Flavonoid metabolites and susceptibility of rat lipoproteins to oxidation

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Benito, Sonia, Susana Buxaderas, and M. Teresa Mitjavila. Flavonoid metabolites and susceptibility of rat lipoproteins to oxidation. Am J Physiol Heart Circ Physiol 287: H2819–H2824, 2004.—Flavonoids are ingested with vegetables and beverages and exert a beneficial effect on cardiovascular disease. Studies in animals in vitro and in humans ex vivo on the resistance of lipoproteins to oxidation are not consistent and the mechanisms by which flavonoids protect against atherosclerosis are a matter of debate. In the present study, we investigated the effects of administering diets containing 0.3% (wt/wt) quercetin, 0.3% (wt/wt) catechin, or 35% (vol/wt) dealcoholated red wine (DRW) for 10 days in healthy rats on markers of oxidative damage in lipoproteins and in plasma. The antioxidant levels in low-density lipoproteins (LDL) or the lag phase, oxidation rate, and maximum level of conjugated dienes during ex vivo LDL oxidation did not differ between control and treated rats. Plasma levels of α-tocopherol and retinol were similar in all groups. The total antioxidant status of the plasma from rats fed either quercetin or DRW diet was higher than in control rats. Only glucuronide and sulfate compounds of quercetin were detected in plasma from rats fed the quercetin-rich diet, and no flavonoids or their metabolites were detected in plasma or LDL from rats fed the catechin- or the DRW-rich diet. No significant differences in malondialdehyde or in conjugated dienes in plasma were observed. These results indicate that although metabolites from quercetin are present in plasma, they are not detected in lipoproteins and do not modify the level of other antioxidants. In conclusion, in the absence of any pathology or of oxidative stress the intake of quercetin, catechin, or DRW did not protect lipoproteins from oxidation ex vivo.

quercetin; catechin; wine; antioxidants; atherosclerosis

THE PROTECTIVE EFFECT OF FLAVONOIDS IN THE DIET IS OF MUCH INTEREST AND THE BIOLOGICAL ACTIVITY OF THESE POLYPHENOLIC COMPOUNDS IS OF GREAT IMPORTANCE IN UNDERSTANDING THE HEALTH BENEFIT DERIVED FROM A DIET RICH IN FLAVONOIDS. FLAVONOIDS LIKE QUERCETIN AND CATECHIN ARE POTENT ANTI-OXIDANTS IN VITRO (35) AND REDUCE THE OXIDATIVE DAMAGE TO LDL MEDIATED BY MACROPHAGES THAT IS INVOLVED IN Atherosogenesis (14, 36). IN VITRO STUDIES SUGGEST THAT THE PROTECTION IS ACHIEVED BY CHELATING Cu2+ IONS (6), SCAVENGING SUPEROXIDE ANION, AND SINGETL OXYGEN OR SPARING ASSOCIATED ANTI-OXIDANTS OF LDL (14).

RED WINE POLYPHENOLS ATTENUATE THE ApoB100 PRODUCTION AND INCREASE LDL RECEPTOR EXPRESSION IN CULTURED HEPATO CYTES (30). IN ADDITION, THE CONSUMPTION OF RED WINE, RICH IN QUERCETIN AND CATECHIN IN THE OXIDATIVE RESISTANCE OF LDL TO OXIDATION EX VIVO ARE SCARCE AND NOT CONSISTENT (13, 18, 19, 27) AND THE MECHANISMS BY WHICH DIETARY FLAVONOIDS MIGHT PROTECT LDL AGAINST OXIDATION ARE UNKNOWN. ALTHOUGH THERE IS EVIDENCE OF INTESTINAL ABSORPTION OF FLAVONOIDS, SUCH AS QUERCETIN (11, 28, 41), ONE STUDY (26) SHOWED THAT FLAVONOIDS ARE METABOLIZED BY ENTEROCYTES AND BY THE LIVER. METABOLITES FROM QUERCETIN AND CATECHIN ARE DETECTED IN PLASMA (1, 15, 26). THE METABOLISM IMPLIES SUBSTITUTION OF PHENOLIC GROUPS, WHICH MODIFIES THE PHYSICAL-CHEMICAL AND BIOLOGICAL PROPERTIES OF FLAVONOIDS AND THUS THEIR EFFECTIVENESS IN VITRO (44). NEVER THELESS, LITTLE IS KNOWN ABOUT THE BIOLOGICAL ACTIVITIES OF THE METABOLITES OF FLAVONOIDS THAT APPEAR IN BLOOD. ALSO, ONLY TWO STUDIES (22, 38) HAVE BEEN PUBLISHED ON THE PRESENCE OF FLAVONOIDs OR THEIR METABOLITES IN LDL IN HUMANS, BUT THERE HAVE BEEN NO SUCH STUDIES IN RATS. FLAVONOIDS ARE MORE HYDROPHILIC THAN THEIR AGLYCONS AND ARE LIKELY TO BE LINKED TO LDL, INCREASING THEIR RESISTANCE TO OXIDATION, EITHER DIRECTLY BY PROTECTING LDL LIPIDS AND PROTEINS OR INDIRECTLY BY REGENERATING LDL ANTI-OXIDANTS.

THE PURPOSE OF THIS STUDY WAS TO ASSESS WHETHER THE FORMS OF FLAVONOIDs PRESENT IN PLASMA AFTER THEIR ABSORPTION AND METABOLISM PROTECT RAT LIPOPROTEINS AND PLASMA AGAINST OXIDATION. THE PRESENCE OF FLAVONOIDS OR THEIR METABOLITES IN LIPOPROTEINS HAS ALSO BEEN STUDIED.

METHODS

ANIMALS AND DIETS. Male Sprague-Dawley rats weighing ~175 g were purchased from Harlan Interfauna Ibérica, Barcelona, Spain. The rats were housed in temperature-controlled rooms (21–23°C), with 40–60% humidity, and exposed to a 12:12-h light-dark cycle. Rats were divided into four groups and for 10 days fed one of the following semipurified diets (Table 1) prepared in our laboratory: 1) control diet, 2) 0.3% (wt/wt) quercetin diet, 3) 0.3% (wt/wt) catechin diet, and 4) 35% (vol/wt) dealcoholated red wine (DRW) diet. A high amount of flavonoids or DRW was used to favor their detection in LDL because most animal and human trials of oral dosages of quercetin aglycon show absorption in the vicinity of 20%. Diets were manufactured and stored at ~20°C under vacuum until use to prevent oxidation and loss of antioxidants. Fresh food was provided once a day. Animals had free access to food and water. The experimental protocols were reviewed and approved by the University of Barcelona Ethical Committee for Animal Experimentation, in accordance with the European Community guidelines.

DRW was prepared from a common commercial wine made in Spain. Alcohol was removed in a rotary evaporator at a maximum temperature of 30°C. Vacuum was applied progressively up to ~70 bars to avoid mechanical stress. Evaporated ethanol was replaced by acidulated distilled water and the pH was adjusted to the original wine pH. Gas chromatography analysis was used to determine traces of ethanol in the DRW. Total polyphenols of the red wine and DRW were measured by the Folin-Ciocalteau colorimetric method, with the use of gallic acid as standard. The phenolic and anthocyanin content of the red wine and the DRW was evaluated by HPLC with diode array detector, as described by Castellari et al. (9). At the end of the...
Table 1. Composition of semipurified diets

<table>
<thead>
<tr>
<th>Components</th>
<th>Control</th>
<th>Quercetin</th>
<th>Catechin</th>
<th>DRW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casin</td>
<td>225</td>
<td>224</td>
<td>224</td>
<td>225</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>446</td>
<td>445</td>
<td>445</td>
<td>446</td>
</tr>
<tr>
<td>Saccharose</td>
<td>223</td>
<td>222</td>
<td>222</td>
<td>223</td>
</tr>
<tr>
<td>Cellulose</td>
<td>31</td>
<td>31</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>L-Lys-Methionine</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>All-rac-α-tocopherol acetate</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>0</td>
<td>3.00</td>
<td>3.00</td>
<td>350</td>
</tr>
<tr>
<td>Water</td>
<td>350</td>
<td>350</td>
<td>350</td>
<td>0.0</td>
</tr>
</tbody>
</table>

DRW, dealcoholated red wine. Casin was vitamin-free delipidated. For flavonoids, the DRW replaced the 350 ml of water added to compact the diet.

dietary period, rats were fasted for 18 h. Blood was withdrawn from the heart into heparinized tubes under sodium urethane (1.5 g/kg ip).

Plasma was obtained by blood centrifugation at 1,700 g at 4°C. Isolation and oxidation of LDL. LDL were isolated from freshly prepared plasma from individual rats. This fraction contained LDL plus VLDLs and was verified by electrophoretic mobility. However, the VLDLs were poorly represented and this fraction will be referred to as LDL. The LDL fraction was obtained by adjusting plasma samples to 1.063 g/ml by the addition of solid NaBr and overlayed with PBS (20 mM sodium phosphate with 0.15 M NaCl, pH 7.4) containing 0.01% EDTA and NaBr. The density of this solution was 1.063 g/ml as verified with a pycnometer. Centrifugation was performed at 100,000 g for 20 h at 4°C. The top fraction (LDL) was passed through a PD10 column (Amersham Pharmacia Biotech; Buckinghamshire, UK) to remove NaBr and EDTA and concentrated with filters (Biomax membrane 10,000 Da, Millipore; Bedford, MA). This purified LDL fraction was immediately used for the kinetic oxidation study. LDLs were also used without the removal of EDTA. LDL aliquots for flavonoids and the determination of metabolites were immediately acidified to pH 4.9 by adding 18 μl of 0.58 M acetic acid and stored at −80°C. For the other measurements (α-tocopherol and retinol), aliquots were stored at −80°C in 10 mM butylated hydroxytoluene in the dark until analysis. Additional aliquots were stored at −80°C for total antioxidant status (TAS) determination. The LDL fractions were used within 1 wk. Protein concentration was measured in aliquots of LDL and plasma by the Bradford method (Bio-Rad; Hercules, CA), using BSA as standard.

LDL oxidation was performed with 50 μg/ml LDL protein freshly isolated and purified in quartz cuvettes. Oxidation was initiated by the addition of CuCl2 (6 μM final concentration) in oxygenated PBS (pH 7.4). The kinetic of the oxidation monitored the increase in the conjugated dienes (CD) absorbance at 234 nm every 10 min at 37°C for up to 5 h in a spectrophotometer equipped with a thermostat-controlled multiple cell positioner. The end of the lag phase (in min) was defined as the intercept of the straight lines derived from the lag phase and the propagation phase. The maximum rate of formation of CD (nmol/g LDL protein/min), defined as the tangent of the steepest part of the oxidation curve, and the maximum amount of CD formed were calculated using a molar extinction coefficient of 2.95 × 104 M/cm.

α-Tocopherol and retinol were measured (4) in 100 μg LDL protein by using 100 μl of 0.1 mM α-tocopherol acetate as internal standard. Lipids were extracted by mixing LDL/water/methanol (0.2:1:1; vol/vol/vol) in a glass tube. Then, 3 ml of n-hexane were added to the mixture, vortex stirred, and centrifuged to separate the organic phase. The organic phase was dried under a continuous stream of N2 and subsequently redissolved in 250 μl of ethanol. α-Tocopherol and retinol were separated on a LiChrospher 100 RP 18 (250 mm × 4.6 mm, 5 μm) supplied by Amersham Pharmacia (Uppsala, Sweden) with 100% methanol as eluent at a flow rate of 1.5 ml/min and their ultraviolet absorption at 290 and 325 nm, respectively, was recorded (16) by HPLC (Merek-Hitachi; Darmstadt, Germany). The internal standard recovery was 95–98%. TAS was assayed in 100 μg of LDL protein using the TAS kit (Randox Laboratories; Crumlin, UK), based on the inhibition by antioxidants of the absorbance of the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS), formed by the interaction of ABTS with the ferrylmyoglobin radical species generated by the activation of metmyoglobin with H2O2.

For the detection of flavonoids and their metabolites, 800 μg of LDL protein from a pool of four rats were used and incubated for 30 min at 37°C in a shaking water bath in the absence or presence of both 5 × 10−6 U/l β-glucuronidase and 2.5 × 10−5 U/l sulfatase from Helix pomatia. For β-glucuronidase one unit is 1 μM of 4-nitrophenol-released from 4-nitrophenol-β-D-glucuronide/min at pH 7 and 37°C. For sulfatase one unit is 1 μmol of 4-nitrophenyl-sulfate hydrolyzed per minute at pH 7.1 and 37°C. The reaction was stopped by the addition of 1 ml of methanol, mixed by vortex for 1 min, sonicated for 30 s, and centrifuged. The supernatant was evaporated under nitrogen stream and redissolved with methanol to a volume of 250 μl. Quercetin and its metabolites were analyzed by HPLC (26). Catechin and its metabolites were detected as described by Piskula and Terao (32). Flavonoids from wine were also determined. The percentage of recovery was estimated to be >90% using pure quercetin or catechin in control samples treated in parallel. Detection limits were 0.25 μM for quercetin and 0.5 μM for catechin. The quality control of the intra- and interassays was >95% of reproducibility.

Assays in plasma. Plasma aliquots (500 μl) for α-tocopherol and retinol, malondialdehyde (MDA), and CD were stored at −80°C for one week with 10 μl of 10 mM butylated hydroxytoluene to prevent further oxidation. Additional 10 μl of plasma were stored at −80°C for TAS determination. Plasma samples (180 μl) for flavonoids and their metabolite determinations were immediately acidified to pH 4.9 by adding 18 μl of 0.58 M acetic acid to prevent a drift of pH and stored at −80°C. All the assays with plasma were performed within 1 wk. α-Tocopherol and retinol (100 μl) and TAS (5 μl), quercetin, catechin, and wine flavonoids or their metabolites (189 μl) were evaluated as described for LDL.

MDA and CD were assayed as markers of oxidative damage. MDA equivalents were measured in 200 μl of plasma by the thiobarbituric acid reactive substance method with the use of tetraethoxypropane as standard (43), in the presence of hydroxytoluene and EDTA to avoid oxidation during the process. Thiobarbituric acid-reactive substances were extracted with butanol (vol/vol). The tubes were centrifuged at 450 g for 10 min, and the absorbance of the upper phase was measured at 535 nm. CD and total lipids were extracted from 100 μl of plasma with chloroform and methanol (vol/vol) by being mixed for 10 min at 4°C and then centrifuged at 1,500 g for 3 min at 4°C. The supernatant was removed and 1 ml of distilled water was added, mixed for 10 min, and centrifuged in the same conditions. The chloroform phase was adjusted to a final volume of 2 ml with chloroform. An aliquot of the organic phase was carefully removed, dried in nitrogen stream at 37°C, resuspended in cyclohexane and CD were measured at 234 nm. Another aliquot was used to evaluate the total lipid concentration in plasma using a colorimetric test (bioMérieux; Lyon, France).

Drugs. All chemicals were of analytic grade and purchased from Sigma (St. Louis, MO), with the exception of starch (Panreac Quimica; Barcelona, Spain) and the mineral and vitamin mix (ICN Biomedicals; Aurora, OH).

Statistical analysis. Data are expressed as means ± SE. Results were compared with the control group by the Student’s t-test for unpaired observations.

RESULTS

DRW and diets. The gas chromatography analysis showed that DRW can be considered a nonalcoholic drink (alcohol
alter the content of total phenols (3.1 ± 0.5 g/l) and phenolic content, mg/l

<table>
<thead>
<tr>
<th>Phenolic content, mg/l</th>
<th>Red Wine</th>
<th>DRW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>3.0 ± 0.8</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Catechin</td>
<td>1.5 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Anthocyanin content, mg/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malvidin</td>
<td>5.9 ± 1.0</td>
<td>6.0 ± 0.8</td>
</tr>
<tr>
<td>Peonidin</td>
<td>10.5 ± 0.5</td>
<td>10.2 ± 0.1</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>7.2 ± 1.0</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td>Malvidin-3-acetate</td>
<td>4.5 ± 0.8</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Malvidin-3-paracumarate</td>
<td>4.5 ± 0.5</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 4 bottles of the same wine. No statistically significant differences between the two wines were observed.

degree < 3 g/l) (Table 2). The dealcoholization process did not alter the content of total phenols (3.1 ± 1.5 and 3.2 ± 0.5 g/l in red wine and DRW, respectively, expressed as gallic acid equivalents) (Table 2). The phenolic compounds and anthocyanin content of DRW were similar to those of red wine before dealcoholization (Table 2).

All groups of rats had a similar mean food intake (30 g/day for the control group) and growth performance (50 g/10 day for the control group).

LDL determination. The intake of quercetin, catechin, or DRW did not affect LDL oxidizability. The lag phase, oxidation rate, and maximum CD level were similar to the control group (Fig. 1). No significant differences in α-tocopherol and retinol levels in LDL were observed in treated rats with regard to the control group (Table 3). The LDL fraction had similar TAS value in all groups (0.10 ± 0.02 nmol/µg protein for the control group) (Table 3).

The HPLC profile of LDL from quercetin-, catechin-, and DRW-fed rats did not reveal the presence of quercetin, catechin, or wine flavonoids, respectively (data not shown). The β-glucuronidase/sulfatase treatment also showed the absence of quercetin, catechin, and flavonoid conjugates in LDL. Similar results were obtained when EDTA was not removed from LDL.

Plasma determinations. No significant differences in α-tocopherol and retinol levels in plasma were observed between the treated and the control group (Table 4). The TAS in plasma from rats fed quercetin-, catechin-, or DRW-rich diets was significantly higher than in control rats (Table 4). MDA and CD levels in plasma did not differ (Table 4).

The HPLC profile of plasma from control rats showed neither quercetin nor its glucuronide and sulfate conjugates (Fig. 2A). The HPLC profile of plasma from rats fed the quercetin-rich diet was characterized by the absence of quercetin and the presence of three metabolites (Fig. 2B, peaks b–d).

The enzymatic treatment revealed that the circulating metabolites in plasma corresponded to glucuro- and/or sulfo con-

jugates of quercetin plus one additional peak (e), probably isorhamnetin (27) (Fig. 2C). Plasma from rats fed catechin- or DRW-rich diets did not show catechin, wine flavonoids, or their respective metabolites.

DISCUSSION

Atherosclerosis is a multifactorial disease and the oxidative modification of LDL is a key step in the formation of an atherosclerotic lesion. Studies carried out on the susceptibility of LDL to oxidation after supplementing diets with red wine gave controversial results (13, 17–19, 27). The extent of the
inhibition of LDL oxidation when observed is not as great as in vitro inhibition (36). Thus the beneficial effects of red wine remain a matter of debate. This report is an integrative study that considers the metabolism of flavonoids to elucidate whether ingested flavonoids can protect against atherosclerosis. Quercetin has a higher antioxidant activity than catechin, although the latter is quantitatively the major flavonoid in red wine (35). Taking into account the half-life of VLDLs and LDL, a 10-day diet was sufficiently enough to assure the exchange of lipoproteins. Moreover, we have observed that this diet induces vasorelaxation ex vivo (3). In this study, we have demonstrated that the resistance of LDL to Cu2+ -catalyzed oxidation ex vivo was similar in the four groups of healthy rats, with special emphasis to the length of the lag phases, which is an accepted measure for the resistance of the LDL to oxidation. This agrees with the lack of effect of dietary flavonoids on the metabolism of flavonoids to elucidate intrinsic factors within the LDL particle such as antioxidants and with the absence of the ingested flavonoids (quercetin, catechin, and DRW) and/or its metabolites linked to the lipoproteins even in a LDL pool of four rats. Quercetin and catechin probably bind to the LDL particle in vitro by forming an ether (glycosidic) bond (19) or by ionic interactions with charged residues on the surface. Moreover, a low integration of polyphenols into LDL has been shown by in vitro experiments (19) and the antioxidant effect of some metabolites of quercetin and catechin on Cu2+ -catalyzed LDL oxidation in vitro is lower than the aglycon (10, 26). Furhman et al. (18) and Nigdikar et al. (27) measured indirectly the content of polyphenols in lipoproteins from volunteers after ingestion of a flavonoid-rich diet by the Folin-Ciocalteau method. According to Waterhouse et al. (42), this method gives unrealistic values when applied to plasma because of interferences. An alternative to evaluate the antioxidant activity in lipoproteins is the measurement of TAS. However, similar values were observed in the four groups of rats. Even if small amounts of flavonoids or their metabolites were incorporated in LDL, they did not modify the resistance to oxidation or the α-tocopherol, retinol, and TAS levels of LDL. Thus their clinical relevance in preventing LDL oxidation in vivo should be questioned.

Several facts may explain the reported contradictory results of the effect of dietary flavonoids on LDL oxidation. It has

Table 3. α-Tocopherol, retinol, and TAS in LDL from rats fed control, quercetin-, catechin-, or DRW-rich diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Quercetin</th>
<th>Catechin</th>
<th>DRW</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol, nmol/mg protein</td>
<td>21.60±1.47</td>
<td>21.96±1.75</td>
<td>24.93±2.63</td>
<td>17.12±1.88</td>
</tr>
<tr>
<td>Retinol, nmol/mg protein</td>
<td>2.34±0.28</td>
<td>2.44±0.34</td>
<td>1.76±0.14</td>
<td>1.60±0.30</td>
</tr>
<tr>
<td>TAS, nmol/µg protein</td>
<td>0.10±0.02</td>
<td>0.14±0.02</td>
<td>0.13±0.02</td>
<td>0.12±0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 rats/group. TAS, total antioxidant status.

Table 4. α-Tocopherol, retinol, TAS, MDA, and CD in plasma from rats fed control, quercetin, catechin, or DRW-rich diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Quercetin</th>
<th>Catechin</th>
<th>DRW</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol, nmol/mg protein</td>
<td>9.98±0.57</td>
<td>10.00±0.80</td>
<td>10.35±0.53</td>
<td>11.45±1.01</td>
</tr>
<tr>
<td>Retinol, nmol/mg protein</td>
<td>2.73±0.18</td>
<td>2.80±0.11</td>
<td>3.19±0.14</td>
<td>2.33±0.16</td>
</tr>
<tr>
<td>TAS, nmol/µg protein</td>
<td>0.74±0.02</td>
<td>0.95±0.03†</td>
<td>0.82±0.02*</td>
<td>0.86±0.05*</td>
</tr>
<tr>
<td>MDA, µM</td>
<td>7.97±0.57</td>
<td>8.31±0.49</td>
<td>7.70±0.97</td>
<td>6.41±0.46</td>
</tr>
<tr>
<td>CD, OD at 234 nm/mg</td>
<td>0.87±0.10</td>
<td>0.92±0.15</td>
<td>1.09±0.09</td>
<td>1.19±0.22</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 8 rats/group. MDA, malondialdehyde; CD, conjugated dienes; OD, optical density. *P < 0.05, †P < 0.001.

Fig. 2. Representative HPLC chromatograms of plasma are shown. A: plasma from a control rat before enzymatic treatment; peak a is impurity. B: plasma from a rat fed the quercetin-rich diet before β-glucuronidase/sulfatase treatment; peaks b–d are nonidentified quercetin metabolites. C: plasma after β-glucuronidase/sulfatase treatment. Peak a is impurity and peak e isisorhamnetin.
been shown (27) that EDTA was not properly removed from LDL after dialysis, although Hayek et al. (19) observed a reduction in LDL oxidation by flavonoids when using this method. In the present study, EDTA was eliminated from lipoproteins by a PD10 column, and the lag phase, oxidation rate and maximum CD formation in Cu^{2+}-mediated LDL oxidation were similar in all groups of rats. However, attenuation of the atherosclerotic lesion and LDL oxidation has been observed after the consumption of red wine or flavonoids in apolipoprotein-E deficient mice (19). The comparison of these results with our results suggests that the oxidative stress associated to atherosclerosis could modulate the antioxidant activity of flavonoids. Another possible explanation for the absence of LDL protection against oxidation is the retention of flavonoids or related compounds by the PD10 column (7). However, the HPLC chromatograms had the same profile, which indicates that flavonoids or their derivatives are not retained by the column. Also, a proteolytic degradation of LDL (34) or loss of LDL-associated polyphenols (8) may take place during the isolation procedure used in ex vivo studies. The measurement of lipoprotein oxidation in an environment more closely resembling conditions in vivo may show different effects.

Because no flavonoids ingested or their metabolites were detected in LDL, we performed assays in plasma to assess their presence and antioxidant activity. We detected metabolites of flavonoids or related compounds by the PD10 column (7). However, the HPLC chromatograms had the same profile, which indicates that flavonoids or their derivatives are not retained by the column. Also, a proteolytic degradation of LDL (34) or loss of LDL-associated polyphenols (8) may take place during the isolation procedure used in ex vivo studies. The measurement of lipoprotein oxidation in an environment more closely resembling conditions in vivo may show different effects.

Because no flavonoids ingested or their metabolites were detected in LDL, we performed assays in plasma to assess their presence and antioxidant activity. We detected metabolites of quercetin in plasma after its ingestion, but neither catechin nor its metabolites were found. Because quercetin metabolites are more strongly bound to albumin than catechin metabolites, their elimination is delayed (24) and the half-life for the quercetin is longer than that of the catechin in human plasma after oral administration (18 h and 4 h, respectively) (3, 29). Furthermore, no polyphenols or metabolites were detected in plasma of fasted rats fed a DRW-rich diet.

Plasma from rats fed quercetin-, catechin- or DRW-rich diets showed a higher TAS than plasma from control rats in accordance with others reports (15, 31), and endogenous α-tocopherol and retinol levels were not modified by the diet. After ingestion, quercetin and catechin (15, 26) and other polyphenols circulate as metabolites, which are more hydrophilic than the aglycon compound and their effectiveness in vivo would depend on their antioxidant activity. In our conditions, the TAS technique is more sensitive in evaluating the presence of flavonoids or their metabolites than HPLC. It is likely that polyphenols or their metabolites could exert their antioxidant action in the blood bound to plasma proteins (5) or arterial wall even if a fraction is bound to LDL. Flavonoid metabolites may inhibit either the free radical formation or the propagation of free radical-mediated reactions. However, our results do not show any differences in MDA and CD levels in plasma between control and treated rats in basol conditions, as expected, because the rats were not submitted to any oxidative stress.

The beneficial effects of red wine or flavonoid-rich food at the cardiovascular level reported in epidemiological studies (20, 33) are difficult to explain at the LDL oxidation level. Recent studies in animal models involve either the antioxidant activity of red wine polyphenols (38), extrinsic factors such as HDL-paraaxonase (19) or a mechanism independent of the inhibition of lipid peroxidation (37, 40) to provide protection against atherosclerosis. Nitric oxide has also been implicated in the in vivo beneficial effects of flavonoids (2, 23). Flavonoids induce vasodilatation by increasing nitric oxide, which is known to be a potent antioxidant of LDL in vitro (12, 21). Both features among others may be involved in the low risk of cardiovascular disease induced by red wine intake.

This study suggests that quercetin, catechin, and flavonoids from DRW or their metabolites do not circulate in plasma linked to lipoproteins or the levels are too low to enhance the resistance of LDL to oxidation ex vivo in healthy rats. It is likely that indirect mechanisms would be involved in the prevention of cardiovascular disease by flavonoids. Better assays in lipoprotein oxidation ex vivo are needed to evaluate the beneficial effects of polyphenols or red wine consumption.

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REFERENCES


