Plant Polyphenols Exhibit Lipoprotein-Bound Antioxidant Activity Using an *in Vitro* Oxidation Model for Heart Disease

Keywords: *Polyphenol antioxidants*

INTRODUCTION

Plants synthesize the well-known antioxidants tocopherol, ascorbic acid, and carotenoids. Flavonoids are polyphenols which are also synthesized in substantial amounts (0.5–1.5%). Plants can use them to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species caused by sunlight (Larson, 1988). The antioxidant activity of flavonoids and other plant phenols in model systems simulating food oxidation has been reviewed by Pratt (1992).

There is increasing evidence that oxidized lower density lipoproteins [low-density lipoprotein (LDL) and very low density lipoprotein (VLDL)] may be involved in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989). Flavonoids have been shown to inhibit LDL oxidation by macrophages and cupric ions (Mangiapane *et al.*, 1992; Rankin *et al.*, 1993). Phenolic acids, flavonoid-like compounds, also acted as antioxidants with LDL oxidized by peroxy radicals generated by an azo initiator (Laranajinha *et al.*, 1994). Resveratrol, a polyphenol found primarily in red wine, inhibited LDL oxidation by cupric ion (Frankel *et al.*, 1993).

There is now considerable epidemiological evidence that dietary flavonoids are protective against heart disease (Hertog *et al.*, 1993, 1995). Since LDL is rendered atherogenic by oxidation in the wall of the artery, only those antioxidants that bind to LDL and VLDL can be beneficial. There is a distinct lack of evidence, with the exception of tocopherol, probucol (Esterbauer *et al.*, 1992), and red wine phenolics (Furman *et al.*, 1995), that antioxidants can bind to low-density lipoproteins. Thus, we have investigated the binding of flavonoids and flavonoid-like polyphenols and their resultant antioxidant activity using a lipoprotein oxidation model.

METHODS

Pure flavonoids and other antioxidants were obtained from either Sigma Chemical Co. or Aldrich Chemical Co. Cyanidin chloride was synthesized and purified (King and White, 1957). LDL plus VLDL was isolated from the plasma of a single normocholesterolemic subject, who was not consuming antioxidant supplements, by an affinity column (Isolabs, Inc.). The procedure for the *in vitro* incubation of the oxidant cupric ion with the lipoproteins and antioxidant, analysis of the lipid peroxidation products, and determination of the concentration of antioxidant for 50% inhbition (IC_{50}) has been previously described (Vinson and Hontz, 1995).

In an *ex vivo* spiking experiment, plasma was incubated with antioxidants in methanol or water at 50, 100, and 200 μM for 1 h at 37 °C to equilibrate. A control with the appropriate solvent was also done. LDL plus VLDL was isolated and immediately subjected to oxidation with cupric ion using the same conditions as for the *in vitro* experiment. The time course of oxidation at 37 °C was followed by the absorbance of conjugated diene formation at 234 nm (Esterbauer *et al.*, 1992). Lag times, which measured bound antioxidant activity, were determined from the graph of absorbance *vs* time. The concentration of antioxidant that increased the lag time to 50% greater than that of the control was determined by a linear regression using 0–200 μM lag times. Representative oxidation curves are shown for epicatechin in Figure 1.

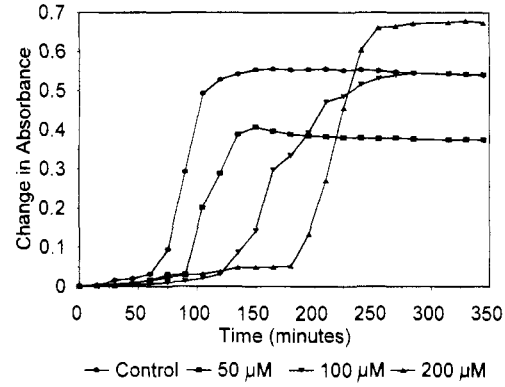

Figure 1. Dose–response effect on plasma spiked with epicatechin at various concentrations followed by LDL plus VLDL isolation and oxidation with cupric ion.

Table 1. Comparison of *in Vitro* Antioxidant Effectiveness (IC_{50}) and Lipoprotein-Bound Antioxidant Activity (CLT_{50}) of Food Phenols

compound	IC_{50} (μM)	CLT_{50} (μM)
epigallocatechin gallate (flavonol)	0.075	41.7
epicatechin (flavonol)	0.187	71.8
cyanidin chloride (anthocyanin)	0.212	120
quercetin (flavonol)	0.224	59.3
chlorogenic acid (phenol acid)	0.296	108
resveratrol (stilbenediol)	0.332	59.1
rutin (flavonol glycoside)	0.512	108
tocopherol (vitamin E)	2.40	54.4
hesperetin (flavanone)	3.66	>200
genistein (isoflavone)	14.3	>200

RESULTS AND DISCUSSION

An extensive study of IC_{50} values of more than 30 natural phenolic antioxidants will be reported elsewhere. We have selected for this study one vitamin and eight polyphenolic compounds which represent the various types of natural phenol antioxidants present in foods. The *in vitro* study produced a sigmoidal dose–response inhibition of lipoprotein oxidation. The IC_{50} data are given in Table 1. Epigallocatechin gallate, the major flavonoid in tea, was by far the strongest *in vitro* antioxidant, i.e., had the lowest IC_{50}. All food phenols except hesperetin and genestein were much better antioxidants than tocopherol.

Typical LDL plus VLDL oxidation curves are shown in Figure 1. They initially exhibit a slow oxidation in which antioxidants are consumed followed by a rapid increase in bound oxidation (propagation phase) and finally a leveling off of oxidation. Lag time is determined graphically by the intersection of the tangents of the propagation curve with the initial oxidation curve. The effect of phenol concentration on lag times, i.e., the bound antioxidant activity, can be seen in Figure 1. The average control value for LDL plus VLDL oxidation was 74 ± 5 min, which agrees well with previously published studies using LDL alone (Esterbauer *et al.*, 1992). Plots of phenol concentration *vs* percent lag time increase produced a straight line for the active antioxidants with correlation coefficients (R^2) greater than 0.905, $p < 0.05$. All compounds produced an increase in lag time with an increase in concentration. Active compounds exhib-

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ited a leveling off of lag time at >200 μM, presumably due to saturation of the lipoprotein binding sites (results not shown). We defined a new criterion (CLT_{50}) to compare the bound low-density lipoprotein antioxidant activities of the compounds. CLT_{50} was the concentration that increased the lag time to 50% greater than that of the control.

As in the *in vitro* study, epigallocatechin gallate had the greatest activity of all the phenols tested. Tocopherol, which had poor *in vitro* antioxidant activity, had good lipoprotein-bound antioxidant activity. This was probably due to its highly lipophilic nature, which increased its binding. Chlorogenic acid and cyanidin chloride, hydrophilic polyphenols, had high CLT_{50} values. Hesperetin and genestein, two lipophilic phenols, probably were bound to lipoproteins but were not good antioxidants. Thus, a good *in vitro* antioxidant was not necessarily a good bound antioxidant since it has to bind before it can be an antioxidant. Indeed, there was no correlation between IC_{50} and CLT_{50}, $p > 0.05$. The situation *in vivo* is best predicted by CLT_{50} since both binding and antioxidant characteristics are necessary.

Lipoprotein-bound antioxidant activity should be a useful criterion by which to choose antioxidants for *in vivo* supplementation. Polyphenolic antioxidants such as found in foods and beverages are able to bind low-density lipoproteins and act as endogenous antioxidants analogous to vitamin E. Thus, if they can be absorbed into the blood they should contribute to the antioxidant pool, inhibit the oxidation of low-density lipoproteins, and thus slow the process of atherosclerosis. These results provide a mechanism for the beneficial effect of flavonoids in foods with respect to heart disease.

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