Grape Extract Inhibits Lipid Peroxidation of Human Low Density Lipoprotein

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Some epidemiological data have linked dietary polyphenols with a lower risk of coronary heart disease. Polyphenols might impair lipoprotein oxidation which is believed to be an important step in initiating atherosclerosis. The purpose of this study was to determine if grape extract known to contain polyphenolic substances can block copper-induced oxidative modification of human low density lipoprotein (LDL). LDL oxidation was monitored spectrophotometrically by measurement of change in absorbance at 234 nm. Incubation of LDL (0.05 mg protein/ml) with 1.66 µM cupric chloride produced a lag phase of 130 min before onset of the propagation phase where polyunsaturated fatty acids undergo conversion to conjugated lipid hydroperoxides. However, in the presence of grape extract at a final concentration equal to an 8000-fold dilution, the lag phase was extended to 185 min. A 4000-fold and 2000-fold dilution of grape extract produced lag phases of 250 and 465 min, respectively. LDL oxidation was essentially blocked for at least 10 h with a 1000-fold dilution of grape extract. In other experiments, incubation of LDL (0.2 mg protein/ml) with 5 µM cupric chloride for 1–4 h increased both thiobarbituric acid-reactive substances and electrophoretic mobility of LDL on agarose gel. In addition, there was loss of immunoreactivity of LDL with a murine monoclonal antibody against human apolipoprotein B-100. However, these oxidative changes to LDL by copper were prevented when diluted grape extract was present during incubation. It is concluded that grape extract contains antioxidants in the form of polyphenols with the capacity to inhibit oxidative modification of LDL.

Key words low density lipoprotein; polyphenol; lipid peroxidation

It is widely accepted that low density lipoprotein (LDL) must be oxidatively modified to exert its full atherogenic effect. During oxidative modification of LDL, constituent polyunsaturated fatty acids undergo lipid peroxidation with subsequent production of reactive aldehydes. These degradative products then react covalently with the apolipoprotein moiety of LDL. Such oxidized LDL particles are not metabolized via the LDL receptor pathway. Instead, they are metabolized via the scavenger receptor pathway operative in macrophages. As cellular accumulation of oxidized LDL-derived cholesterol occurs, macrophages are eventually transformed into foam cells, whose presence marks the initial onset of atherosclerosis.

Much attention has been focused on identifying dietary factors capable of inhibiting oxidative modification of LDL. For example, ascorbic acid and vitamin E5) and also β-carotene6) can each inhibit LDL oxidation in vitro. Thus, when also considering epidemiological findings,5) these three dietary antioxidants might be of value in opposing atherosclerotic disease. However, actual clinical testing is in order before specific recommendations can be made regarding dietary antioxidant supplementation as a possible means of attenuating or even preventing atherosclerosis.6)

Lately, some attention has been given to other dietary antioxidants that might have an impact in countering atherosclerosis. Two recent reports have brought the polyphenols into the limelight. Frankel et al.7) and Kanner et al.8) demonstrated that polyphenolic substances in red wine possess antioxidant activity and can inhibit oxidative modification of LDL. Based on these findings, they believe that polyphenolic substances may protect against coronary heart disease in the French population that drink red wine regularly. It is logical to assume that polyphenolic substances in red wine originate naturally from grapes. Therefore, we asked the question would extract of grapes, i.e., grape juice, inhibit oxidative modification of LDL as well. Accordingly, the aim of this study was to evaluate the capacity of pure Concord grape juice to block copper-induced oxidative modification of human LDL. We demonstrate that polyphenolic antioxidants present in wine capable of inhibiting LDL oxidation9) are apparently present in wine's non-alcoholic counterpart, grape juice. As suspected, grape juice was able to similarly inhibit LDL oxidation.

MATERIALS AND METHODS

Isolation of Human LDL Plasma was recovered after low-speed centrifugation of fresh human blood collected in the presence of disodium ethylenediamine tetraacetic acid (EDTA) (1.5 mg/ml). LDL was isolated from plasma by discontinuous density gradient ultracentrifugation as previously described.8) The background solvent density of plasma was raised to 1.21 g/ml by mixing with potassium bromide (0.3265 g/ml). Using a Beckman L7-65 ultracentrifuge and 50.3 Ti rotor, density-adjusted plasma was centrifuged at 114000 x g for 40 h at 18 °C to float all lipoprotein class particles. After recovery of the total lipoprotein fraction and adjustment to a final density of 1.21 g/ml, 3.4 ml of the preparation was transferred into Beckman 50 Ti rotor tubes and overlaid with 8.5 ml of a sodium chloride solution containing 0.01% EDTA (d = 1.006 g/ml). Tubes were centrifuged at 105000 x g for 4 h. LDL appeared as a distinct yellow-orange band in the middle of the tubes and was recovered by suction after side-puncturing the tubes with a needle/syringe assembly.

Agarose Gel Electrophoresis Purity of the isolated

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LDL was verified by agarose gel electrophoresis using a Ciba-Corning electrophoresis system/lipoprotein gel kit and following the manufacturer's instructions. A single lipoprotein band was observed on 1% agarose gel after staining with Fat Red 7B. This electrophoretic approach was also used to evaluate changes in electrophoretic mobility of LDL after oxidative modification.

Oxidative Modification of LDL  Prior to use, LDL was dialyzed extensively at 4°C against 10 mM phosphate-buffered saline (PBS), pH 7.4, to remove EDTA. After filtration through a 0.45 micron filter, LDL was stored at 4°C under nitrogen gas and used within 2 weeks. Protein content of the LDL was determined as described by Markwell et al.9) Bovine serum albumin was used as the protein standard. LDL (0.2 mg protein/ml) was incubated with 5 μM cupric chloride in the absence and presence of pure whole-pressed Concord grape juice (After The Fall Products, Inc., Brattleboro, Vermont). This bottled product is claimed to contain no additives including sugar, preservatives, and artificial flavors. Upon opening the bottle for the first time, a portion of the grape juice was removed and filtered through a 0.45 micron filter. The concentration of total phenolics in the filtered grape juice was determined colorimetrically,10) using gallic acid as the standard. Filtered grape juice was stored at 4°C under nitrogen gas and used within 2 weeks. Deionized water was used to dilute grape juice to give final concentrations in the incubation mixture equal to a 100-fold to 8000-fold dilution. LDL not treated with copper or grape juice served as the control. Incubation was performed at 37°C for 1–4 h, depending upon which of the four experimental methods was going to be subsequently used to assess extent of LDL oxidation.

Assay of Thiobarbituric Acid-Reactive Substances (TBARS) TBARS levels were determined spectrophotometrically.11) To 0.1 ml aliquots of post-incubation mixture and also tetramethoxypropane standards (0–4 nmol) were added 1 ml of 20% trichloroacetic acid and 1 ml of 1% thiobarbituric acid containing EDTA (79 mg/100 ml). Tubes were placed in a boiling water bath for 30 min. After cooling, tubes were centrifuged at 1500 × g for 15 min. Absorbance of the supernate was measured at 532 nm.

Immunodot-Blotting Aliquots of the post-incubation mixture was diluted 50-fold to give a final LDL protein concentration of 4 μg/ml. Diluted samples (100 μl) were applied in duplicate to a nitrocellulose membrane sheet positioned in a Bio-Rad Dot-Blot Apparatus. Non-specific protein binding sites on the membrane sheet were blocked with 3% gelatin in tris-buffered saline or TBS. After washing with TBS containing 0.05% Tween 20 (TTBS), the membrane sheet was incubated overnight with 1000-fold dilution of a murine monoclonal antibody to human apolipoprotein B-100 (clone 6-H12, Organon Teknika Corp., Durham, NC). This step was followed by washing with TTBS. The membrane sheet was then incubated for 2 h with 3000-fold dilution of a goat anti-mouse immunoglobulin G/alkaline phosphatase conjugate, supplied as part of an Immun-Blot kit (Bio-Rad, Melville, NY). Finally, after washing with TTBS and then TBS, the membrane sheet was incubated with the supplied color reagent system. Purple immunodot-blot were scanned on a Bio-Rad GS model GS-670 Imaging Densitometer in the reflectance mode.

Continuous Monitoring of Conjugated Diene Formation Formation of conjugated dienes during LDL oxidation was monitored continuously using the spectrophotometric method of Estebauer et al.,12) which is based on measurement of change in absorbance at 234 nm over time. Concentrations of LDL and copper were 0.05 mg protein/ml and 1.66 μM, respectively. Absorbance readings were recorded at 5 min intervals for 10 h.

RESULTS

The concentration of total phenolic substances in the filtered grape juice was 3.4 mg (18 μmol) gallic acid equivalents per ml. Four experimental methods were used to critically evaluate the capacity of this grape juice to inhibit oxidative modification of LDL. Initially, TBARS levels were measured (Table 1). Incubation of LDL with copper resulted in formation of about 3 nmol TBARS/mg LDL protein, compared to no detectable amounts in control LDL samples. However, when diluted grape juice was present during incubation of LDL with copper, formation of TBARS was inhibited. Although a concentration of grape juice equivalent to a 4000-fold dilution in the final incubation volume had only a marginal effect, a 2000-fold dilution of grape juice inhibited TBARS formation by 44%. A 1000-fold dilution of grape juice essentially prevented any TBARS formation. No detectable amounts of TBARS were found with a 500-fold dilution of grape juice. In the second experimental method, the capacity of diluted grape juice to inhibit LDL oxidation was evaluated spectrophotometrically by continuously monitoring change in absorbance at 234 nm (Fig. 1). Incubation of LDL with copper produced a lag phase of 130 min before onset of the propagation phase where polysaturated fatty acids undergo conversion to conjugated lipid hydroperoxides. However, in the presence of grape juice at a final concentration equal to an 8000-fold dilution, the lag phase was extended to 185 min. A 4000-fold and 2000-fold dilution of grape juice produced lag phases of 250 and 465 min, respectively. LDL oxidation

<table>
<thead>
<tr>
<th>Sample</th>
<th>TBARS (nmol/mg LDL protein)</th>
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<tbody>
<tr>
<td>Control LDL</td>
<td>N.D.</td>
</tr>
<tr>
<td>LDL/copper</td>
<td>32.8</td>
</tr>
<tr>
<td>LDL/copper/4000-fold dilution of grape juice</td>
<td>28.4</td>
</tr>
<tr>
<td>LDL/copper/2000-fold dilution of grape juice</td>
<td>18.4</td>
</tr>
<tr>
<td>LDL/copper/1000-fold dilution of grape juice</td>
<td>0.3</td>
</tr>
<tr>
<td>LDL/copper/500-fold dilution of grape juice</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

LDL (0.2 mg protein/ml) was incubated at 37°C for 2 h with 5 μM cupric chloride and various concentrations of grape juice equal to 4000-fold to 500-fold dilutions in the final incubation volume. To 0.1 ml aliquots of post-incubation mixture and tetramethoxypropane standards (0–4 nmol), was added 1 ml of 20% trichloroacetic acid and 1 ml of 1% thiobarbituric acid containing EDTA (79 mg/100 ml). Tubes were placed in a boiling water bath for 30 min. After cooling, tubes were centrifuged at 1500 × g for 15 min. Absorbance of the supernate was measured at 532 nm. Values are the average of duplicate determinations. N.D. none detected.
was essentially blocked for at least 10 h with a 1000-fold dilution of grape juice.

The third experimental method, viz., agarose gel electrophoresis, produced results (Fig. 2) in accord with data from the previous two methods. Based on migration distance of lipoprotein particles from the origin (indicated by arrow) to the anode, electrophoretic mobility of copper-treated LDL (lane 2) was increased relative to control LDL (lane 1, 8). Electrophoretic mobility of LDL treated with copper in the presence of either a 4000-fold (lane 3) or 2000-fold (lane 4) dilution of grape juice was the same as that of LDL treated with copper alone (lane 2). Presence of a 1000-fold dilution of grape juice during incubation of LDL with copper (lane 5) prevented slightly the increase in electrophoretic mobility. However, the effect of grape juice was readily apparent at 500-fold (lane 6) and 100-fold (lane 7) dilutions.

Lastly, an immunochemical method was used to assess the protective effect of grape juice against copper-induced LDL oxidation. The basic principle here is that the structure of an antigen (apolipoprotein B-100) must be reasonably intact in order for it to react with a specific antibody (murine monoclonal antibody against human apolipoprotein B-100). Hence, an immunodot-blot assay was performed to indirectly detect structural damage or alteration of the apolipoprotein B-100 moiety of LDL after being incubated with copper in the absence and presence of grape juice. The resulting immunodot-blots on nitrocellulose membrane sheet are shown in Fig. 3. Columns A1/A2, B1/B2, and C1/C2 represent paired duplicate immunodot-blots after 1, 2, and 4 h of incubation, respectively. Row 1 represents control LDL, while row 2 represents copper-treated LDL. Rows 3–8 represent LDL treated with copper in the presence of various dilutions of grape juice (8000-fold to 100-fold). In essence, control LDL produced a good immunodot signal. In contrast, the immunoreactivity of LDL with the murine monoclonal antibody decreased with increasing time of prior incubation of LDL with copper, as evidenced by loss of immunodot signal. However, this loss of immunoreactivity was gradually prevented by the presence of increasing concentrations of diluted grape juice during incubation of LDL with copper.

When the immunodot-bLOTS on the nitrocellulose membrane were scanned by a densitometer, data appearing in Table 2 were generated. Overall, the relative densitometric intensity (RDI) values confirm visual interpretation of the immunodot-bLOTS. Immunodot-bLOTS corresponding to control LDL produced relatively high RDI values indicative of good immunoreactivity. In contrast, immunodot-bLOTS corresponding to LDL incubated with copper alone produced lower RDI values indicative of loss of immunoreactivity. However, immunodot-bLOTS corre-

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**Fig. 1.** Effect of Various Dilutions of Grape Juice on Copper-Induced Oxidation of Human LDL Determined Spectrophotometrically by Continuous Monitoring of Increase in Absorbance at 234 nm by Conjugated Dienes

LDL (0.05 mg protein/ml) was incubated at 25°C with 1.66 μM cupric chloride in the absence (plot A) or presence of grape juice equal to 8000-fold (plot B), 6000-fold (plot C), 4000-fold (plot D), 2000-fold (plot E), and 1000-fold (plot F) dilutions in the total incubation volume. Length of time (lag phase) before onset of the propagation phase where conjugated lipid hydroperoxides are formed was 130, 185, 220, 250, 465 min for plots A, B, C, D, and E, respectively. Note that grape juice at 1000-fold dilution essentially blocked lipid peroxidation in LDL for at least 10 h (plot F).

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**Fig. 2.** Agarose Gel Electrophoresis of Control Human LDL and LDL Treated with Copper in the Absence and Presence of Grape Juice

LDL (0.2 mg protein/ml) was incubated for 4 h at 37°C with 5 μM cupric chloride and various concentrations of grape juice equal to 4000-fold to 100-fold dilutions in the final incubation volume. Samples were applied to 1% agarose gel. Staining was performed with Fast Red 7B. Lane 1—control LDL. Lane 2—LDL/copper. Lane 3—LDL/copper/4000-fold dilution of grape juice. Lane 4—LDL/copper/2000-fold dilution of grape juice. Lane 5—LDL/copper/1000-fold dilution of grape juice. Lane 6—LDL/copper/500-fold dilution of grape juice. Lane 7—LDL/copper/100-fold dilution of grape juice. Lane 8—control LDL. The point of origin of sample migration is indicated by the arrow.
Fig. 3. Evaluation by Immunodot-Blotting of the Effect of Grape Juice on Copper-Induced Oxidative Modification of Human LDL.

LDL (0.2 mg protein/ml) was incubated at 37°C with 5 μM cupric chloride and various concentrations of grape juice equal to 8000-fold to 100-fold dilutions in the final incubation volume. After incubation times of 1, 2, and 4 h, samples were applied to nitrocellulose membrane followed by immunodetection as described under Materials and Methods. Columns A1 and A2 duplicate dots after 1 h of incubation. Columns B1 and B2 duplicate dots after 2 h of incubation. Columns C1 and C2 duplicate dots after 4 h of incubation. Row 1 control LDL. Row 2 - LDL/copper. Row 3 - LDL/copper/8000-fold dilution of grape juice. Row 4 - LDL/copper/4000-fold dilution of grape juice. Row 5 - LDL/copper/1000-fold dilution of grape juice. Row 6 - LDL/copper/100-fold dilution of grape juice. Row 7 - LDL/copper/500-fold dilution of grape juice. Row 8 - LDL/copper/1000-fold dilution of grape juice.

Table 2. Results from Densitometric Scanning of Immunodot-Blots on Nitrocellulose Membrane Sheet Shown in Fig. 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>RDI Time of incubation</th>
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<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Control LDL</td>
<td>3.92</td>
</tr>
<tr>
<td>LDL/copper</td>
<td>2.61</td>
</tr>
<tr>
<td>LDL/copper/8000-fold dilution of grape juice</td>
<td>2.80</td>
</tr>
<tr>
<td>LDL/copper/4000-fold dilution of grape juice</td>
<td>2.80</td>
</tr>
<tr>
<td>LDL/copper/2000-fold dilution of grape juice</td>
<td>2.80</td>
</tr>
<tr>
<td>LDL/copper/1000-fold dilution of grape juice</td>
<td>3.34</td>
</tr>
<tr>
<td>LDL/copper/500-fold dilution of grape juice</td>
<td>3.66</td>
</tr>
<tr>
<td>LDL/copper/100-fold dilution of grape juice</td>
<td>3.62</td>
</tr>
</tbody>
</table>

Numbers represent the average RDI values from duplicate dots.

Responding to LDL incubated with copper in the presence of diluted grape juice produced RDI values noticeably higher than those of immunodot-blots corresponding to LDL incubated with copper alone. Hence, it can be concluded upon comparison of the RDI values that diluted grape juice was able to significantly prevent loss of immunoreactivity when LDL was incubated with copper to induce oxidative modification.

DISCUSSION

Appreciable interest has been generated by recent studies suggesting that consumption of polyphenolic substances in red wine may oppose atherogenesis by blocking oxidative modification of LDL. In one of these previous studies, isolated human LDL was incubated with cupric ions to induce oxidative modification. There was elevation of hexanal and conjugated dienes resulting from lipid peroxidation. However, when LDL was incubated with cupric ions in the presence of diluted red wine, levels of hexanal and conjugated dienes were reduced. Red wine equal to a 500-fold dilution completely blocked copper-induced oxidation of LDL. Polyphenolic substances extracted from red wine were found to possess the potent antioxidant activity. It was concluded that these polyphenolic substances did not act by binding cupric ions, suggesting that they may act instead by donating hydrogen atoms to neutralized lipid radicals. Plant polyphenols with antioxidant potential are known to react with aqueous peroxyl radicals.13

In the present study, extract of grapes (grape juice) from which wine can be derived also inhibited copper-induced oxidative modification of LDL. After incubation of LDL with copper, there were increases in conjugated dienes, TBARS, and agarose gel electrophoretic mobility. These changes are known to occur upon oxidative modification of LDL.12.14 In addition, copper treatment caused loss of immunoreactivity of LDL with a specific murine monoclonal antibody against human apolipoprotein B-100, indicating structural damage or alteration of the apolipoprotein. A similar immunochromical finding has been reported following oxidative modification of LDL.15 In contrast, when LDL was incubated with copper in the presence of diluted grape juice, oxidative modification was inhibited in a dose-dependent manner. In other words, addition of grape juice to the incubation mixture prevented formation of TBARS and conjugated dienes, increase in electrophoretic mobility, and loss of immunoreactivity.

It was beyond the scope of our work to identify the exact compound(s) in Concord grape juice that inhibited LDL oxidation. The inhibitory effect was most likely provided by polyphenolic substances, which were measured as total phenolics.10 Whole grapes contain polyphenolic substances capable of acting as antioxidants.19 Preliminary work in our laboratory has shown that filtered homogenates prepared from several whole grape varieties can inhibit LDL oxidation. Four monomeric anthocyanins with potent antioxidant activity have been identified in Muscat Bailey A grapes,16 and Concord grapes also contain various anthocyanins.17 Moreover, the flavonoids, quercetin and myricetin, have been detected in grape juice.18 Therefore, these and possibly other related polyphenolic antioxidants might be responsible for the protective effect of Concord grape juice against LDL oxidation.

Various dietary factors other than polyphenols have the capacity to inhibit oxidative modification of LDL in vitro. The most notable are ascorbic acid and vitamin E31 and
also β-carotene. In contrast to ascorbic acid, both vitamin E and β-carotene along with some other lipophilic antioxidants such as lycopene, cryptoxanthin, canthaxanthin, zeaxanthin, lutein, phytofluene, and ubiquinol-10 can be found integral to LDL. Furthermore, it is believed that LDL isolated from plasma may contain additional lipophilic antioxidants other than the aforementioned ones. Whether polyphenols are actual constituents of LDL is unknown. Information concerning the bioavailability of polyphenols consumed in the diet is lacking, as pointed out. On the other hand, there is evidence suggesting that significant absorption of polyphenols occurs after consumption to have a beneficial effect. Recently, it was reported that consumption of red wine results in enhanced serum antioxidant activity. Also, LDL isolated from red wine drinkers was found to be more resistant to oxidative modification in vitro. In both of these studies, serum or LDL polyphenol levels were not quantified. On the other hand, a more recent study has reported elevation of total polyphenols associated with LDL from subjects drinking red wine. It would be of interest to determine if consumption of grape juice or other polyphenol-containing fruit juices can have a similar effect as red wine in enhancing resistance of LDL to oxidation. Therefore, the physiological significance of the present work is in need of further investigation, especially in view of the recent study, establishing an inverse relationship between coronary heart disease mortality and the levels of polyphenols consumed in the diet.

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REFERENCES

1) This study was presented at the annual meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, April 1994, [FASEB J. 8, A449 (abstract No. 2599), 1994].