

Original Paper

Red Wine Inhibits the Cell-Mediated Oxidation of LDL and HDL

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Key words: low density lipoprotein, high density lipoprotein, wine, macrophage, oxidation

Objective: We compared the *in vitro* effects of red wine, white wine and ethanol on the cell mediated oxidation of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) by three frequently-used assays.

Methods: LDL and HDL isolated from normolipidemic human serum were incubated with J774.A1 macrophages in DMEM with copper, with or without red wine, white wine or ethanol (equivalent to 0.2 mg ethanol/ml). Lipoprotein oxidation was assessed by conjugated diene formation as measured by changes in absorbance at 234 nm (ΔA_{234}), thiobarbituric-acid-reactive-substance (TBARS) production and trinitrobenzene-sulfonic-acid (TNBS) reactivity.

Results: Red wine (0.2 mg ethanol/mL) inhibited LDL oxidation as indicated by an 85.7% decrease in absorbance at 234 nm, a 96.5% decrease in TBARS production and complete prevention of the decrease in TNBS reactivity. White wine and ethanol did not have any significant effect at 0.2 mg/mL. White wine at 1.0 mg ethanol/mL inhibited TBARS production from LDL by 84.1%. Red wine (0.2 mg ethanol/mL) inhibited HDL oxidation as indicated by a 78.9% decrease in ΔA_{234} , an 81.7% decrease in TBARS production and by no change in TNBS reactivity. White wine and ethanol had no effect at 0.2 mg/mL. White wine at 1.0 mg ethanol/mL inhibited TBARS production from HDL by 66.4%.

Conclusions: These results indicate that red wine inhibits the cell mediated oxidation of lipoproteins, that white wine is not as effective as red wine and that the effect of the red wine is not due to its ethanol content.

INTRODUCTION

The oxidative modification of low density lipoprotein (LDL) and its unregulated and accelerated uptake by artery wall macrophages have been implicated in the development of atherosclerosis [1]. LDL oxidation has been demonstrated *in vitro* [2–4], and there is evidence that LDL oxidation occurs *in vivo* [5–7]. While the oxidation of high density lipoprotein (HDL) has been the subject of relatively few experiments, the results of *in vitro* studies suggest that HDL oxidation also occurs *in vivo* [8,9]. Oxidation of HDL diminishes its capacity to mediate cholesterol efflux from various cell types [10–12] and we have previously demonstrated a significant negative correlation between the extent of HDL oxidation and cholesterol efflux [13]. The results of epidemiological studies indicate an inverse relationship between plasma concentrations of the

antioxidant vitamins and mortality from coronary artery disease, suggesting that dietary antioxidants are anti-atherosclerotic agents [14]. Dietary supplementation with antioxidant vitamins decreases the *ex vivo* susceptibility of LDL to oxidation [15–17]. We have reported previously that dietary administration of antioxidant vitamins decreases the susceptibility of HDL to oxidation *ex vivo* and decreases the loss of its cholesterol efflux capacity which results from oxidation [18].

Epidemiologic studies have indicated that moderate alcohol consumption results in diminished risk of coronary artery disease [19]. The interest in red wine as an anti-atherosclerotic agent stems from epidemiological evidence that the occurrence of coronary artery disease in France, where consumption of red wine is high, is lower than in other countries where serum lipoprotein concentrations, dietary saturated fat and alcohol consumption are comparable. This observation is referred to as the “French paradox” [20,21]. These reports suggest that wine

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has a greater protective effect against coronary artery disease than can be attributed to its alcohol content. However, consumption of alcohol in any form may be beneficial, and there may be several mechanisms mediating the beneficial effects [22]. Red wine contains several flavonoids, which are polyphenols that exert chain-breaking anti-oxidant activity by donating a hydrogen to react with hydroxyl, superoxide and lipid peroxy radicals [23,24], suggesting that one beneficial effect of red wine is to inhibit the *in vivo* oxidation of lipoproteins. Fuhrman et al. [25] reported that red wine, but not white wine, inhibited the formation of thiobarbituric acid reactive substances *in vitro* during the copper mediated oxidation of LDL. Frankel et al. [26] measured the formation of conjugated dienes and hexanal from the copper catalyzed oxidation of LDL and demonstrated that polyphenols extracted from red wine inhibit oxidation more effectively than α -tocopherol. The phenolic compounds inhibited oxidation by scavenging lipid peroxy radicals and not by chelating copper [26].

In the present study, we demonstrate that the J774.A1 macrophage cell line stimulates lipoprotein oxidation in the presence of copper and use these cells to study the effects of red wine, white wine and ethanol on the oxidation of LDL and HDL.

MATERIALS AND METHODS

Chemicals

Phosphate buffered saline (PBS), Dulbecco's minimum essential medium (DMEM), bovine calf serum, tetramethoxypropane, thiobarbituric acid, trinitrobenzene sulfonic acid, butylated hydroxytoluene (BHT), lipid determination kits and Hank's balanced salt solution were obtained from Sigma Chemical (St. Louis, MO). Trichloroacetic acid and copper nitrate solution (1 mg/mL) were from Fisher Scientific (Springfield, NJ). Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). Total antioxidant status assay kit was from Wak-Chemie Medical GMBH (Bad Soden, Germany). Cabernet Sauvignon (12.5% alcohol by volume or 99 mg ethanol/mL) and Chardonnay (13.0% alcohol by volume or 103 mg ethanol/mL) were from Robert Mondavi (Woodbridge, CA). The sulfite contents of these wines were equal. Additional wines are mentioned below when used.

Cell Culture

Murine monocyte-derived J774.AI macrophages were maintained in culture in DMEM with phenol red and 10% bovine calf serum, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C under 5% CO₂ in air. Cells (2×10^5 /mL) were plated in DMEM with 10% bovine calf serum with antibiotics. Medium was also added to wells without cells. After eight hours, the wells were washed with serum-free Hank's balanced salt solution without phenol red before incubation with LDL and HDL.

Lipoprotein Preparation

Serum was obtained from a fasting male volunteer whose serum lipid concentrations were triacylglycerol, 63 mg/dL; total cholesterol, 204 mg/dL; HDL cholesterol, 61 mg/dL. The subject maintained a constant diet and level of physical activity and did not consume any antioxidant vitamin supplements, wine or other alcoholic beverages. Serum concentrations of vitamins C and E in this subject were determined previously [described in ref. 18] and were 10.0 μ g/mL and 10.8 μ g/mL respectively. Total antioxidant status of the serum of this individual was 1.60 mM. The reported total antioxidant status of a normal adult reference population was 1.46 ± 0.14 mM [27]. LDL (1.019 g/mL < d < 1.063 g/mL) and HDL (1.063 g/mL < d < 1.21 g/mL) were isolated by sequential ultracentrifugation from serum [28] as previously described [29]. After isolation, the lipoproteins were dialyzed and filtered. Protein content of the fractions were estimated by the bicinchoninic acid method. Phospholipid, cholesterol and triacylglycerol content were determined by enzymatic methods. The chemical composition (weight %) of LDL was 27% protein, 35% cholesterol, 23% phospholipid and 15% triacylglycerol and of HDL was 52% protein, 23% cholesterol, 23% phospholipid and 2% triacylglycerol. Vitamin E concentrations in lipoprotein fractions isolated from this subject were determined previously [17,18]. The value for LDL was 5.7 μ g/mg and for HDL was 2.7 μ g/mg protein.

Lipoprotein Oxidation

LDL and HDL were dialyzed at 4°C against PBS with calcium, magnesium and 10 mM sodium bicarbonate, pH 7.4. Incubations were done at 37°C in serum-free DMEM without phenol red. Our preliminary experiments (data not shown) and results reported by others [30] demonstrate that copper is required to catalyze lipoprotein oxidation in this medium. LDL (0.2 mg protein/ml) was incubated without and with macrophages (500,000 cells/well) in 6-well plates or in 24-well plates (200,000 cells/well) for 16 hours in serum-free DMEM containing 2 μ M copper. HDL (0.5 mg protein/mL) was incubated without and with macrophages as above for 24 hours in serum-free DMEM containing 5 μ M copper. Wine or ethanol were added in amounts equivalent to 0.2 mg ethanol/mL or as indicated for concentration dependency studies. Samples of media were taken for assays of LDL and HDL oxidation. Incubation with wines or ethanol had no effect on the appearance of cells or their adherence to the incubation wells as determined by light microscopy.

Assays of LDL and HDL Oxidation

Lipoprotein oxidation in samples of media was quantified by conjugated diene formation measured by change in absorbance at 234 nm (ΔA_{234}), thiobarbituric acid reactive substance (TBARS) production and loss of trinitrobenzene sulfonic acid (TNBS) reactivity as previously described [29].

Change in absorbance at 234 nm was determined after a 1/10 dilution [31]. TBARS are expressed as nanomoles malondialdehyde (MDA) equivalents per milligram protein compared with tetramethoxypropane standards [32]. TNBS reactive lysine amino groups is expressed as a percent of that in unincubated, unmodified lipoprotein [33]. Samples of incubation media were dialyzed against 0.01% EDTA, 150 mM sodium chloride and 10 mM sodium bicarbonate, pH 7.6 before being assayed for Δ A234 and TNBS reactivity.

Statistics

Analyses of data were performed using the SAS system. Comparisons were done by an analysis of variance. Where significant effects were found, a Duncan's analysis was done for individual comparisons. Results are expressed as mean \pm standard error of the mean, or as noted.

RESULTS

The oxidation of LDL in the absence and presence of J774 macrophages for 16 hours was determined. In the presence of cells, LDL oxidation measured by Δ A234, TBARS production and loss of TNBS reactivity was 2.3, 7.3 and 3.0 fold higher respectively, compared to incubations in the absence of cells (Fig. 1). The results of the incubation of LDL in the presence and absence of red wine, white wine and ethanol at 0.2 mg ethanol/ml are also shown in Fig. 1. Addition of red wine inhibited conjugated diene formation by 85.7% as indicated by a decrease in Δ A234 ($p < 0.0001$). White wine and ethanol had no effect (Fig. 1A). There was a 96.5% reduction in cell mediated TBARS production with the addition of red wine ($p < 0.0001$). White wine produced a 20.1% reduction, while the addition of ethanol resulted in a 7.4% increase in TBARS production, neither significantly different compared to control cells incubated in the absence of wine and ethanol (Fig. 1B). Red wine completely prevented the decrease in TNBS reactivity, whereas 36.0%, 27.4% and 32.9% reductions ($p < 0.006$ compared to red wine) were measured in the media from control incubations and incubations in the presence of white wine and ethanol, respectively (Fig. 1C).

The effect of the incubation of LDL at various concentrations of red wine and white wine on cell mediated oxidation was determined (Fig. 2). TBARS production in the presence of red wine was significantly lower ($p < 0.001$) than that in the presence of white wine in the range of 0.05–0.5 mg ethanol/mL. There was no difference between red wine and white wine at 1.0 mg/mL. The concentrations for 50% inhibition of LDL oxidation were approximately 0.04 mg ethanol/mL for red wine and 0.7 mg ethanol/mL for white wine. TBARS production from the cell-mediated oxidation of LDL in the presence of 1 mg/mL ethanol was 110.5% of control, but this difference was not significant. The effects of red wine and white wine at 0.2

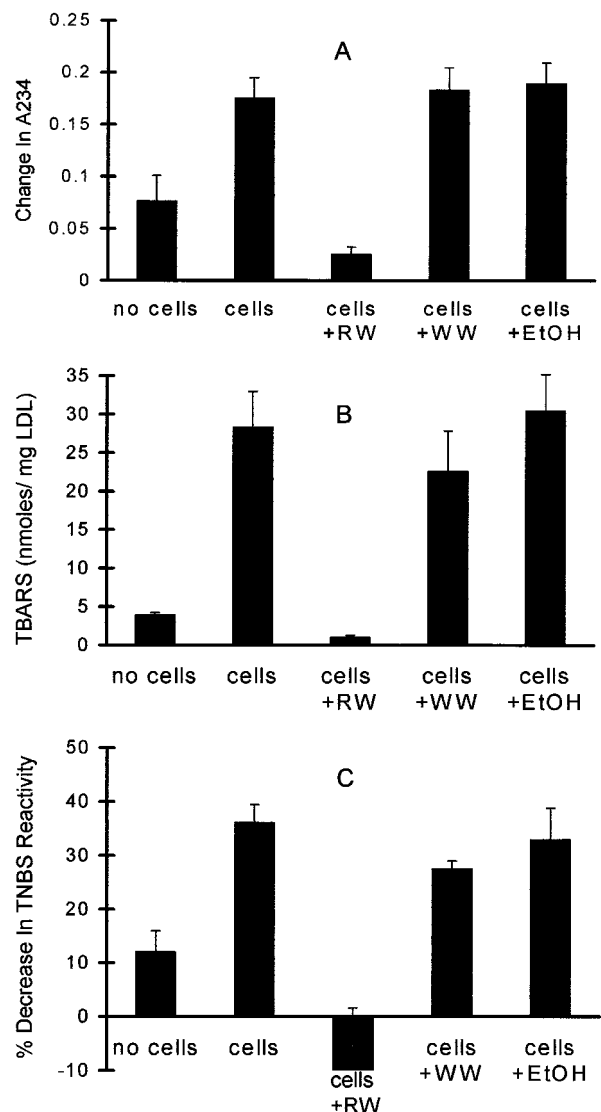


Fig. 1. Effects of red wine, white wine and ethanol (equivalent to 0.2 mg/mL ethanol) on LDL oxidation. LDL (0.2 mg protein/mL) was incubated with and without cells in 6 well plates (500,000 cells/well) for 16 h at 37°C in serum-free DMEM containing 2 μ M copper. (A) Change in absorbance at 234 nm (Δ A234) is expressed relative to unmodified LDL. (B) TBARS are expressed as nmoles malondialdehyde (MDA) equivalents/mg LDL protein. (C) percent decrease in TNBS reactivity relative to the unmodified LDL (0%). The mean absorbance of the unmodified LDL (50 μ g) at 340 nm = 0.164 \pm 0.005. Results are means \pm SEM of four experiments done in triplicate. Red wine vs. control, white wine and ethanol (A), $p < 0.0001$; (B), $p < 0.0001$; (C), $p < 0.0006$. RW = red wine; WW = white wine; EtOH = ethanol.

mg ethanol/mL on the time course of LDL oxidation over 24 hours are shown in Fig. 3. Red wine almost completely prevented the increase of Δ A234 (Fig. 3A) as well as the production of TBARS (Fig. 3B), while white wine had a minimal effect. The effects on TBARS production at 16 hours of three

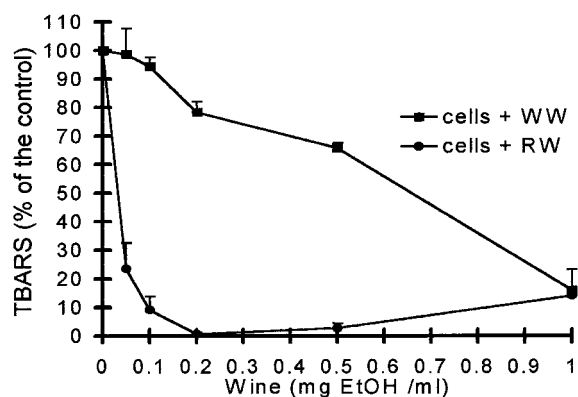


Fig. 2. Concentration-dependent effect of red wine and white wine on cell mediated oxidation of LDL. Incubation conditions were as described in the legend to Fig. 1 except for incubation in 24-well plates (200,000 cells/well). The mean TBARS production by the cell control (represented as 100%) = 15.1 ± 2.4 nmoles MDA/mg LDL protein. The points are the means ± SEM of four determinations done in triplicate. Red wine vs. white wine in the range of 0.05–0.5 mg EtOH/mL, $p < 0.001$, at 1.0 mg/mL, NS. RW = red wine; WW = white wine.

other red wines (Ruby Cabernet, Pinot Noir and Merlot at 0.2 mg ethanol/mL) and two other white wines (Sauvignon Blanc and another Chardonnay at 1.0 mg ethanol/mL) were similar to those obtained with the Mondavi Cabernet Sauvignon and Chardonnay respectively (data not shown).

HDL oxidation determined by TBARS production was 4.4-fold higher, and there was a 13% decrease in TNBS reactivity in the presence of cells compared to a 0.4% decrease in the absence of cells, indicating a stimulation of HDL oxidation by J774 macrophages. However, there was no significant difference in ΔA_{234} in the presence of cells under these incubation conditions (Fig. 4). The effects of red wine, white wine and ethanol on the cell mediated oxidation of HDL are also shown in Fig. 4. Red wine decreased ΔA_{234} by 78.9% compared to no addition ($p < 0.0001$). White wine and ethanol had no effect (Fig. 4A). TBARS production decreased by 87.1% ($p < 0.0001$) by the addition of red wine. The 9.7% decrease and 4.0% increase with white wine and the ethanol respectively were not significant (Fig. 4B). TNBS reactivity decreased by 13.0%, 11.0% and 17.9% in the control incubations and in the presence of white wine and ethanol, while red wine prevented the decrease ($p < 0.007$, Fig. 4C). HDL oxidation was also studied at various concentrations of red wine and white wine. Results shown in Fig. 5 again demonstrate the greater antioxidant properties of red wine at all concentrations. TBARS production from the cell-mediated oxidation of HDL in the presence of 1 mg/mL ethanol was 92.1% of control. Preincubation of macrophages for 24 hours with red wine, white wine and ethanol (all 0.2 mg/mL ethanol) had no effects on their subsequent ability to oxidize LDL and HDL (data not shown).

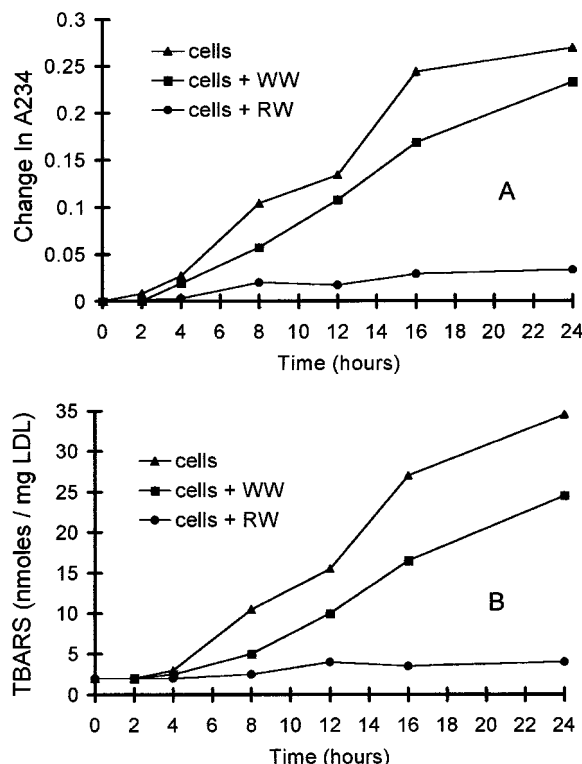


Fig. 3. Time course of LDL oxidation. Incubation conditions were as described in the legend to Fig. 1 except for incubation in 24-well plates (200,000 cells/well). (A) Change in absorbance at 234 nm (ΔA_{234}) is expressed relative to unmodified LDL. (B) TBARS are expressed as nmoles MDA equivalents/mg LDL protein. Results are from one duplicate experiment. RW = red wine; WW = white wine.

DISCUSSION

The oxidation of lipoproteins in the vessel wall can be mediated by endothelial cells, smooth muscle cells and monocyte-macrophages [1]. In the present study, J774.A1 macrophages, which are commercially available and easy to maintain, were used to study cell-mediated lipoprotein oxidation. Assays based on the production of TBARS and the decrease in TNBS reactivity both indicated a stimulation of the oxidation of LDL and HDL by these cells. These cells also increased conjugated diene production (ΔA_{234}) from LDL but not HDL (Fig. 4). In our previous time course studies of HDL oxidation in the absence of cells [13], we found that TBARS production follows conjugated diene formation. It is possible that under the experimental conditions in this study, J774 macrophages accelerated the conversion of dienes to TBARS, thus preventing their accumulation.

Our results indicate that red wine inhibits the cell mediated oxidation of lipoproteins *in vitro*, that white wine is not as effective as red wine and that the effect of the red wine is not due to its ethanol content. The antioxidant components of red wine include the polyphenol flavonoids catechin and quercetin and the phytoalexin resveratrol. These components have been

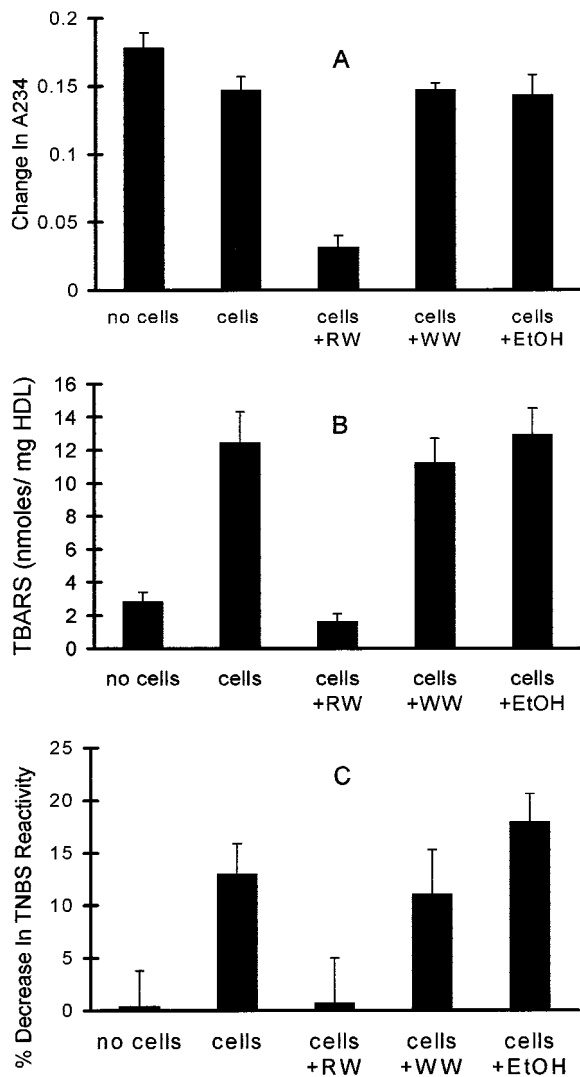


Fig. 4. Effects of red wine, white wine and ethanol (equivalent to 0.2 mg/mL ethanol) on HDL oxidation. HDL (0.5 mg protein/mL) was incubated with and without cells in 6 well plates (500,000 cells/well) for 24 h at 37°C in serum-free DMEM containing 5 μ M copper. (A) Change in absorbance at 234 nm (ΔA_{234}) is expressed relative to unmodified HDL. Results are means \pm SEM of four experiments done in triplicate. (B) TBARS are expressed as nmoles malondialdehyde equivalents/mg HDL protein. (C) % decrease in TNBS reactivity relative to the unmodified HDL (0%). The mean absorbance of the unmodified HDL (50 μ g) at 340 nm = 0.243 \pm 0.040. Red wine media vs. control, white wine and ethanol (A) $p < 0.0001$; (B), $p < 0.0001$; (C) $p < 0.007$. RW = red wine; WW = white wine; EtOH = ethanol.

shown to inhibit copper-catalyzed or cell-mediated oxidation of LDL *in vitro* [34–36]. Our experiments do not elucidate the mechanism of this antioxidant activity. Possible mechanisms by which wine could inhibit oxidation include chelation of copper or scavenging of lipid peroxy radicals. In studies performed in the absence of cells, red wine at a concentration of 0.1% inhibited copper catalyzed oxidation of LDL, while white

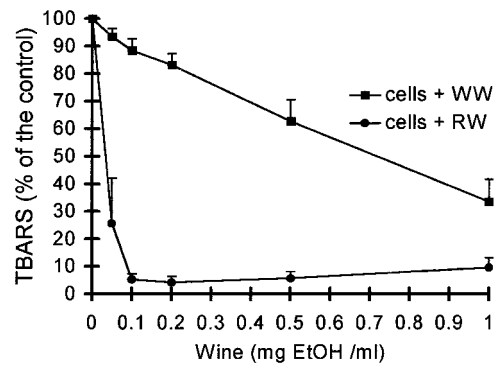


Fig. 5. TBARS production from HDL oxidation at various concentrations of red and white wines. The incubation conditions were as described in the legend for Fig. 4 except for incubation in 24-well plates (200,000 cells/well). The mean TBARS production by the cell control (represented as 100%) = 9.7 \pm 0.7 nmoles MDA/mg HDL protein. The results are the means \pm SEM of four determinations done in duplicate. Red wine vs. white wine in the range of 0.05–0.5 mg EtOH/mL, $p < 0.007$, at 1.0 mg EtOH/mL, $p = 0.0534$ RW = red wine; WW = white wine.

wine at 1.0% had no inhibitory effect [37]. Red wine added *in vitro* also decreased the susceptibility of plasma to oxidation initiated by a free radical generator [25,37].

While there is strong evidence that red wine inhibits lipoprotein oxidation *in vitro*, the data on the effects of consumption of red wine on the *ex vivo* susceptibility of lipoproteins to oxidation are conflicting [38]. Flavonoids are capable of entering phospholipid bilayers [24] and thus could associate with lipoproteins. Kondo et al. [39] reported that administration of red wine at an amount equivalent to 0.8 g/kg for 14 days resulted in an increased resistance of LDL to oxidation compared to samples obtained before administration of the wine. Similarly Fuhrman et al. [25] showed that plasma and LDL obtained from subjects following the consumption of 400 ml of red wine for 14 days was less susceptible to oxidation. Consumption of white wine, which does not have as high a content of flavonoids [23], did not have similar effects [25]. In contrast, deRijke et al. [37] reported that consumption of red wine with a reduced ethanol content did not result in a decrease in the *ex vivo* susceptibility of LDL to oxidation. The extent of inhibition of *ex vivo* lipoprotein oxidation by red wine would depend on whether the wine flavonoids remain associated with lipoprotein particles during their isolation, as has been shown to occur with vitamin E [17,18]. However, it may not be necessary for flavonoids to bind to lipoproteins *in vivo* because they could inhibit oxidation when present in serum or the arterial wall. Assays based on the susceptibility of whole serum to oxidation may be more appropriate, and it has been reported that consumption of red wine increases the total antioxidant capacity of serum [40]. At present such assays are not as well characterized as those with isolated lipoproteins, and further studies are needed to characterize the antioxidant effects of the consumption of red wine.

CONCLUSIONS

Our results indicate that red wine, at a concentration attained in serum with moderate consumption, almost completely inhibits LDL and HDL oxidation mediated by J774 macrophages *in vitro*. While it is established that oxidized LDL exists *in vivo* and contributes to the formation of the atherosclerotic plaque, the extent of the *in vivo* oxidation of HDL and its role in atherogenesis are unknown. *In vitro* oxidation of HDL diminishes its capacity to mediate cholesterol efflux from macrophages and the administration of antioxidant vitamins inhibits the *ex vivo* oxidation of HDL and the loss of its efflux capacity. If oxidation of HDL diminishes its antiatherogenic properties, our results would suggest that red wine consumption may be beneficial by inhibiting the oxidation of both HDL and LDL.

ACKNOWLEDGMENT

The authors thank Shelley Greenhaus for clinical assistance.

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Received July 1998; revision accepted September 1998.