Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation in vitro: identification and mechanism of action of some cinnamic acid derivatives from red wine

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1. An antioxidant effect of phenolic substances in red wine to reduce oxidizability of low-density lipoprotein has been proposed as the basis for a relatively lower incidence of coronary disease in populations with high red wine intake. We have now investigated the possible antioxidant effects of various beverages, including red wines, white wines, beers and red grape juices (diluted 1:500), on metal ion-dependent (copper) and -independent (aqueous peroxyl radicals) oxidation of isolated human low-density lipoprotein. We also tested the effects of these beverages on copper-initiated oxidation of lipoproteins in serum.

2. The higher the polyphenolic content of a beverage, the greater was its antioxidative effect measured as change in lag time in the different oxidation systems. Upon stripping the polyphenolics from the drinks, the lag times returned to control levels in isolated low-density lipoprotein; however, the low concentrations of phenolics remaining after stripping had a lesser but still significant effect on oxidation of lipoproteins in serum. The inhibitory effect of these phenolics appeared to be more pronounced for metal ion (copper)-induced oxidation than for those induced by aqueous peroxyl radicals, suggesting that both copper-binding and free radical-trapping activities may be involved. A mixture of the carboxylic acids representative of those present in red wine exhibited no significant effect on lag time of metal ion-dependent and -independent low-density lipoprotein oxidations. Ethanol, at concentrations of 0.1-0.5%, had no effect on either copper-induced or aqueous peroxyl radical oxidations.

3. Extracts of acid-hydrolysed red wine were separated by thin-layer chromatography and the most active antioxidant fractions identified. GC-MS and HPLC analysis of these fractions resulted in the identification of several cinnamic acid derivatives, such as coumaric acid, caffeic acid and protocatechuic acid. Dose-response studies using the pure compounds indicated that caffeic acid was the most active antioxidant with an IC₅₀ <1 μmol/l for copper-initiated low-density lipoprotein oxidation. Caffeic acid (1 μmol/l) significantly inhibited lipid hydroperoxide formation while sparing α-tocopherol consumption. Caffeic acid at the same concentration also inhibits aqueous peroxyl radical-induced oxidation of low-density lipoprotein, sparing α-tocopherol. There was no evidence of caffeic acid preventing the binding of copper to low-density lipoprotein.

4. We conclude that phenolics in both alcoholic and non-alcoholic beverages can give dose-dependent protection against oxidation of low-density lipoprotein. Caffeic acid and protocatechuic acid are two compounds likely to contribute to these effects. These findings may be relevant to the putative cardiovascular-protective effects of high phenolic content alcoholic beverages such as red wine; however, the widespread occurrence of antioxidants such as caffeic acid in fruits and vegetables suggests that these protective principles are not limited to red wine.

INTRODUCTION

A high dietary intake of saturated fat is usually associated with increased mortality rates from coronary heart disease. However, in some parts of France this is less apparent, and it has been suggested that this cardiovascular protection is provided, at least to some extent, by the consumption of red wine [1]. Although there are biologically plausible reasons for ascribing this protection to the

Key words: caffeic acid, grape juice, lipoprotein oxidation, phenolic compounds, wine.

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane)dihydrochloride; ANOVA, analysis of variance; FOX, ferrous oxidation-Xylenol Orange; LDL, low-density lipoprotein; TMS, trimethylsilyl.

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alcohol content of this wine [2], a study by Criqui and Ringel [3] suggests that wine has a stronger protective effect against coronary heart disease than either beer or spirits. More recent evidence implies that such an effect may be mediated by phenolics in wine, preventing oxidation of low-density lipoproteins (LDL) [4-6].

Oxidized LDL (oxLDL) is thought to play an important role in atherogenesis by virtue of a variety of biological effects [7]. OxLDL is taken up by macrophages at an enhanced rate, resulting in the formation of foam cells in the subendothelial space. Products secreted by lipid-laden macrophages (foam cells) may cause further recruitment and retention of macrophages, leading to the formation of fatty streaks. OxLDL has many other biological properties, such as cytotoxicity to endothelial cells, which suggest its involvement in the initiation of atherogenesis [8]. Although a number of cell types have been shown to oxidize LDL in vitro [9], the precise mechanism of how LDL is oxidized in vivo and the role of both endogenous and exogenous antioxidants in protecting it against oxidation are still not clearly understood [10].

Studies by Frankel et al. [11] in vitro have shown that diluted red wine (with the ethanol removed) inhibits copper-induced LDL oxidations as measured by hexanal formation. It has been suggested that the antioxidative effects are due to phenolics in the red wine, although it has not been determined whether dicarboxylic acids in the wine might act as metal ion chelators in this model of oxidation. To study these observations further, we investigated the effect of carboxylic acids at the concentrations found in red wine on LDL oxidations. Using a range of beverages, including wine, grape juice and beer, of varying phenolic content we wished to determine specifically whether antioxidant action could be totally accounted for by the phenolic constituents and by what mechanism this inhibition occurs. To do this, the effect of beverages with and without phenolics removed on both metal ion-dependent and -independent lipoprotein oxidation was studied. To examine the oxidation of lipoproteins in a more physiological environment, some oxidations were carried out in diluted serum. In addition, we identified some cinnamic acid derivatives in red wine which act as potent inhibitors of LDL oxidation and investigated their mode of action as antioxidants.

MATERIALS AND METHODS

Serum and LDL were obtained from healthy men and women (aged 25-45 years) who were non-smokers, low to moderate drinkers (<2 standard drinks/day) and taking no drugs or antioxidant supplements. Blood samples were collected after an overnight fast.

We tested the effects of two red grape juices and their corresponding red wines on serum and isolated LDL oxidation. These beverages were prepared and analysed by the Agriculture Department of Western Australia (Table 1). A third red grape juice prepared by more vigorous crushing and of higher polyphenolic content was also assessed. We also examined the effects of two white wines, Emu Lager beer (4.9% v/v alcohol), Swan Light beer (0.9% v/v alcohol) and redistilled ethanol. All grape juices, beers, wines and phenolic-striped beverages were diluted 1:500 in the final reaction mixture. These dilutions were chosen to correspond to those used previously [11]. Redistilled ethanol was diluted to give 0.1% and 0.5% ethanol by volume in the final reaction mixture.

LDL purification

LDL was isolated from EDTA (1 mg/ml) plasma by density-gradient ultracentrifugation as previously described [12]. Briefly, plasma density was increased to 1.07 by addition of sodium chloride and then a four-step gradient was constructed over the plasma using the following densities (kg/l): 0.5 ml, 1.063 (NaCl); 0.5 ml, 1.04 (NaCl); 0.5 ml, 1.02 (NaCl); and 0.9 ml, double-distilled water. Samples were ultracentrifuged at 296000 g (average) for 4h using a Centrikon T-1190 Ultracentrifuge (Kontron Instruments, Milan, Italy). The LDL band was collected by aspiration and passed through a Pharmacia P10 Sephadex column to remove the excess salt and the majority of the EDTA. The LDL was stored at 4°C in the dark under nitrogen and the controlled oxidation studies carried out within 1 week. Agarose gel electrophoresis (Paragon LIPO-gel system, Beckman) indicated that the LDL was pure and free of contamination by other lipoproteins or albumin.

LDL oxidation

The in vitro oxidation procedure used was similar to that described by Esterbauer et al. [13] for Cu2+ oxidations and by Frei and Gaziano [14] for 2,2'azobis-(2-aminopropane)hydrochloride (AAPH)
oxidations, as used previously in our laboratory [15, 16]. Just before the oxidation experiments the isolated LDL was passed through a second Pharmacia PD10 Sephadex column to remove any remaining EDTA. The cholesterol concentration of the LDL was measured using a standard enzymic method (Monotest, Boehringer Mannheim, Germany) and the LDL diluted with PBS to a standard concentration of 0.3 mmol/l cholesterol. Oxidations were initiated by the addition of freshly prepared copper chloride (final concentration 8 μmol/l) or AAPH (Polysciences, Warington, PA, U.S.A.) at a final concentration of 3 mmol/l. AAPH is a water-soluble compound that thermally decomposes to generate peroxyl radicals at a constant and known rate. AAPH oxidations were not carried out in the presence of a metal ion chelator. Oxidation kinetics was determined by monitoring the change in absorbance at 234 nm using a DU650 UV-VIS spectrophotometer (Beckman Instruments, CA, U.S.A.) with absorbance readings made every 20 min over 240 min at 37°C. The plot of absorbance against time was divided into a lag phase and a propagation phase. The lag time was defined as the intercept between the tangent of the absorbance curve during the propagation phase and the baseline and was expressed in minutes. All oxidations were carried out on individual LDL samples, not on pooled LDL.

All beverages tested were initially diluted 1:2.5 with PBS, then 10 μl of diluted test beverage was added to 2 ml of LDL solution such that the final dilution of beverage was 1:500. The same volume (10 μl) of PBS was added to control oxidations. For each test beverage a control oxidation was carried out on the same LDL preparation.

For preparation of dose-response curves for pure compounds, caffeic acid, protocatechuic acid and coumaric acid, each compound was dissolved in ethanol and diluted in water before being added to isolated LDL to give final concentrations of 0.1–10 μmol/l. The ethanol concentration in the final mixture did not exceed 0.1%. Oxidations were initiated with 8 μmol/l Cu²⁺ and monitored for 4 h as described above. Per cent inhibition was calculated as the change in lag time compared with control oxidation.

In one set of experiments, oxidation of LDL (0.1 mg/ml or approximately 0.3 mmol/l cholesterol) was monitored by formation of lipid hydroperoxides and consumption of α-tocopherol in the presence and absence of 1 μmol/l caffeic acid. Oxidations were initiated with either 5 μmol/l Cu²⁺ or 3 mmol/l AAPH.

Serum oxidation

Serum was prepared from blood taken after a 12-h fast and left in the dark at room temperature for 30 min. Copper-induced serum oxidation (0.67% serum diluted in PBS) was carried out as described by Regnstrom et al. [17] using 12.5 μmol/l Cu²⁺. Oxidation kinetics was determined by measuring the absorbance at 234 nm every 20 min over 240 min at 37°C, as for LDL oxidations. Only red wine 2 and grape juice 3 were studied in the serum oxidation assay.

Carboxylic acids

Wine is known to contain large amounts of dicarboxylic acids such as tartaric, malic, succinic and citric acids as well as monocarboxylic acids such as lactic and acetic acids. Analysis of red wine 1 obtained from the Agriculture Department of Western Australia indicated that the concentrations of these acids were as follows: citric 0.38 g/l, tartaric 4.54 g/l, malic 2.05 g/l, succinic 1.26 g/l, lactic 0.16 g/l and acetic acid 0.24 g/l. To test the antioxidant effects on copper-induced LDL oxidation, a mixture of these carboxylic acids at the above concentrations was made and diluted 1:500 in the final reaction volume.

Phenolic stripping

To determine whether the antioxidant effects of red wine and grape juice were due to the phenolic content, we examined the effects of phenolic stripping on LDL and serum oxidations. A 1-ml aliquot of beverage was passed through a 2-ml bed of polyvinylpolypyrrolidone (Sigma Chemicals) made up in water containing 12% ethanol and prewashed three times with the same mixture. The polyphenolic content of these beverages was determined before and after phenolic stripping, and on average more than 90% were removed by this procedure.

Polyphenolic measurement

Total polyphenolic quantitation was carried out using the method published by the Association of Official Analytical Chemists [18]. This method is a colorimetric assay using Folin-Denis reagent and estimates polyphenolics at 760 nm in relation to a tannic acid standard curve.

Extraction and separation of red wine phenolics

Red wine 2 (8 ml) was hydrolysed with 2 mol/l hydrochloric acid at 100°C for 1 h to break glycosidic bonds and the released aglycones were extracted into ethyl acetate. The crude extract was separated on silica gel plates (Merck) developed in diethyl ether containing 1% acetic acid. Plates were examined under UV light and six major absorbing bands were scraped, recovered into organic solvent, evaporated under nitrogen and reconstituted in ethanol (100 μl). The inhibitory activity of each band, at dilutions of 1:200 and 1:400, was assessed against
copper-induced LDL oxidation. The most active bands were those at Rf 0.40 and 0.57, as well as the most polar fraction remaining at the baseline, which was not further investigated. The two active bands were analysed as the methyl trimethylsilyl (TMS) ether derivatives by CG-MS. A number of cinnamic acid derivatives were identified by matching mass spectra with library spectra; these include coumaric acid, caffeic acid and protocatechuic acid. Their identity was confirmed by comparison with authentic standards (Sigma). In addition, 4-hydroxyphenethylalcohol was identified in fraction Rf 0.40. No flavonoids were identified in the two fractions. The level of these compounds in red wine was analysed by reversed-phase HPLC using a 250×4 mm Vydac C18 column and a gradient mobile phase starting with ammonium dihydrogen phosphate buffer pH 2.6 (95%) and acetonitrile (5%) and finishing with 80% acetonitrile–20% buffer. Compounds were detected by UV absorbance monitored at 255, 280 and 300 nm simultaneously using a Hewlett Packard 1050 series detector.

**Tocopherol analysis**

LDL was mixed with an equal volume of cold methanol, and the mixture was extracted with hexane. The hexane phase was dried under nitrogen and reconstituted in ethanol. The extract was analysed for α-tocopherol content by reversed-phase HPLC using electrochemical detection as previously described [15]. The mobile phase was methanol–ethanol (50:50, v/v) containing 2.5 g/l sodium perchlorate with a flow rate of 1 ml/min on a 25-cm Nucleosil C18 column (Alltech).

**Lipid hydroperoxide analysis**

In some experiments the formation of lipid peroxides in response to either Cu²⁺ or AAPH oxidation was determined by the Ferrous Oxidation–Xylenol Orange (FOX) assay [19]. Briefly, aliquots of the LDL incubation (50 μl) were mixed with 1 ml of FOX reagent, incubated at room temperature for 30 min and the absorbance read at 560 nm. Quantitation was made by comparison with standards of hydrogen peroxide of known concentration.

**Copper binding to LDL**

EDTA-free LDL (0.2 mg protein/ml) in PBS was incubated with 5 μmol/l Cu²⁺ both in the presence and in the absence of 5 μmol/l caffeic acid for 15 min at 37°C. Samples were passed through a Pharmacia PD-10 column to separate LDL-bound copper and free copper. Copper concentrations in LDL were measured by atomic absorption spectrophotometry.

**Statistics**

Values are quoted as means±SEM. Multiple regression and one-way analysis of variance (ANOVA) were used to study the effect of different beverages on LDL oxidation kinetics, using the Statistical Package for the Social Sciences.

**RESULTS**

**Beverage total phenolic content**

The polyphenolic content of each beverage tested is listed in Table 1. Red grape juice 1 was fermented on the pomace (skins and seeds) of the grapes to produce red wine 1; similarly, red grape juice 2 was used to produce red wine 2. Red grape juice 3 was produced by a different, more vigorous, technique, enabling the wine-maker to produce wine from this juice without the need for the pomace simply by allowing it to rest in large barrels for a few days. This more vigorous extraction technique resulted in a grape juice with very high phenolic content.

**Effect of grape juice and wine on copper- and AAPH-induced LDL oxidation**

The effect of different grape produce drinks on the kinetics of copper-initiated LDL oxidation is shown in Fig. 1. It is clear that the higher the polyphenolic concentration of the beverage, the greater the inhibition of LDL oxidation. Red wines 1 and 2 gave very similar results. For simplicity, only results for red wine 1 are presented. The mixture of carboxylic acids, mimicking that found in red wine, had no significant effect on LDL oxi-
Beverage phenolic content and lipoprotein oxidation inhibition

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100 120 140 160 180 200 220 240
Time (min)

Fig. 2. Mean change (±SEM) in absorbance at 234 nm in LDL after initiation of peroxidation with 3 mmol/l AAPH at 37°C. Control oxidation was LDL plus PBS vehicle (□, n = 10). LDL plus 1:50 diluted red wine 1 (n = 9); LDL plus 1:500 diluted high phenolic content red grape juice 3 (n = 2).

Table 2. Lag time results obtained from in vitro oxidations of individual isolated LDL samples initiated with either (a) copper or (b) AAPH according to the procedure outlined in Materials and methods. All beverages were diluted 1:500 in the final reaction mixture. Statistical significance: *P < 0.001 compared with lag time of control oxidation. Values are means (SEM).

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Copper-induced LDL oxidations</th>
<th>AAPH-induced LDL oxidations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag time (min)</td>
<td>Range n</td>
</tr>
<tr>
<td>Control</td>
<td>54 (8.0)</td>
<td>34-67 4</td>
</tr>
<tr>
<td>Carboxylic acids</td>
<td>68 (9.7)</td>
<td>41-86 4</td>
</tr>
<tr>
<td>Control</td>
<td>62 (6.0)</td>
<td>54-74 3</td>
</tr>
<tr>
<td>Red grape juice 1</td>
<td>166* (29)</td>
<td>109-200 3</td>
</tr>
<tr>
<td>Control</td>
<td>58 (5.1)</td>
<td>34-74 7</td>
</tr>
<tr>
<td>Red wine 1</td>
<td>&gt;240*</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>57 (1.7)</td>
<td>54-59 3</td>
</tr>
<tr>
<td>Red grape juice 3</td>
<td>&gt;240*</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>65 (5.9)</td>
<td>54-74 4</td>
</tr>
<tr>
<td>White wine</td>
<td>129* (10.9)</td>
<td>107-142 4</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of removing more than 90% of phenolics from red wine and grape juice on oxidation of LDL initiated with copper as described in Fig. 1. The results are of three experiments done in duplicate. Control oxidation (□) was LDL plus PBS. LDL plus 1:500 diluted red wine 2; LDL plus 1:500 diluted phenolic-stripped red wine 2; LDL plus 1:500 diluted high-polyphenolic red grape juice 3; LDL plus 1:500 diluted phenolic-stripped red grape juice 3.

Effect of phenolic stripping on inhibition of LDL oxidation

Upon stripping the phenolics from both red wine 2 and red grape juice 3, all inhibitory effects on copper-induced LDL oxidation were abolished (Fig. 3), with lag times returning to control values. Similar results were obtained with AAPH-induced oxidation with both phenolic-stripped beverages, giving lag times similar to control values: red wine 2 (phenol stripped) lag time 69 ± 5 min compared with a control lag time of 59 ± 8 min; and phenol-stripped
Table 3. Effect of two different beers on lag time of isolated LDL oxidations. Conditions as described in Table 2. Statistical significance: *P < 0.001. Values are mean (SEM), n = 5.

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Lag time (min)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper-induced LDL oxidations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>57 (6.4)</td>
<td>38-78</td>
</tr>
<tr>
<td>Full-strength beer</td>
<td>109 (1)</td>
<td>76-137</td>
</tr>
<tr>
<td>Light beer</td>
<td>106 (11)</td>
<td>83-137</td>
</tr>
<tr>
<td>AAPH-induced LDL oxidations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54 (8.3)</td>
<td>38-85</td>
</tr>
<tr>
<td>Full-strength beer</td>
<td>85 (15)</td>
<td>62-142</td>
</tr>
<tr>
<td>Light beer</td>
<td>79 (11)</td>
<td>62-120</td>
</tr>
</tbody>
</table>

Grape juice 3 lag time 77 ± 16 min compared with a control lag time of 51 ± 4 min (n = 3).

Effect of beer phenolics on LDL oxidation

Table 3 summarizes the lag time results for LDL oxidations in the presence and absence of light or full-strength beer at dilutions of 1:500. Both beers, which have similar phenolic content, significantly delayed copper-induced LDL oxidations. AAPH-induced oxidations were less inhibited.

Effect of ethanol on LDL oxidation

Ethanol at concentrations of 0.1–0.5% had no significant effect on the oxidation of LDL initiated by either copper or AAPH (results not shown). The concentration of ethanol in the diluted red wine samples was less than 0.03%.

Serum oxidations

The effects of high-phenolic beverages on copper-induced oxidation of diluted serum are shown in Fig. 4. Red wine 2 and red grape juice 3 both totally inhibited oxidation. When phenolics were stripped from these beverages, oxidation took place, but lag times were still significantly longer than for control oxidations.

The identification of antioxidant constituents of red wine

Several cinnamic acid derivatives (Fig. 5) were identified in TLC fractions separated from red wine that showed strong antioxidant activity. 4-Hydroxyphenethylalcohol was also identified, but this compound showed little antioxidant activity in doses up to 10 μmol/l. The quantities of each compound in the red wine were estimated by HPLC analysis as follows: coumaric acid 4.1 mg/l, caffeic acid 5.0 mg/l and protocatechuic acid 7.2 mg/l. The pure compounds were individually tested for antioxidant activity in doses ranging from 0.1 to 10 μmol/l in copper-initiated LDL oxidations, with per cent inhibition calculated as the change in lag time compared with control oxidations. The dose-response curves (Fig. 6) show that caffeic acid (IC_{50} 0.6 μmol/l) is the most effective antioxidant in this system followed by protocatechuic (IC_{50} 0.85 μmol/l) and coumaric acid (which has virtually no activity in the concentration range tested).

The effects of caffeic acid on tocopherol consumption and on lipid peroxide formation in copper- or AAPH-mediated oxidation of LDL

Exposure of LDL to Cu^{2+} caused rapid depletion of α-tocopherol that was nearly complete within 20 min (Fig. 7a). The formation of lipid hydroperoxides increased rapidly after approximately 50 min and continued to increase up to 160 min. In the presence of 1 μmol/l caffeic acid α-tocopherol depletion was less rapid and was not completed until
Beverage phenolic content and lipoprotein oxidation inhibition

Fig. 6. Dose-response curves for the pure compounds caffeic acid (■), protocatechuic acid (▲) and coumaric acid (●), dissolved in ethanol and diluted in water before being added to isolated LDL to give final concentrations of 0.1–10 μmol/l. Oxidations were initiated with 5 μmol/l Cu²⁺ and monitored over a period of 4 h as described in the Materials and methods section. Per cent inhibition was calculated as the change in lag time compared with control oxidation. Results are the mean of three individual experiments performed in duplicate.

40 min. The onset of rapid formation of lipid hydroperoxides was also significantly delayed until after 120 min. In these experiments, the molar ratio of Cu²⁺ to caffeic acid was 5:1. It is therefore unlikely that the extent of inhibition seen is due primarily to copper binding. The effect of caffeic acid on LDL oxidation induced by the watersoluble free radical initiator AAPH (3 mmol/l) is shown in Fig. 7b. Under the conditions of these experiments, caffeic acid at a relatively low concentration was able to prolong the consumption of α-tocopherol and lead to a significant decrease in lipid hydroperoxide production. It is not possible to compare directly the copper- and AAPH-induced oxidations as they are initiated by totally different mechanisms[20]. Both results are consistent with caffeic acid acting as a free radical scavenger and a co-antioxidant for α-tocopherol[21].

Effect of caffeic acid on copper binding to LDL

The results of copper binding to LDL in the presence or absence of caffeic acid are presented in Table 4. Caffeic acid did not decrease copper bound to LDL, but in fact led to a threefold increase in the ratio of copper to LDL particles.

DISCUSSION

The results of the present study demonstrate that phenolic constituents of various beverages can protect both isolated LDLs and serum from oxidation in vitro. The extent of inhibition depends upon the phenolic content of the beverage and the oxidation system used. In order to differentiate antioxidant effects due to copper binding or free radical trapping, we used two different models of LDL oxidation: the copper-induced oxidation of LDL and oxidation induced by peroxyl radicals generated during the thermal decomposition of AAPH. It was evident that red wine and some grape juices con-
tained high levels of phenolic compounds and that these appeared to be more effective in inhibiting copper-induced oxidation of LDL than AAPH-induced oxidation. The inhibitory effects could be diluted out and were not due to the dicarboxylic acids present in red wines. Conclusive evidence that the inhibitory activity was due to the phenolic constituents was gained by selectively removing them from the beverages with subsequent loss of activity.

The observation that the beverage phenolics appear to be more effective at inhibiting copper-induced oxidations is not surprising given that phenolic compounds can actively bind copper ions as well as trap free radicals [22]. As there is some evidence that redox-reactive copper ions may exist in atherosclerotic lesions [23], and that the copper-binding protein ceruloplasmin contains redox-active copper and may oxidatively modify LDL [24], the potency of these naturally occurring substances in inhibiting copper-induced oxidations may have physiological relevance. There is also some evidence that in lipoproteins lipid peroxidation may be promoted by \( \alpha \)-tocopherol, which can act as a phase and chain transfer agent [25]. In this model 'co-antioxidants' may be of critical importance in protecting or removing the tocopherol radical from an LDL particle [26]. Ubiquinol-10 is possibly one such endogenous co-antioxidant [26], and it may be possible that natural antioxidants derived from the diet can play an important role as co-antioxidants.

When ethanol is consumed there is some evidence that it has pro-oxidant effects [27], which have been ascribed to an alcohol-induced increase in NADH-dependent production of reactive oxygen intermediates [28] or iron mobilization favouring free radical generation [29]. Ethanol up to a final concentration of 0.5% by volume had no effect on isolated LDL oxidation in this study. In a recent controlled crossover study conducted by us, during which regular beer drinkers consumed a high-alcohol or a very low-alcohol beer, an increased susceptibility of LDL to oxidation was observed during high-alcohol consumption [30]. The two beers were the same as used in this study and had similar phenolic content. Our results and those of others [31] lead us to propose that the antioxidant phenolics in alcoholic beverages act counter to the potential pro-oxidant effect of ethanol, such that low-phenolic beverages like white wine or beer may have a net pro-oxidant effect while red wine could have a net antioxidant influence.

The results obtained with diluted serum are of some interest as the lipoproteins are oxidized in a more physiological setting than isolated LDL. There is evidence that the sub endothelial fluid contains many of the antioxidants found in plasma [32]. The high-phenolic juice and red wine were able to inhibit serum oxidation completely, and even the small amount of phenolics remaining after stripping of these beverages significantly prolonged the lag time (Fig. 4). In the isolated LDL assay these phenolic-stripped beverages had no significant effect on lag time. The reason for this is not clear but could be related to the fact that the lipoprotein concentration in the diluted serum would be less than that used in the isolated LDL system. In any case, oxidations in serum may provide a sensitive assay to examine potential antioxidants in a physiological system.

There are now several studies which suggest that red wine can protect LDL against oxidation in vitro [11, 33]. There are also several studies showing that specific phenolic compounds such as flavonoids can protect LDL against oxidation [34–36]. The major question remaining is whether these phenolic substances can be absorbed into the bloodstream and exert biological activity. There is some recent evidence that this may be the case. Dogs administered red wine or grape juice intravenously showed inhibited platelet activity and thrombosis in stenosed coronary arteries in vivo [37]. In a study in rats given red wine or a grape seed extract containing tannins, there was inhibition of alcohol-induced lipid peroxidation and blunting of the rebound platelet activation occurred after alcohol withdrawal [38]. In humans given 400 ml of red wine per day for 2 weeks, susceptibility of LDL to copper-induced lipid peroxidation was reduced, an effect not seen after consumption of the same amount of white wine, which actually increased the propensity of LDL to undergo oxidation [31]. In another study in which subjects were given 200 ml of red wine per day for 10 days, no effect on susceptibility of LDL to oxidation was seen [39]. In the study by Fuhrman et al. [31], polyphenol levels associated with LDL after 2 weeks of red wine consumption were 28 mg/g protein, which is very similar to the levels used in our current study. For example, the high-phenolic juice and red wine would have provided, on average, 20 mg of phenolics per gram of LDL protein in the oxidation experiments.

In an attempt to determine which phenolic compounds in red wine are responsible for inhibition of LDL oxidation, we analysed red wine, after hydrolysis of glycosides, for its major antioxidant components. Our results indicate that caffeic acid and protocatechuic acid are two important antioxidant components of red wine. Although much of the scientific interest to date has focused on flavonoids, the phenolic non-flavonoid compounds may contribute significantly to the antioxidant capacity of red wine. These compounds have already been shown to have activity towards peroxyl radicals with significant inhibition of LDL oxidation in low micromolar concentrations [40, 41]. Compounds with the catechol-type structure are better than monoprotic phenols in inhibiting peroxyl-initiated oxidation of LDL [21]. This is probably because of the ability of the formed antioxidant radicals to be resonance stabilized. Catechol structures are also found in catechin, a major flavonoid component of red wine.
(120–390 mg/l) [42], as well as in the polymeric tannins. Catechin itself has been shown to inhibit copper-catalysed oxidation of LDL in a dose-dependent manner, giving complete inhibition at 68 μmol/l [36]. The method used to assess inhibition was based on total formation of thiobarbituric acid-dependent manner, giving complete inhibition at

The change in lag time compared with control. which one might expect to be similar. The caffeic acid content of red wines has been reported to be in the range 5–15 mg/l [42, 43]. By HPLC analysis we estimate that in red wine 2 the caffeic acid content was 5 mg/l and the protocatechuic acid content 7 mg/l. The combined level of these two compounds would be approximately 80 μmol/l in red wine, which at the 1:500 dilutions used in our experiments would give 0.2 μmol/l. Although this concentration is at the lower end of the dose–response curve for inhibition of isolated LDL oxidation by these compounds, the total antioxidant capacity of red wine is likely to be the result of the combined activity of a wide range of compounds.

In our experiments with caffeic acid, copper binding does not appear to be a major contributor to its antioxidant effects. Caffeic acid has been shown to bind to proteins such as albumin, altering its conformation [44]. It is conceivable that caffeic acid may also bind to apoprotein B100, altering the binding of copper ions. We have found that, in the presence of caffeic acid, the amount of copper associated with LDL actually increases and yet LDL oxidation is still inhibited. The predominant mechanism for protection of LDL oxidation by caffeic acid appears to be free radical trapping, thus sparing ș-tocopherol from oxidation (Fig. 7).

The characterization of at least some of the specific constituents of red wine responsible for its antioxidant effect will enable us to target the absorption of these compounds and their possible association with lipoproteins in vivo. Further studies are also needed to determine if the same substances in red wine or grape juice that confer its antioxidant properties are also the same compounds responsible for the observed inhibition of platelet function. The possibility that plant phenolics may be absorbed and contribute to the antioxidant protection of LDL as well as influencing platelet and vascular function [37, 38, 45] has potential implications for the role of these substances in human nutrition and disease.

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