Antioxidant and Prooxidant Activities of Elderberry (*Sambucus nigra*) Extract in Low-Density Lipoprotein Oxidation

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Spray-dried elderberry juice, containing high amounts of anthocyanin glucosides, was investigated for its antioxidant and prooxidant potentials and mechanisms. A strong, concentration-dependent prolongation of the lag phase of copper-induced oxidation of human low-density lipoprotein (LDL) was found, but the maximum oxidation rate was unchanged. Peroxyl-radical-driven LDL oxidation showed both prolongation of lag time and reduction of maximum oxidation rate. In the case of copper-mediated oxidation, the anthocyanins were able to reduce α-tocopheroxyl radical to α-tocopherol. Clear prooxidant activity in copper-mediated oxidation was observed, depending on the time of addition of extract: whenever the extract was present from the beginning or added within the first 10 min, the antioxidant effect prevailed. Addition at later times led to a considerable reduction of lag phase and increase of maximum oxidation rate. No such effect was found in peroxyl-radical-mediated LDL oxidation; on the contrary, the extract was even able to reduce the propagation rate considerably.

Keywords: *Antioxidants; anthocyanins; lipid peroxidation; low-density lipoprotein; Sambucus nigra*

INTRODUCTION

Atherogenesis is assumed to be causally related to the oxidation of low-density lipoprotein (LDL) in the arterial wall (Halliwell, 1994; Esterbauer et al., 1997). Therefore, oxidation resistance of LDL and also the antioxidant potential of plasma and whole blood play an important role in the assessment of the risk for developing atherosclerosis. Dietary antioxidants may be important in moderation of the risk, and a wealth of potentially beneficial compounds in foodstuffs have been identified, many of them phenolic substances found in grape juice or wine and also in green and black tea (Rice-Evans et al., 1996; Cao et al., 1997). Polyphenolic flavonoids have elicited widespread interest because of their presence in "healthy" diet and their multifunctionality, which includes reducing properties in addition to their radical scavenging capabilities. Whereas the antioxidant and prooxidant properties of flavonoids have been extensively studied (Cao et al., 1997; Otero et al., 1998), also with respect to their importance in explaining the "French paradox", studies investigating the antioxidant potential of anthocyanins, and specifically elderberry constituents as antioxidants, are scarce. Anthocyanins (the aglycons) have recently been shown to prevent lipid peroxidation in vitro (liposomes, rat liver microsomes, and LDL) and to be able to scavenge oxygen radicals (Tsuda et al., 1996a,b; Satué-Gracia et al., 1997).

LDL oxidation, like lipid peroxidation in general, is a radical chain reaction that generates conjugated diene (CD) hydroperoxides as the initial main product, the formation of which can easily be followed spectrophotometrically (Puhl et al., 1994). It proceeds in three phases under the conditions employed in this study: after addition of the oxidant (either copper ions or AAPH), a phase of very slow oxidation, termed the lag phase, follows, which is mainly due to the content of endogenous antioxidants. The rate of oxidation then increases considerably (propagation phase). Subsequently, a decomposition phase follows during which the dienes formed react further to give the late products of lipid peroxidation. Both the duration of the lag phase, t_{lag}, and sometimes also the maximum rate of oxidation during propagation, V_{prop}, are used as indices for the oxidation resistance of LDL [for a detailed review see Esterbauer and Ramos (1995)].

Antioxidants can have different effects, depending on their chemical nature. Lipophilic substances, such as vitamin E (α-tocopherol, TocOH) or carotenoids, tend to accumulate in the plasma lipoproteins, for example, LDL (Esterbauer et al., 1991), upon supplementation. This group of antioxidants is supposed to act as highly efficient scavengers of such lipid peroxyl radicals which are formed within the lipoprotein particles as a consequence of the radical chain reaction of lipid peroxidation (Esterbauer and Ramos, 1995). Hydrophilic antioxidants, which cannot enter the lipid moiety of LDL, would thus be less efficient as they are principially unable to encounter most of these lipophilic radicals. Such compounds, however, may act in a synergistic manner with the lipophilic antioxidants by regenerating them. For α-tocopheroxyl radical ($TocO^•$) it has been reported that, for example, ascorbate and also phenolic substances (Ortero et al., 1997, Cao et al., 1997; Hayakawa et al., 1997) are able to reduce it to TocOH, thus increasing the oxidation resistance of LDL considerably (Kagan et al., 1992).

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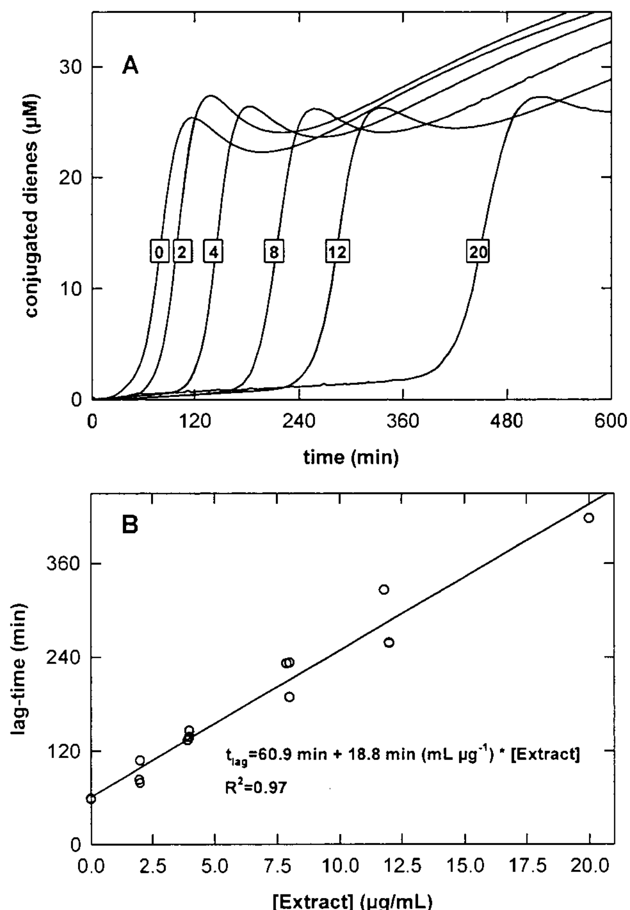

Figure 1. Effect of elderberry extract on copper-mediated LDL oxidation: (A) typical pattern of oxidation curves (note that maximum rate of oxidation remains constant for different concentrations of the extract); (B) linear dependence of the lag time of Cu-mediated oxidation on the extract concentration. 0.1 μM LDL was oxidized with 1.6 μM Cu^{2+} in the presence of 0−20 μg/mL of elderberry extract, as indicated by the labels.

In this study we report antioxidant and prooxidant properties of the constituents of a water-soluble elderberry extract, containing high concentrations of anthocyanin glucosides, and investigate their mechanism of action in both copper- and peroxyl-radical-driven lipid peroxidation.

MATERIALS AND METHODS

Spray-dried elderberry juice (cv. Rubini) was obtained from IPRONA AG (Lana, Italy) as gelatinous capsules containing 200 mg of dry matter each. The composition is given under Results. To prepare the extract for our investigations, the content of one capsule was weighed and dissolved in phosphate-buffered saline (PBS), 10 mM sodium phosphate, and 160 mM sodium chloride, pH 7.4, to give a total concentration of 4.00 mg/mL. The extract solution was kept at 4 °C in the dark prior to use for a maximum of 1 week, which we found safe in the case of the rather stable anthocyanin glucosides (Satué-Gracia et al., 1997) (this is corroborated by the rather narrow distribution of data points in Figure 1B). LDL cholesterol content was used to determine LDL concentration by means of the CHOD-PAP test kit (Boehringer, Mannheim, Germany), assuming a total cholesterol content of 31.6% in LDL. 2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH) was from PolyScience (Warrington, PA). All other chemicals were of analytical grade, from either Merck (Darmstadt, Germany) or Sigma (Vienna, Austria), unless indicated otherwise.

Determination of Anthocyanins, Ascorbate, and α-Tocopherol. Anthocyanins were determined according to the method of Wrolstad (1976): absorbance was measured at 512 nm (pH 1.0) and corrected for polymeric anthocyanins by measuring absorbance at pH 4.5; the difference was used for calculation of concentration on the basis of cyanidin 3-glucoside. The assay was calibrated with cyanidin 3-glucoside (molar absorption coefficient = 26900 cm mol^{-1}; Roth, Karlsruhe, Germany).

Ascorbate was determined by a validated HPLC method based on a standard method (Ball, 1994). After dissolving the spray-dried fruit juice in the mobile phase (0.2 M KH_2PO_4, pH 2.4) and sonication for 1 min, 20 μL of the sample was injected onto a reversed-phase column (LiChrospher 60 RP18, precolumn LiChroCART 4-4, both from Merck), with a flow rate of 1.0 mL/min, UV detection at 254 nm; retention time of ascorbate was 3.16 min. Limit of quantitation is 50 ng/g.

For tocopherol analysis, 2 mL samples ($n = 2-4$ per data point, shown as mean ± SEM) containing 0.5 mg of LDL were withdrawn from the reaction mixture and immediately mixed with the same volume of ice-cold ethanol containing 1 mg/mL of BHT and EDTA (10 μL of aqueous solution containing 100 mg/mL). Two milliliters of hexane was added, and the mixture was vortexed for >1 min. After phase separation, 1.4 mL of the hexane phase was collected and dried under nitrogen. The residue was dissolved in 0.15 mL of ethanol/ethyl acetate (10:1, v/v) and separated by HPLC on a Lichrospher 100 RP-18 (5 μm) column (Merck), using methanol/acetonitrile/ethanol/water (60:50:20:2, v/v) and 0.01% ammonium sulfate as the mobile phase (1 mL/min). α- and γ-tocopherol were monitored by a fluorescence detector (Jasco 821 FP) set at 292/335 nm (excitation/emission wavelength) (Vuilleumier et al., 1983, with modifications) using external standardization. Limit of quantitation is 0.01 μM (in plasma).

LDL Preparation. Human LDL was prepared from pooled plasma by single-step density gradient ultracentifugation in a Beckman NVT65 rotor, as described (Ramos et al., 1995). Prior to use, LDL was stored in a sterile evacuated glass vial (TechneVial, Mallinckrodt) at 4 °C in the dark for up to 1 week. To remove EDTA, LDL was gel-filtered on an EconoPac 10DG column (Bio-Rad, Richmond, CA) equilibrated with PBS, both for copper- and AAPH-mediated oxidation.

LDL Oxidation. For oxidation, 0.1 mM LDL was incubated at 37 °C in PBS. To start oxidation, either 1.6 μM $CuSO_4$ or 1 mM AAPH′ was added. Oxidation was monitored by measuring the absorption of CD at 234 nm (Puhl et al., 1994) at intervals of 4 min in a Beckman DU-640 spectrophotometer equipped with a Peltier-thermostated six-cell holder. *Sambucus nigra* extract was added to the solutions a few minutes prior to start of oxidation, unless stated otherwise. For determination of the consumption of α-tocopherol, aliquots of 1 mL were withdrawn at the indicated time points and processed as described under Determination of Anthocyanins, Ascorbate, and α-Tocopherol.

Determination of Lag Time and Propagation Rate. Duration of lag phase, t_{lag} was determined as the time coordinate of the intersection of the tangents to the increase in CD during the lag and propagation phases, as described (Puhl et al., 1994). Formation of CD was calculated from their absorbance at 234 nm, using the molar absorption coefficient ϵ_{234} = 29500 M^{-1} cm^{-1}. In the case of AAPH-mediated oxidation the linear increase of absorbance due to decomposing AAPH was subtracted. Data were evaluated using MS-Excel 5.0 and StatGraphics 2.1 for Windows.

RESULTS

The main constituents of the *S. nigra* extract investigated here are anthocyanins (90 mg/g), mainly cyanidin 3-glucoside and cyanidin 3-sambubioside (Macheix et al., 1990); other polyphenols were not found. The content of ascorbate was below the quantification limit (<50 ng/g).

Copper-Mediated LDL Oxidation. Incubation of 0.1 μM LDL with increasing concentrations of the extract led to a proportional increase of lag time (Figure

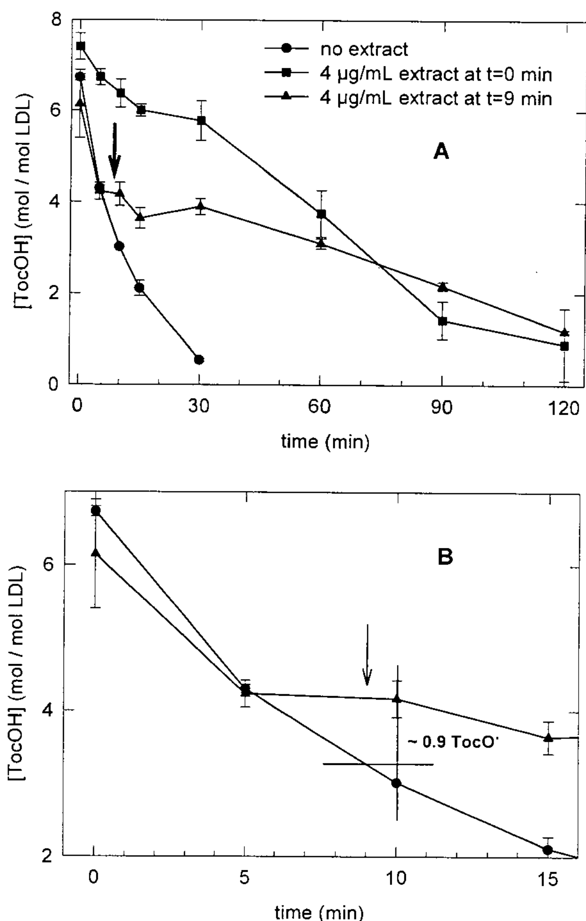

Figure 2. Consumption of TocOH. 0.1 μM LDL was oxidized with 1.6 μM Cu^{2+}, either without extract or in the presence of 4 μg/mL of extract, added either immediately before or 9 min after (arrows) start of oxidation. Data points are shown as means (n = 2..4) ± SEM.

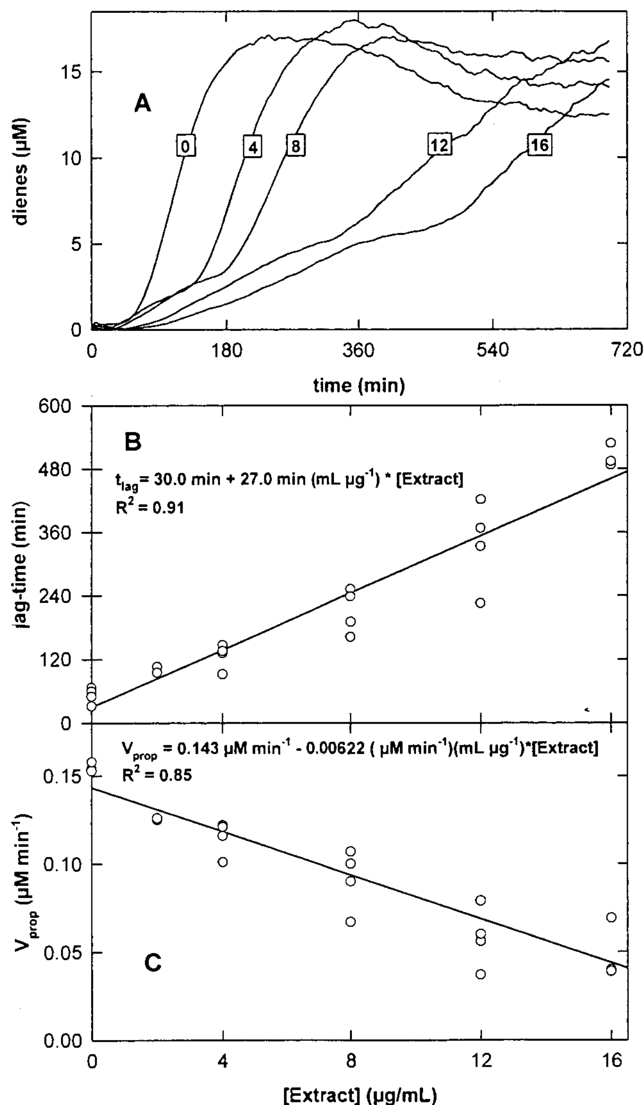

Figure 3. Effect of elderberry extract on AAPH-mediated LDL oxidation: (A) typical pattern (note that the maximum rate of diene formation is *reduced* by increasing concentrations of extract); (B) dependence of lag time on the extract concentration; (C) dependence of V_{prop}. 0.1 μM LDL was oxidized with 1 mM AAPH and CD formation monitored by following their absorbance at 234 nm in the presence of 0–16 μg/mL elderberry extract, as indicated by the labels.

1A) when oxidized with 1.6 μM Cu^{2+}. A linear relationship between $[extract]_0$ and duration of lag phase (t_{lag}) was observed over the extract concentration range investigated (0–20 μg/mL, where 4 μg/mL roughly corresponds to 1 μM cyanidin 3-glucoside) (Figure 1B). One microgram of extract per milliliter led to an increase in t_{lag} of 18.8 ± 0.96 min (R^2 = 0.97, P < 0.01). Maximum rate of CD formation (V_{prop}) and peak CD concentrations remained unaffected over the whole range of $[extract]_0$ applied. This excludes the possibility that the effect is solely a consequence of redox inactivation of copper by chelation, for example, by formation of a metal complex with anthocyanins (Satué-Gracia et al., 1997; Skalindi and Naczk, 1995) because this would have lowered the propagation rate (Ziouzenkova et al., 1998).

Consumption of α-Tocopherol in the Absence and Presence of Extract. Consumption of TocOH is much slower in the presence of extract than in its absence, indicating a protective effect of the extract (Figure 2A) on the consumption of TocOH, both when extract is present from the beginning and when it is added 9 min after the start of the oxidation. In Figure 2B the effect of delayed addition of extract on TocOH consumption is shown: until addition of extract, TocOH is consumed rapidly. When the extract is added 9 min after the start of the oxidation, 1 min later [TocOH] is much higher than in absence of extract. Actually, [TocOH] is nearly as high as 5 min before, indicating considerable regeneration of TocOH from TocO•. By comparing [TocOH] after 10 min in the oxidations without extract and with extract added after 9 min, we estimate that 0.9 TocO•/LDL was available for regeneration during this time.

AAPH-Mediated LDL Oxidation. In a second series of experiments we checked the potential of the extract to inhibit peroxyl-radical-driven lipid peroxidation reactions in LDL using AAPH, a thermolabile radical starter that generates peroxyl radicals at a constant rate. Figure 3A shows the effect of increasing concentrations of extract in the medium, where 0.1 μM LDL was oxidized using 1 mM AAPH. Duration of lag phase increased linearly proportional to extract concentation (Figure 3B), with an increase in t_{lag} of 27.0 ± 1.90 min (μg of extract)$^{-1}$ mL^{-1} (R^2 = 0.91, P < 0.01). In this case also V_{prop} was reduced considerably by increasing concentrations of extract, indicating a prolonged action of the constituents of the extract. Although the actual relationship between V_{prop} and extract concentra-

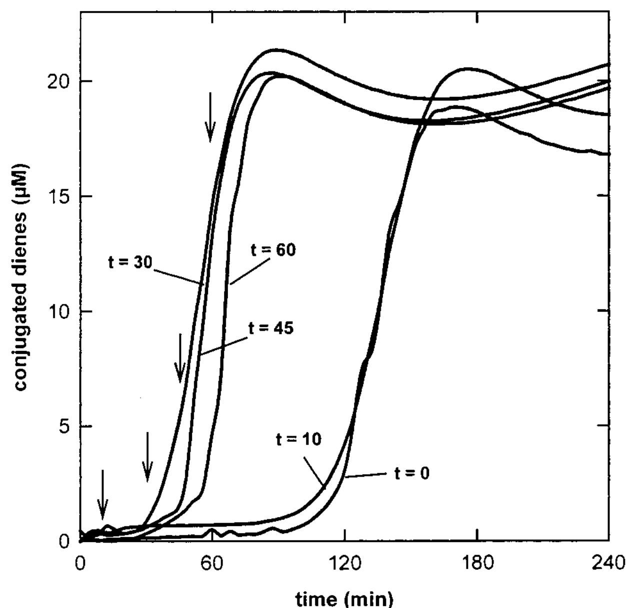

Figure 4. Influence of delayed addition of elderberry extract on copper-mediated LDL oxidation. 4 μg/mL extract was added immediately before (0 min) or at the indicated times (labels, arrows) after addition of 0.1 μM Cu^{2+} and formation of CD monitored.

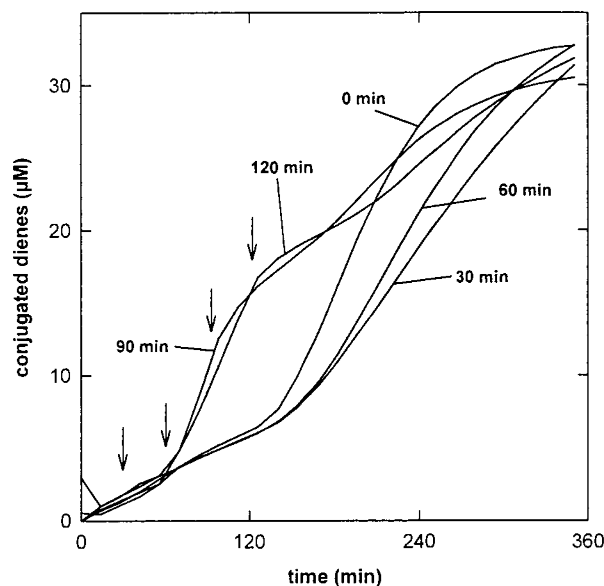

Figure 5. Influence of delayed addtion of elderberry extract on AAPH-mediated LDL oxidation. 4 μg/mL extract was added immediately before (0 min) or at the indicated times (labels, arrows) after addition of 1 mM AAPH and formation of CD monitored.

tion is probably more complex, V_{prop} was tentatively approximated to be linearly dependent on extract concentration (Figure 3C), showing a decrease of V_{prop} of $6.22 \times 10^{-3} \pm 5.98 \times 10^{-4}$ μM min^{-1} (μg of extract)$^{-1}$ mL^{-1} ($R^2 = 0.85$, $P < 0.01$) (see Discussion).

Prooxidant Effects. Elderberry extract can also exhibit prooxidant properties in vitro, depending on the time of addition of the extract. Figure 4 shows the effect of addition of 4 μg/mL elderberry extract at different times after the start of the reaction with copper. When extract was added immediately before addition of copper (0 min) or 10 min after the start of oxidation, the addition of extract did not enhance oxidation; that is, the lag phase continued for a long time after the addition of extract. When extract was added 20 min and later after the start of oxidation, a transition to prooxidant behavior could be observed, characterized by a pronounced increase of the oxidation rate immediately after the addition of extract. The increase of oxidation rate became more pronounced the later the extract was added during oxidation.

A similar experiment was performed to check whether such prooxidant effects could be found also in AAPH-mediated LDL oxidation (0.1 μM LDL, 1 mM AAPH): 4 μg of extract/mL was added between 0 and 120 min after the start of oxidation (Figure 5). Until 60 min after the start of oxidation, addition of extract led to a slight increase in t_{lag}, proportional to the delay of addition, together with a slight, proportional, decrease in V_{prop}. When extract was added later, that is, when propagation had already started, V_{prop} was considerably reduced (Figure 4, 90 and 120 min) as a consequence.

DISCUSSION

Antioxidant Mechanism in Copper-Mediated Oxidation−Regeneration of α-Tocopherol. The strong antioxidant effect of the constituents of *S. nigra* extract led to a considerable increase in t_{lag} when investigated with copper-mediated oxidation. No change in V_{prop} could be observed in this case. Figure 2A shows that in the presence of the extract, consumption of TocOH was retarded, as well.

As shown in Figure 2B, like ascorbate (Kagan et al., 1992), the anthocyanin glucosides in elderberry extract are able to regenerate α-tocopherol (TocOH) from α-tocopheroxyl radical (TocO•) with considerable efficiency. About 0.9 molecule of TocO•/LDL is thus present or being formed between 9 and 10 min after the start of oxidation and is regenerated when extract is added (Figure 2B). Regeneration by anthocyanins proceeds continuously until TocOH is entirely consumed, consistent with the reduced rate of TocOH loss as compared with oxidation in the absence of extract.

We have recently proposed a mechanism for copper-mediated LDL oxidation in which interlocked redox cycling of Cu^{2+}/Cu^{+} and TocOH/TocO• contributes to initiation of lipid peroxidation (Abuja et al., 1997). The TocOH/TocO• redox cycle delivers ∼2.5 times more primordial radicals than the Cu^{2+}/Cu^{+} redox cycle, with decreasing efficiency as TocOH is eventually consumed by

$$\mathrm{TocO^{\bullet} + LOO^{\bullet} \rightarrow LOOToc} \qquad (1)$$

where LOO• is lipid peroxyl radical and LOOToc is peroxytocopherone (Yamauchi et al., 1994) or other nonradical products. Reaction 1 is the only reaction by which net consumption of TocOH occurs.

Anthocyanins partake in this redox cycling by shifting the balance between TocOH and TocO• to the reduced form, thus slowing net TocOH consumption by reaction 1 and lowering the concentration of available primordial radicals, TocO• and LOO•. Both effects are responsible for the antioxidant effect of anthocyanins in copper-mediated LDL oxidation. It should be noted that the regenerating effect of reduction of TocO• *does not mean that the actual concentration of radical scavengers is increased*, as both TocOH and TocO• are able to scavenge LOO•.

The regeneration of TocOH by anthoyanins is not complete, as can be seen from the slow decrease of

[TocOH] in Figure 2A, which is consistent with some $TocO^•$ escaping reduction by anthocyanins and combining with $LOO^•$, as in reaction 1. This is reflected by slow formation of LOOH in the presence of antioxidants of the extract.

The antioxidant constituents of the extract are confined to the aqueous phase because loading of LDL with the extract [in contrast to lipophilic antioxidants, such as TocOH (Esterbauer et al., 1991)] was not possible; that is, no effect on oxidation was observable after incubation of plasma with the extract and subsequent reisolation of LDL (results not shown). This is most likely a consequence of glycosylation of the anthocyanins.

In copper-mediated oxidation the anthocyanin glucosides are apparently consumed before propagation starts, because no prooxidant effect occurs in copper-mediated LDL oxidation whenever the extract is present from the beginning (Figure 1): if phenols were still present at the end of the lag phase, increased V_{prop} would ensue, as observed in the case of delayed addition (Figure 4). Probably some additional consumption of anthocyanins occurs by direct copper reduction in the aqueous phase during lag time (see Antioxidant Mechanism in AAPH-Mediated Oxidation: Scavenging of Water-Soluble Radicals; and Prooxidant Mechanism: Reduction of Cu^{2+}). Note that ascorbate is present only in minute quantities in the spray-dried powder, if at all, and can, therefore, not be responsible for the effects described above.

Antioxidant Mechanism in AAPH-Mediated Oxidation: Scavenging of Water-Soluble Radicals. Behavior in copper-mediated oxidation is complemented by the results obtained with a water-soluble radical starter, AAPH, which delivers aqueous peroxyl radicals at a constant rate. The lag phase is prolonged for a considerable time, just as in copper-mediated LDL oxidation, but V_{prop} is reduced as well. Thus, apart from being able to regenerate $TocO^•$, the extract can directly intercept aqueous peroxyl radicals, lowering the rate of initiation, R_i. Interestingly, this also indicates that the anthocyanins are not entirely consumed by the end of the lag phase, implying that regeneration of $TocO^•$ is slower than scavenging of aqueous peroxyl radicals.

As shown in Figure 3B,C, we approximated both t_{lag} and V_{prop} with linear models, although the extract concentration is expected to decrease exponentially over time, due to the scavenging of AAPH-derived peroxyl radicals, which in turn leads to a steady increase of R_i over time. We thus tried an exponential model for the dependence of t_{lag} on $[extract]_0$, which fit, however, slightly worse than the linear one. As the time at which V_{prop} is reached depends also on $[extract]_0$, a more or less linear dependence results, for which we tentatively approximated the data, to give an impression of the magnitude of the effect. A more complex model does not appear to be useful in this case.

Prooxidant Mechanism: Reduction of Cu^{2+}. Prooxidant effects of antioxidants have been frequently discussed. Generally, antioxidant and prooxidant properties of any substance depend on the conditions and the partners in the reaction (Buettner, 1993).

Simulations show that the concentration of $TocO^•$ is maximal in the first third of the lifetime of TocOH (Abuja et al., 1997; Abuja and Esterbauer, 1995), ∼30 min in the absence of extract (Figure 2). Therefore, we expect maximum $TocO^•$ concentration during the initial 10 min, after which time the concentration becomes progressively lower, until, after consumption of TocOH, it becomes zero. Figure 4 illustrates the consequences: without any extract at all, the lag phase would last some 60 min (cf. Figure 1A); it is considerably reduced, to 36 min, when the extract is added 30 min after the addition of copper, indicating that only very little $TocO^•$ is available for regeneration to TocOH. A very similar effect was found when extract was added at 20 min (omitted in the graph for clarity). Addition at even later times, when TocOH is absent, leads to nearly immediate onset of propagation after addition and increased V_{prop}. Similar phenomena were also observed recently for caffeic acid (Yamanaka et al., 1997a) and catechins (Yamanaka et al., 1997b). Anthocyanins and other phenolic antioxidants probably reduce Cu^{2+} to Cu^+ when bound to LDL, which alone could account for their prooxidant potential. We actually observed direct copper reduction by 4 μg of extract/mL by monitoring Cu^+ formation by chelation with bathocuproine (results not shown).

Whenever the constituents of the extract are present before the start of the oxidation, their main action is to prevent oxidation as described above. This explains why in Figure 1 the propagation rate remains unaffected: at the end of the lag phase the aqueous antioxidants from the extract are already consumed (possibly also by reduction of Cu^{2+} in the aqueous phase) and cannot act as prooxidants anymore. If the extract is added late enough during oxidation, it is not entirely consumed by regeneration of the remaining small concentrations of $TocO^•$ and anthocyanins will become prooxidant.

In peroxyl-radical-mediated LDL oxidation, prooxidant effects upon delayed addition of the extract are completely absent, supporting the interpretation that reduction of copper is the cause. Addition of extract after the onset of oxidation leads to a prolongation of the lag phase proportional to the delay, but smaller in magnitude, as long as elderberry extract is added *before the end of the lag phase*. Moreover, whenever extract is added during the propagation phase, such as in Figure 5 (addition at 90 and 120 min), it is even able to *reduce the propagation rate* instead of enhancing oxidation, indicating a particularly high antioxidant potential of the anthocyanidin glycosides against aqueous peroxyl radicals. This is corroborated by investigations of Tsuda et al. (1996a,b), who demonstrated that the cyanidin 3-*O*-β-D-glucoside shows considerable reactivity against peroxyl radicals, superoxide, and hydroxyl radicals. As the initial reaction products with peroxyl radicals may themselves have antioxidant properties (Tsuda et al., 1996b), anthocyanins might be able to scavenge more than two radicals.

Conclusions. The consequences of the prooxidant potential of elderberry juice, limited to copper-mediated LDL oxidation in the absence of TocOH, are probably marginal in vivo: at any time only a very small fraction of LDL will be devoid of TocOH. Such LDL is to be regarded as already partially oxidized, and it matters only little whether, in the presence of phenolic compounds, it will be completely oxidized in a shorter period of time than in their absence. Metal catalysis accounts for only a part of the prooxidant influences LDL encounters in the arterial wall; therefore, the prooxidative properties of the extract can be neglected,

whereas their antioxidant potential contributes to the preservation of LDL in its unoxidized state.

As can be seen from our results, a steady-state concentration of the elderberry constituents of only 4 μg/mL would already give considerable antioxidant protection, both from copper-induced LDL oxidation and from the attack of peroxyl radicals. Glycosylated flavonoids and anthocyanidins have recently been found to be absorbed without deglycosylation (Paganga and Rice-Evans, 1997), so it may be inferred from our results that supplementation with higher doses of such compounds might contribute markedly to the antioxidant capacity of plasma. Nevertheless, differential absorption, metabolization, and excretion of the compounds may substantially change their relative concentrations in plasma; therefore, further investigations will be necessary to link the in vitro antioxidant potential of this extract to its in vivo relevance.

ABBREVIATIONS USED

LDL, low-density lipoprotein; CD, conjugated dienes; AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); PBS, phosphate-buffered saline; V_{prop}, maximum rate of CD formation; t_{lag}, duration of lag phase; BHT, butylated hydroxytoluene; TocOH, α-tocopherol; TocO•, α-tocopheroxyl radical; LOOH, lipid hydroperoxide; LOO•, lipid peroxyl radical; LOOToc, peroxytocopherone; R_i, rate of initiation.

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