Flavonoids and their effects on LDL oxidation

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Summary

Oxidation of low density lipoprotein (LDL) has been implicated in the development of atherosclerosis and there is increasing interest in dietary sources of antioxidants which have the potential to protect LDL from oxidation and thus reduce heart disease risk. Interest in the role of phytochemicals in this protective process is increasing. Phytochemicals (eg red wine phenolic compounds and soy isoflavonoids) have potent antioxidant activity in vitro, however, results of their effects in vivo are controversial, at least with respect to their protection against LDL oxidation.

Introduction

Free radical oxidation has been implicated in a variety of diseases including atherosclerotic cardiovascular disease. Free radicals are chemical species which have one or more unpaired electrons and as such they are extremely reactive and have very short half-lives. They are essential to life in that some have important biological functions, while others are intermediates in crucial biochemical reactions such as respiration. However, because of their high reactivity, free radicals can cause pathological problems by being involved in undesired reactions with biological molecules such as lipids, proteins and DNA (1). It would be expected that the free radical stress would be diminished if antioxidants were increased in the circulation and in tissues. A commonly used method to indirectly detect the effect of increased antioxidant activity in the circulation is to isolate LDL from blood and subject it to oxidative stress in vitro. Oxidation of LDL in vivo has been implicated in the development of atherosclerosis (2). Reactive oxygen species can interact with the polyunsaturated bonds of fatty acids in LDL forming lipid peroxides which are damaging to the LDL particle (2). As a consequence of this modification, oxidised LDL is taken up into macrophages in an unregulated manner (3), resulting in the formation of lipid-laden foam cells which are components of atherosclerotic plaque.

Many studies have examined the effect of the antioxidant vitamins (e.g. vitamins E, C and β-carotene) on LDL oxidation however there is increasing interest in the role of the lesser known phytochemicals in this process. Plants contain numerous compounds with antioxidant potential and two such phytochemicals will be discussed here, namely the phenolic compounds found in wine and the isoflavonoids found in soy.

Red wine phenolic compounds

There is increasing interest in the potential health benefits of wine, particularly in relation to its antioxidant properties. It has been suggested that wine consumption might, in part, account for the 'French Paradox' (4). This paradox relates to the fact that mortality from coronary heart disease (CHD) in France is lower than in the United States of America, even though risk factors for heart disease, such as plasma cholesterol levels, blood pressure and smoking, are similar in both countries (4). A number of studies have reported on the antioxidant properties of red wine (4, 5, 6) and we have recently demonstrated that it is the phenolic compounds in red wine which confer antioxidant protection as measured by in vitro oxidation of human LDL in the presence of copper ions. Copper-mediated oxidation of LDL measures the production of conjugated dienes (intermediates in the production of lipid hydroperoxides) by spectrophotometric absorbance at 234 nm (7). The time during which there is no conjugated diene produced, after

addition of the pro-oxidant (Cu), is referred to as the lag time and is a measure of protection by antioxidants. Table 1 shows that red wine increases the lag time of copper-mediated LDL oxidation, indicating an antioxidant effect of the wine. Red wine which had been stripped of phenolic compounds demonstrated no protective effect, nor did ethanol at the same concentration as in the wine. These results indicate that it is the phenolic compounds in red wine which confer the antioxidant protection (8).

Table 1. Lag time before the onset of copper-mediated oxidation of LDL in the presence of components of red wine during incubation for two hours at 37°C

	Lag time (min)
LDL	58.5 ± 1.1
LDL + red wine (6 mg phenol/L 12.5% ethanol)	>120
LDL + ethanol (12.5%)	59.0 ± 0.8
LDL + stripped wine (12.5% ethanol)	59.3 ± 0.5

The antioxidant effect of red wine on LDL oxidation was dose dependent (Figure 1). White wine also contains phenolic compounds albeit at lower concentrations than in red wine. However, when both red and white wines were diluted to give similar phenolic concentrations (0.16 and 0.17 mg/L respectively) they both led to similar increases in lag time before the onset of oxidation (increases of 33.4 ± 1.7% and 38.3 ± 6.1% for red and white wine respectively) (8). To demonstrate that the protective effect was not due to chelation of copper ions by the compounds in wine, LDL oxidation experiments were also conducted using an azo compound, 2,2'-Azobis - (2-amidinopropane) hydrochloride (AAPH), as pro-oxidant. In this system the production of thiobarbituric acid reactive substances (TBARS) is used as a measure of oxidation (9). The results of this experiment also indicated a dose dependent antioxidant effect of red wine (data not shown). Red wine was fractionated into different classes of phenolic compounds according to their hydrophilic properties as described previously (10). Four fractions were obtained; Fraction 1 contained phenolic acids (29 ± 4% of total phenolics in red wine), Fraction 2 contained catechins and monomeric anthocyanidins (25 ± 4% of total phenolics), Fraction 3 contained flavonols (14 ± 1% of total phenolics) and Fraction 4 contained polymeric anthocyanadins (33 ± 3% of total phenolics). At equivalent phenolic concentrations (0.2 mg/L)

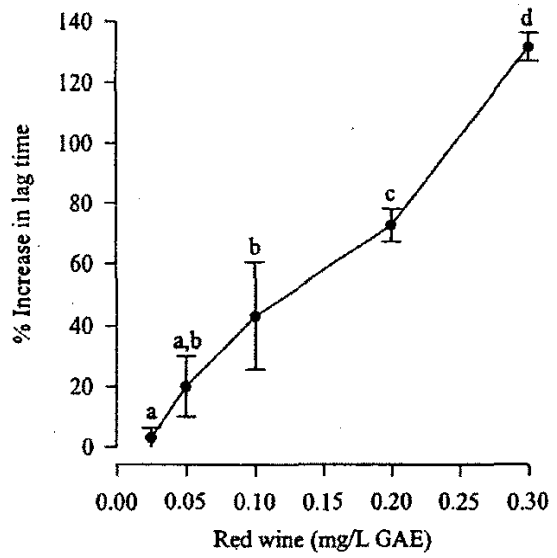

Figure 1
The effect of red wine on the lag time of copper-mediated LDL oxidation in vitro. The LDL (50 mg protein/L) were oxidised in the presence of 5 μM copper and indicated amounts of red wine phenolic compounds expressed as mg/L gallic acid equivalents (GAE). All samples were measured in duplicate. All values are mean ± SD of n=3 experiments. Values with differing alphabetic superscripts are significantly different from each other at $P<0.05$

all fractions inhibited copper-mediated LDL oxidation as shown by the increase in lag time (65.07 ± 18.93%, 92.03 ± 24.21%, 37.03 ± 15.81% and 42.40 ± 12.46% for fractions 1, 2, 3 and 4 respectively) compared to the increase in lag time of 74.87 ± 17.06% with whole red wine

at a concentration of 0.2 mg/L phenolics. Similar results were obtained for inhibition of azo-initiated LDL oxidation by red wine fractions (8).

Support for the protective effects of wine consumption in vivo comes from epidemiological and dietary intervention studies. A number of epidemiological studies have indicated an inverse relationship between the consumption of red wine and mortality from CHD (4, 11-14) and some human dietary intervention studies have claimed beneficial effects of red wine consumption on parameters of lipid oxidation and levels of antioxidants in plasma (15-20) while others have been unable to see any beneficial effects (21, 22). In studies which showed a protective effect associated with wine consumption, wine intake was of the order of 300-600 ml/d (equivalent to 4-9 ml/d/kg body weight) (15, 18-20).

We have recently completed a study in rabbits in which red wine intake was comparable to that used in human studies. Rabbits (n=6) weighing 3.35 ± 0.19 kg consumed approximately 22.5 ml red wine (containing 7.5% ethanol) /d (equivalent to 6.7 ml/d/kg body weight) for three months. This study examined the effects of red wine or ethanol consumption on LDL oxidation and development of atherosclerosis in rabbits made hypercholesterolaemic by feeding a diet containing 0.5% w/w cholesterol. After three months of supplementation the results of copper-mediated oxidation of LDL showed that lag time was significantly reduced in rabbits supplemented with red wine compared to controls. Lag time in the ethanol group was not significantly different from the control and red wine groups (160.8 ± 16.8 min, 112.1 ± 11.1 min and 124.2 ± 9.6 min for control, red wine and ethanol groups respectively, ANOVA P=0.044). There were no significant differences in the development of fatty streaks, as assessed by lipophilic staining of the aortic arch, between the three groups. A recent study (23) which examined the effect of resveratrol (a polyphenolic compound found in grape skins and red wine) was also unable to detect a protective effect on LDL oxidation in hypercholesterolemic rabbits even though resveratrol, like red wine, has been shown to inhibit LDL oxidation in vitro (24). In fact with resveratrol atheroma development was significantly increased (23).

The levels of phenolic compounds in plasma and/or in LDL have not yet been measured in our study, and were not reported in the study by Wilson et al (23). It is possible that phenolic compounds do not associate to any great extent with LDL in plasma and therefore do not exert any beneficial effect with respect to LDL oxidation. However, we have demonstrated, in vitro, that pre-incubation of plasma with red wine prior to isolation of LDL for oxidation studies does result in some association of protective compounds with LDL since there was a 50% increase in lag time after pre-incubation ($P<0.01$) (8). From this degree of protection achieved when plasma was pre-incubated with red wine containing 75 mg/L phenolics, and extrapolating from the dose response curve (Figure 1), which showed a 50% increase in lag time with approximately 0.15 mg/L phenolics, we have estimated that a 500-fold greater concentration of red wine is required in the pre-incubation experiments, compared with direct addition of red wine, suggesting that only about 0.2% of the phenolic compounds in plasma associated with LDL. This is based on the assumption that there is no significant oxidation of phenolics during the incubation with plasma, or non-specific binding of the phenolics to plasma proteins. Our findings are supported in a recent report (25) in which isolated phenolic acids were pre-incubated with plasma prior to isolation of LDL for oxidation studies. The concentration of phenolic acids required to give equivalent protection against LDL oxidation in pre-incubation studies was 200-600 times greater than required when phenolic acids were added directly to LDL incubations. However, one study (15) has provided evidence of incorporation of red wine phenols into lipoproteins in vivo and subsequent antioxidant protection. The level of phenolic compounds in LDL was 1-2 mg/50 mg LDL protein following supplementation of healthy subjects with red wine (15).

Soy Isoflavonoids

The isoflavonoid genistein, present in high concentrations in soybeans, is known to have antioxidant properties. Much of the research impetus into the effects of genistein have focussed on its anti-cancer properties since it has been shown to inhibit growth of cancer cells (26) and inhibit angiogenesis (27). It has been suggested that soy consumption is one dietary factor which may contribute to the lower rates of some cancers in Asian countries (28). Genistein exhibits antioxidant effects in vitro including the inhibition of ADP and NADPH dependent lipid peroxidation in rat liver microsomes (24), suppression of NADH oxidase respiratory chain in rat liver mitochondria (30) and inhibition of the coupled oxidation of β-carotene and linoleic acid (31). Record et al (32) have demonstrated genistein's antioxidant activity in liposomes following a number of pro-oxidant challenges including UV exposure, peroxyl radical lipid peroxidation and hydroxyl radical generating systems. Genistein is also reported to be an effective scavenger of hydrogen peroxide (32, 33). There have been some reports in the literature concerning genistein's antioxidant effect on LDL oxidation (34-36). A recent report (36) describes the kinetics of the effect of genistein on copper-mediated LDL oxidation and shows that 1-10 μM genistein inhibited conjugated diene formation in human serum. Another study (34) was able to show that genistein inhibited copper-mediated LDL oxidation in vitro, but was unable to demonstrate this protective effect when LDL was isolated from plasma which had been pre-incubated with genistein, suggesting that genistein was not incorporated into LDL, although the concentration of genistein in LDL was not measured. We have recently completed studies (unpublished) in which we have examined the effect of genistein on copper-mediated and azo-initiated LDL oxidation in vitro. We have also measured the incorporation of genistein into LDL during pre-incubation of genistein with plasma. Copper-mediated LDL oxidation was inhibited by genistein as shown by an increase in lag time at concentrations of genistein ≥1 μM ($P<0.001$) (Figure 2). Azo-initiated LDL oxidation was also suppressed by genistein at concentrations of 100 and 200 μM, but not at 50 μM. For example after 5 h of incubation of LDL with AAPH the concentration of TBARS (expressed as nmol MDA/mg LDL protein) in the absence of genistein was 15.1 ± 1.2 and in the presence of 50, 100 and 200 μM genistein was 12.5 ± 0.6 (nsd), 11.1 ± 0.4 ($P<0.05$) and 8.2 ± 0.1 ($P<0.05$) respectively.

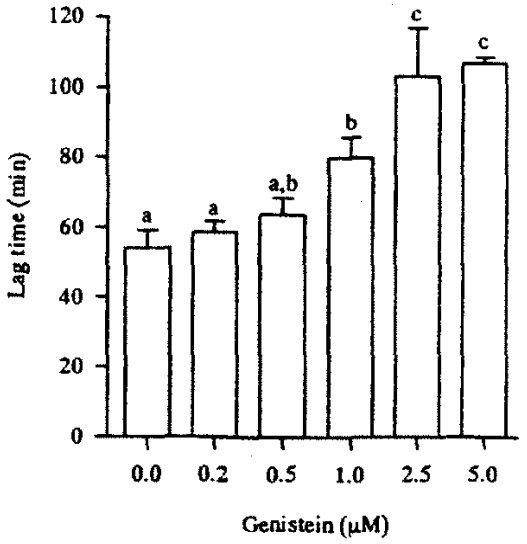

Figure 2
The effect of genistein on the lag time (min) of copper-mediated LDL oxidation in vitro. The LDL (50 mg/L protein) were oxidised in the presence of 5 μM copper and indicated amounts of genistein (μM). All samples were measured in duplicate. All values are mean ± SD of n=3 experiments. Values with differing alphabetic superscripts are significantly different from each other at $P<0.05$

Pre-incubation of plasma with genistein prior to isolation of LDL did not lead to any antioxidant protection. The lag times before the onset of copper-mediated oxidation in control LDL and LDL isolated from plasma incubated with 25, 50 and 100 μM genistein, were 60.5 ± 9.2 min, 60.9 ± 11.3 min, 63.0 ± 6.3 min, and 58.6 ± 8.9 min respectively. The concentrations of genistein measured by HPLC with dual UV and ECD detection in LDL isolated after pre-incubation of plasma with 25, 50 and 100 μM genistein were 0.7 ± 0.2, 2.1 ± 0.1 and 3.7 ± 0.8

nmol/mg LDL protein respectively. However, it is apparent that these concentrations are not sufficient to confer any significant protection from oxidation in vitro. These concentrations of genistein are approximately 30-fold lower than those which were effective in the experiments shown in Figure 2. However, it should be noted that they are higher than the concentrations of endogenous antioxidants such as lycopene (0.3 nmol/mg LDL protein) and ubiquinol-10 (0.2 nmol/mg LDL protein), although lower than α-tocopherol (11.6 nmol/mg LDL protein) (37). The concentration of genistein in human plasma after a single soy based meal (equivalent to 3.6 mmol genistein/kg body weight) was 3.2 μM 10 h after consumption (King RA, Bursill DB, unpublished). This level is about 10-30 fold lower than the concentrations used in our pre-incubation experiments. Further evidence that levels of genistein in plasma, after consumption of soy products, may not be sufficient to confer protection against LDL oxidation comes from a study in which subjects were given 80 mg of a soy concentrate daily (containing 46 mg genistein) for up to 10 weeks. Oxidation of LDL was unchanged in this study although another important measure of arterial health, systemic arterial compliance, was significantly improved after soy supplementation (38).

Conclusion

In conclusion our studies have shown that red wine phenolic compounds have antioxidant activity in vitro. However, studies in cholesterol-fed rabbits showed that consumption of red wine for 12 weeks did not confer any protection against atherosclerosis development or LDL oxidation. In fact there was an apparent pro-oxidant effect of red wine in LDL oxidation in this model as indicated by a reduction in lag time. We have also shown that genistein has antioxidant activity in vitro but in a human dietary intervention study with soy isoflavones no protection of LDL from oxidation was observed. However, arterial compliance was improved in this study indicating a beneficial effect of soy isoflavones. It is apparent that in vitro antioxidant activity of these isoflavones may not necessarily translate into antioxidant protection in vivo at least in relation to the susceptibility of LDL to oxidation.

Acknowledgments

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