBARS, nmol MDA/mL</td>
<td>10.39±0.37</td>
<td>4.99±10*</td>
</tr>
<tr>
<td>LDL Oxidation</td>
<td></td>
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</table>

Liver enzymes were increased by the hypercholesterolemic diet (baseline AST, 32±2 U/L, and ALT, 41±3 U/L; hyperlipemic AST, 64±9 U/L, and ALT, 68±9 U/L, P<0.05). Interestingly, wine-fed hyperlipidemic animals did not show any impairment in liver enzymes (AST, 52±2 U/L [HD], and 58±9 U/L [LD]; ALT, 34.25±6 U/L [HD], and 65±13 U/L [LD]). No detectable levels of ethanol were found in blood as expected, because the last red wine ingestion was the day before the experimental procedure.

Plasma levels of total cholesterol and HDL cholesterol were significantly increased in the LD group (Table 2).

### LDL Oxidation

The LDL oxidation, measured as maximal conjugated dienes, was significantly lower (P<0.0015) in the LD group than in the HD group (Table 3). Lag time was not significantly increased by red wine ingestion. The maximal velocity of conjugated diene formation was significantly reduced for the LD group but was not affected in the HD group with respect to the control group. The thiobarbituric acid–reactive substances test confirmed the lower oxidative capacity of LDL from the HD group respect to the HD group (P<0.001). No differences in these parameters were found for normolipemic animals fed or not fed red wine.

### Tissue Factor Expression in Induced Monocytes

Quantification of DNA resulting from the reverse transcription–PCR for TF showed that isolated peripheral blood monocytes, differentiated by adhesion, from wine-fed animals have both a lower TF mRNA content and a lower capacity to induce TF mRNA expression in the presence of LPS (Figure 5).

#### Discussion

Our results demonstrate that chronic intake of red wine with meals induces a significant inhibitory effect of platelet deposition on damaged vessel wall and that this inhibitory effect is more evident with the high dose of wine than with the lower doses. One single dose of red wine in association with the meal in normolipemic animals showed a reduction in platelet deposition, although it did not reach significance in
all conditions tested. These results indicate that moderate intake of red wine could exert a beneficial antithrombotic effect.

Thrombotic risk in the control hyperlipemic animals increased with perfused lesion severity, perfusion time, and shear rate conditions, as described previously. Groups ingesting red wine showed the same platelet deposition pattern but with lower growth, suggesting that the reduction in platelet deposition will probably be the result of a passivation effect of red wine in platelets. There is evidence that flavonoids present in red wine inhibit cAMP and cGMP phosphodiesterases, increasing platelet levels of cAMP and cGMP. As a result, platelet cytosolic calcium ([Ca]i) levels would decrease, reducing the level of in vivo platelet activity. Activation of platelets implies different responses, including shape change, adhesion, aggregation, and secretion. Shape change of platelets could be regulated by both calcium-dependent and -independent pathways. We have found that red wine ingestion reduced the translocation of RhoA into membranes (active site), accompanied by an increase in the levels of cytoplasmic RhoA (inactive), corresponding to a reduction in platelet deposition.

Alcohol and polyphenols have been shown to inhibit in vitro platelet aggregation when added to platelets obtained from healthy volunteers and challenged with thrombin and ADP. However, the high concentrations required (≥130 μmol/L) are not reached by feasible moderate intake of wine. Alcohol intake has also been shown to affect platelet activity indirectly, through an HDL-mediated stimulation of the production of prostacyclin, and inhibit the production of thromboxane A2, perhaps by inhibiting platelet cyclooxygenase activity, or may be promoting an increase in platelet membrane fluidity. Nevertheless, a diet rich in saturated fat, like that ingested by our animals, reduces platelet surface area and membrane fluidity compared with platelets from normolipemic animals. This could be the reason why the ingestion of red wine cannot significantly reduce platelet aggregation in our study, results that are in concordance with those found by Rand et al. using thrombin as aggregating agent.

This study also shows for the first time that ingestion of red wine reduces TF expression in LPS-stimulated monocytes from normolipemic animals. Resveratrol, a wine phenolic component, showed inhibitory effects on TF expression in human endothelial cells and monocytes in in vitro studies using high doses of the isolated compound.

In the pathogenesis of atherosclerosis, modified LDL particles may become oxidized, then promoting foam-cell formation and enhancing the atherosclerotic lesion. Antioxidant therapies had been proposed to stop or delay this process. Phenolic compounds of the red wine had been shown to exert these activities in vitro and in vivo, but in vivo studies are performed with healthy volunteers ingesting about 400 mL of red wine for only 2 or 4 weeks. In our hyperlipemic experimental model, the group ingesting a low dose of wine (LD group) showed better changes in oxidative parameters than the groups ingesting the higher amount of wine. In vivo, the antioxidant protective effect of red wine can be overshadowed by the pro-oxidant effect of the ethanol present in wine, and in groups ingesting 30 and 40 g of alcohol, the antioxidant protective effect of polyphenols may be overshadowed by the pro-oxidant effect of ethanol. The balance between alcohol and polyphenols of a wine may be critical for its in vivo effect on LDL. The wine used in the study contains high amounts of resveratrol (76 μmol/L) and catechins (3.36 g/L), phenolic compounds with demonstrated cardioprotective effects. A moderate and regular con-
sumption of red wine could lead to a modification of cell function that is evidenced in the beneficial effect of red wine on the thrombotic response.

Considering that thrombosis is an integral part of atherosclerosis and coronary artery disease, it is possible that the decreased risk of CHD associated with moderate intake of red wine could be caused by a combination of vascular and anti-thrombotic effects. The ability of red wine to reduce thrombotic risk, the reduction in monocyte TF expression, the reduction in membrane Rho-A protein translocation, and the global antioxidant effect are major steps to confirm that a moderate intake of red wine can be included as a real cardioprotective factor. Results obtained in oxidative modification of LDL indicated that a low ingestion of red wine, preferably a wine rich in polyphenols, is better than a higher amount of wine, whereby the pro-oxidant effects of ethanol are overshadowing the beneficial effects. Interestingly, the platelet aggregation response to wine-ethanol has been shown to differ according to the type of diet.29,30 We have demonstrated that a moderate dose of red wine taken with a diet rich in saturated fats is able to produce beneficial antiatherothrombotic effects. Therefore, patients at risk, especially those with an exclusive western diet, will receive the most benefit from the moderate red wine intake.

Limitations of the Study

With the experimental design used in this study, we can only draw conclusions about the anti-thrombotic effects of the chronic intake of a moderate amount of red wine. We cannot identify whether alcohol or non-alcoholic components of red wine are the protective components or whether ingestion of white wine or hard alcoholic beverages (spirits) will produce effects similar to those of red wine.

Acknowledgments

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