Free Radical Biology & Medicine, Vol. 31, No. 12, pp. 1570–1581, 2001

0891-5849/01/$–see front matter

PII S0891-5849(01)00737-7

Original Contribution

ANTIOXIDANT MECHANISMS OF ISOFLAVONES IN LIPID SYSTEMS: PARADOXICAL EFFECTS OF PEROXYL RADICAL SCAVENGING

RAKESH P. PATEL,*†‡ BRENDA J. BOERSMA,‡§ JACK H. CRAWFORD,* NEIL HOGG,‖ MARION KIRK,§ BALARAMAN KALYANARAMAN,‖ DALE A. PARKS,†‡¶ STEPHEN BARNES,‡§ and VICTOR DARLEY-USMAR‡

*Department of Pathology, Molecular and Cellular Division, †Center for Free Radical Biology, ‡Purdue-UAB Botanical Center, and §Department of Pharmacology, University of Alabama at Birmingham, Birmingham, AL, USA; ‖Biophysics Research Institute, Medical College of Wisconsin, Milwaukee, WI, USA; and ¶Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, AL, USA

(*Received* 19 *July* 2001; *Accepted* 18 *September* 2001)

Abstract—Oxidation of lipids has been implicated in the pathophysiology of atherosclerosis. It has been suggested that scavenging of lipid peroxyl radicals contribute to the antiatherosclerotic effects of naturally occurring compounds such as the isoflavones. This group of polyphenolics includes genistein and is present in relatively high concentrations in food products containing soy. Soy isoflavones are capable of inhibiting lipoprotein oxidation in vitro and suppressing formation of plasma lipid oxidation products in vivo. However, key aspects of the antioxidant mechanisms remain unknown. In this study the antioxidant effects of genistein and other soy isoflavones on lipid peroxidation initiated by mechanistically diverse oxidants was investigated. Although isoflavones inhibited lipid peroxidation stimulated by both metal-dependent and independent processes, the concentration required for these effects were relatively high compared to those found in vivo. Interestingly, however, isoflavones were not consumed and remained in the native state over the time during which inhibition of lipid peroxidation was observed. This was also the case under conditions where synergistic inhibition of LDL oxidation was observed with ascorbate. Furthermore, in an oxidation system driven solely by peroxyl radicals, isoflavones were found to be relatively poor peroxyl radical scavengers. Consistent with the apparent lack of reactivity with lipid-derived oxidants, isoflavones were also relatively resistant to oxidation mediated by the potent oxidant peroxynitrite. The potential antioxidant mechanisms of isoflavones are discussed in the context of possible reactivities of isoflavone-derived phenoxyl radicals.

Keywords—Free radicals, Genistein, Atherosclerosis, Low density lipoprotein, Peroxyl radicals, Antioxidant

INTRODUCTION

Atherosclerosis is a common disease of the artery wall that is precipitated by a wide range of environmental and genetic factors [1–7]. Much attention has recently been focused on the potential antiatherosclerotic effects of components in the diet [8–11]. For example, epidemiological evidence supports a protective action of dietary antioxidants such as α-tocopherol and ascorbic acid against atherosclerosis and its associated vascular dysfunction [9,10,12,13]. More recent studies have highlighted the potential cardioprotective effects of polyphenolic compounds available from a wide variety of sources including red wine and soy products [14–23]. Where specific compounds, such as the isoflavone genistein, have been investigated, oral intake in humans is associated with an increased resistance of low-density lipoproteins (LDL) to oxidation, and an inhibition of the accumulation of plasma lipid oxidation products [11,22]. Epidemiological studies also support the concept that dietary intake of polyphenolics is associated with decreased risk for cardiovascular disease.

As a molecular mechanism that could underlie these antiatherosclerotic effects, the antioxidant properties of the polyphenols have been highlighted with a specific emphasis on the inhibition of LDL oxidation [17,24–26]. The oxidative hypothesis for atherosclerosis proposes that the accumulation of macrophage-derived foam cells in atherosclerotic lesions is dependent upon pro-oxidant

Address correspondence to: Victor M. Darley-Usmar, Department of Pathology, University of Alabama at Birmingham, Volker Hall Room G019, 1670 University Boulevard, Birmingham, AL 35294-0019, USA; Tel: (205) 975-9686; Fax: (205) 934-1775; E-Mail: darley@path.uab.edu.

reactions in the vasculature [2]. A key event in this process is the oxidative damage to LDL, which leads to the production of oxidized lipids. Oxidized lipids can elicit a wide variety of biological responses that could contribute to lesion development, including the formation of an LDL particle that is taken up by a family of scavenger receptors [2]. Oxidative damage to LDL in vivo could arise from a number of different mechanisms including the lipoxygenase or cyclooxygenase enzymes, peroxidases, heme, or copper-containing proteins, or the interaction of free radicals such as superoxide and nitric oxide [3,27–32].

Dietary factors are thought to be important in determining the ease with which LDL becomes oxidized and the presumption is that consumption of antioxidants decreases the proatherogenic potential of the lipoprotein. This hypothesis is supported by studies in which LDL isolated from subjects at risk of developing coronary heart disease was found to be more readily oxidized [33–35]. The measurement of LDL oxidizability most frequently involves addition of copper to the isolated lipoprotein. The operational parameter defined in these experiments is the "lag phase." This value is a property of the oxidation system and is governed by such factors as the lipid composition of the particle, the concentration of chain-breaking antioxidants and the levels of seeding or endogenous lipid hydroperoxides [32,36].

A number of studies have shown that consumption of soy is antiatherogenic and the bioactive components in this regard are the isoflavones [11,14,15,22]. These polyphenols have the potential to scavenge lipid-based peroxyl radicals, and it is possible that prevention of lipid peroxidation is an important mechanism underlying the protective effects of soy consumption. This contention is supported by the inhibition of copper-dependent LDL oxidation by addition of purified forms of the isoflavones, genistein, daidzein, and biochanin A in vitro [17,24–26]. In addition, dietary supplementation of human subjects with a soy product containing isoflavones results in LDL more resistant to copper-dependent oxidation [11,22].

While these studies are consistent with a role for isoflavones in inhibiting lipid peroxidation and so exerting an antiatherogenic effect, problems with this hypothesis can be identified. For example, the concentrations required in the in vitro studies for significant inhibition of LDL oxidation (20–30 μM) far exceed those found in the vasculature (300–800 nM) [11,15,22]. In addition, the antioxidant mechanisms of isoflavones remain to be fully defined. Interestingly, a recent study suggests alternative mechanisms of action of the isoflavones towards LDL oxidation that do not involve scavenging of lipid-based radicals, but in which the compounds stabilize apoB and the LDL structure [24]. Also, synergistic interactions between isoflavones and ascorbate have been described [24].

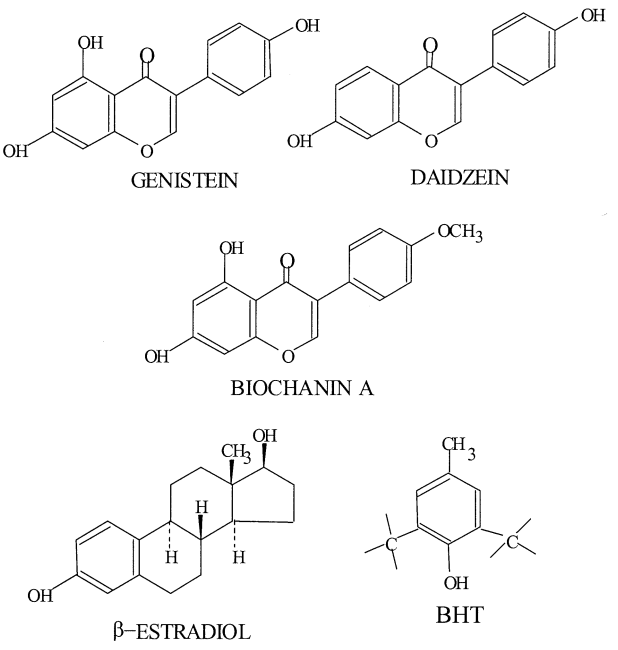

Fig. 1. Structures of the compounds used in the study.

In the present study, we have investigated the activity of the isoflavones as inhibitors of lipid peroxidation using a number of model systems including liposomes and LDL (structures of the compounds used are shown in Fig. 1). Our data indicate that the isoflavones in simple in vitro systems exhibit a low capacity to react with oxidants and inhibit lipid peroxidation. Interestingly however, and in contrast to classical peroxyl radical antioxidants, isoflavones are not consumed during this process. The possible mechanisms that may account for these observations are discussed.

MATERIALS AND METHODS

Materials

Genistein was extracted and purified as described previously [37]. Daidzein was obtained from LC Labs (Woburn, MA, USA). Biochanin A was purchased from Aldrich Chemicals (Milwaukee, WI, USA). 2,2′-Azobis-amidino-propane hydrochloride (AAPH) was purchased from Polysciences (Warrington, PA, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Peroxyl radical scavenging assay

Lipid peroxidation was initiated in phosphatidylcholine liposomes by addition of AAPH. Experiments were

conducted with liposomes (5 or 10 mg/ml) and AAPH (11 or 22 mM, respectively) in 50 mM sodium phosphate, pH 7.4 containing DTPA (50 μM) at 37°C as previously described [38]. Oxidation was assessed by measuring oxygen uptake using a Clark type oxygen electrode.

Oxidation of low-density lipoprotein

Isolation of low-density lipoprotein from human blood was performed by collection into EDTA (final concentration 0.27%) and centrifugation for 20 min at 1000 $\times$ *g* to obtain platelet-poor plasma. LDL was then isolated from the plasma of individual donors by differential centrifugation using the method described [39]. After dialysis against calcium- and magnesium-free phosphate-buffered saline (PBS) containing NaCl (140 mM), KCl (2.7 mM), $Na_2 2HPO_4$ (8.13 mM), KH_2PO_4 (1.47 mM) and DTPA (10 μM), the LDL was sterilized by filtration through a 0.22 μm filter and stored at 4°C until use. The protein concentration was measured using the Bradford protein assay reagent (BioRad) and was typically 1–2 mg/ml. In this study the concentration of LDL is reported in terms of mg protein/ml.

Samples of LDL were diluted to 50–125 μg/ml with PBS with (in the case of myoglobin, SIN-1 or lipoxygenase to chelate contaminating metals) or without (in the case of copper) 10 μM DTPA and incubated at 37°C. Oxidation was monitored spectrophotometrically by measuring the formation of conjugated dienes in the LDL particle at 234 nm. The lag phase, which was used as a measure of the oxidizability of the LDL, was determined as previously described [36]. Lipid hydroperoxides were measured by the FOX (ferrous oxidation by xylenol orange) assay [40]. Solutions to be analyzed for LOOH (100 μl) were added to the FOX reagent (900 μl) and left in the dark for 30 min (room temperature) and then the absorbance at 560 nm measured against an optical reference of buffer (100 μl) and FOX solution (900 μl). Concentrations were determined using the calculated extinction coefficient of ε_{560} = 43970 M^{-1} cm^{-1}. Conjugated dienes in LDL were measured assuming an extinction coefficient at 234 nm of 24,400M^{-1} cm^{-1}. Oxidation of LDL was quenched by addition of butylated hydroxytoluene (BHT, 10 μM). Ethanolic solutions of BHT were added to samples of LDL such that the ethanol concentration was constant at 1% (v/v). Relative electrophoretic mobility (REM) of LDL was measured using the Beckman Paragon electrophoresis apparatus.

Measurement of phytoestrogens

All HPLC analyses were performed using a Beckman HPLC 125 solvent module with a diode array model 168 detector (Beckman Instruments, Fullerton, CA, USA). Samples were extracted from lipid using diethylether and aliquots (40 μl) of the reaction mixtures were analyzed by reverse-phase HPLC, using an Aquapore octyl RP-300, C-8, 22 cm $\times$ 4.6 mm i.d., 7 μm column pre-equilibrated with 10% aqueous acetonitrile in 0.1% trifluoroacetic acid (TFA). The column was eluted at a flow rate of 1.5 ml/min with the following mobile phase composition: 0–10 min, linear gradient (10–50%) of acetonitrile in 0.1% TFA; 10–12 min, linear gradient (50–90%) of acetonitrile in 0.1% TFA; and 12–15 min, isocratically with 90% aqueous acetonitrile in 0.1% TFA. For mass spectrometry reaction mixtures were separated by HPLC using a 10 cm $\times$ 4.6 mm i.d., C-8 Aquapore reverse-phase column pre-equilibrated with 10 mM ammonium acetate (NH_4OAc). The mobile phase composition was: 0–10 min, linear gradient (0–50%) of acetonitrile in 10 mM NH_4OAc; 10–12 min, isocratically with 50% aqueous acetonitrile in 10 mM NH_4OAc; 12–15 min, linear gradient (50–90%) of acetonitrile in 10 mM NH_4OAc; and 15–17 min, isocratically with 90% aqueous acetonitrile in 10 mM NH_4OAc. The column eluent was passed into the electrospray source in the negative ion mode of a PE-Sciex (Concord, Ontario, Canada) API III triple quadrupole mass spectrometer. The voltage on the electrospray needle was -4900 V and the orifice potential was set at -60 V. Negative ion spectra were recorded over a *m/z* range of 200–400. Selected $[M\text{-}H]^-$ molecular ions were analyzed by collision-induced dissociation with 90% argon-10% nitrogen, and the daughter ion mass spectra recorded over a range from *m/z* 20 to the *m/z* of the selected parent ion. Data were analyzed using software provided by the manufacturer on Macintosh Quadra 950 and PowerPC 9500 computers (Apple Computers, Cupertino, CA, USA).

EPR and peroxynitrite

Peroxynitrite ($ONOO^-$) was synthesized as reported previously [41] and quantified spectrophotometrically at 302 nm (pH =12, ε_{302}= 1670 $M^{-1}cm^{-1}$) in 1 M NaOH. HOCl concentrations were determined spectrophotometrically at 290 nm (pH =12, ε_{290}= 350 $M^{-1}cm^{-1}$). Electron paramagnetic resonance (EPR) measurements were taken on a Varian E109 X-band spectrometer. The oxidizing agent (peroxynitrite or HOCl) was added to a methanol:water (88:12) solution containing phytoestrogen (1.2 mM) and magnesium chloride (120 mM). Samples were immediately placed in a flat cell and positioned in the cavity of the EPR spectrometer for spectral acquisition. EPR spectra were simulated using WINSIM by David Duling from NIEHS [42].

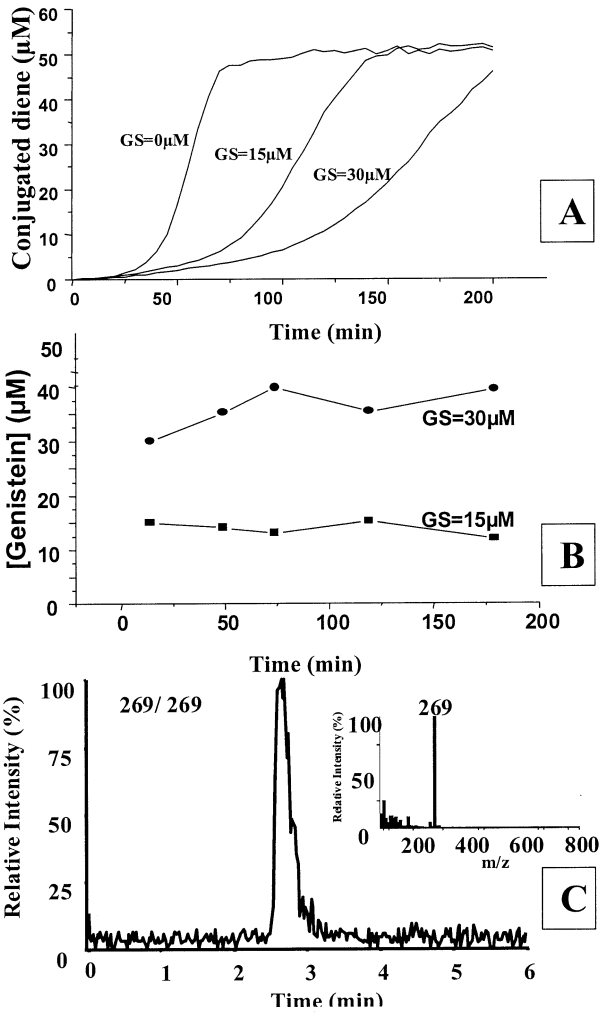

Fig. 2. The effects of genistein on oxidation of LDL promoted by copper. The effect of different concentrations of genistein (as indicated) on copper (II) sulfate (5 μM)-dependent oxidative modification of LDL (50 μg protein/ml) in PBS at 37°C was assessed by monitoring formation of conjugated dienes (Panel A). Panel B shows the changes in genistein concentrations over the time during which antioxidant effects were measured. Samples were extracted at the indicated times and analyzed by HPLC. Panel C shows representative mass spectra of genistein extracted from experiments in which an antioxidant effect was observed. Shown are the selected ion monitoring for the m/z = 269 ion for native genistein after LC-MS and the associated mass spectrum (inset, panel C).

RESULTS AND DISCUSSION

Inhibition of lipid peroxidation by genistein

To test the ability of isoflavones to protect LDL against lipid peroxidation the isolated lipoprotein was exposed to a number of oxidants in the absence and presence of genistein. Isoflavones have been shown previously to inhibit copper-dependent LDL oxidation and this is confirmed in Fig. 2 [17,24,25]. As shown, genistein at concentrations of 15 and 30 μM inhibited oxidative modification of LDL as assessed by right shifts in conjugated diene time courses. Similar effects were observed for formation of LOOH and increases in REM (not shown). In parallel experiments, the changes in genistein concentration were measured by HPLC chromatography after extraction, over the same time course. Surprisingly, while genistein is capable of inhibiting oxidation, the compound remains in the native state with no detection of any oxidation product (Fig. 2B). This is in marked contrast to classical peroxyl radical scavenging compounds (e.g., α-tocopherol, BHT), which are consumed and oxidized during the time period over which an antioxidant effect is observed [13]. To further substantiate these observations and rule out the possibility that an oxidation product of genistein has similar elution profiles and spectral characteristics to native genistein, extracted samples were also analyzed by LC-MS. Figure 2C demonstrates that indeed over the time during which genistein inhibits copper-dependent lipid peroxidation, it does not become oxidized and remains in its native form. Similar results were observed using copper-mediated oxidation of liposomes (data not shown) indicating this phenomenon is not due to specific interactions between the isoflavone and apoB.

It may also be noted that change in the maximum rate of formation of conjugated dienes is evident in the presence of genistein. In the copper oxidation system, this phase of the conjugated diene assay is sensitive to the availability of the transition metals to catalyze decomposition of the lipid peroxides [43]. It is instructive to note that classical peroxyl radical scavenging compounds have no effect on the rate of oxidation once they are consumed [12]. Inhibition as seen in Fig. 2 is consistent with chelation of copper during the assay and this has been noted for isoflavones, although the effect is extremely weak [44]. Other mechanisms leading to the same effect could include competition for specific binding sites on the protein where it is postulated that peroxides and the copper interact [24].

Effects of genistein on LDL oxidation mediated by metal ion-independent mechanisms

Mechanisms in which metal chelation is a key aspect could account for the antioxidant effects and the lack of consumption of isoflavone shown in Fig. 2. To explore this further therefore, we tested the effects of genistein on LDL oxidation mediated by metal ion-independent mechanisms using met myoglobin (metMb) and SIN-1. MetMb-dependent lipid peroxidation is insensitive to low molecular weight chelators [28]. In this case, it is postulated that lipid peroxides interact with the heme protein promoting a cyclic oxidation reaction. SIN-1 promotes oxidative damage to LDL in a metal indepen-

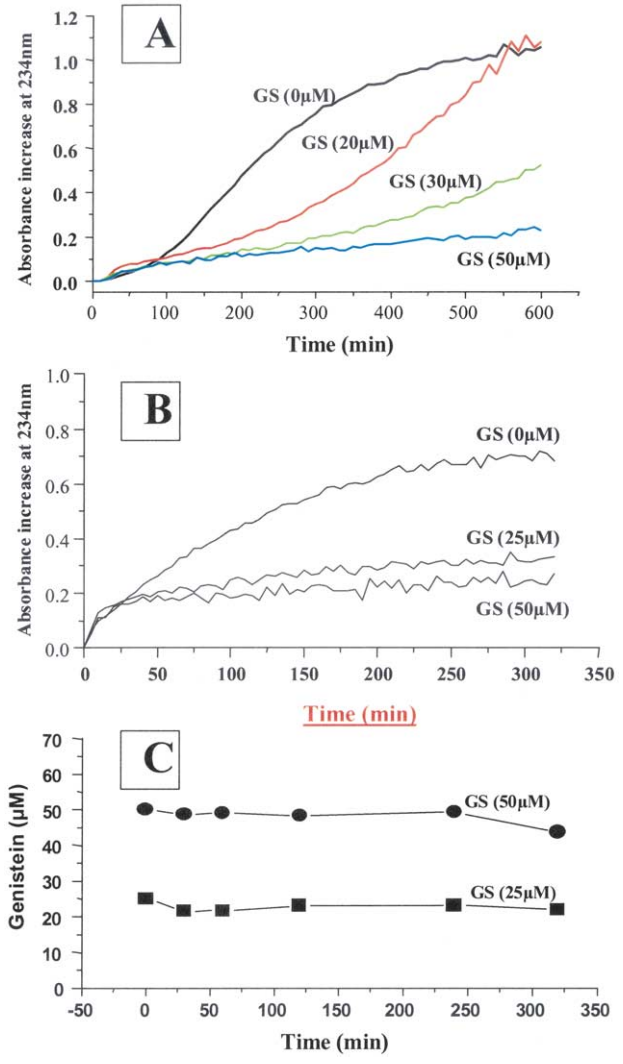

Fig. 3. Effect of genistein on Mb and SIN-1 mediated oxidative modification of LDL. (A) Metmyoglobin (5 μM) was incubated with LDL (50 μg/ml) in PBS + 10 μM DTPA at 37°C with increasing concentrations of genistein (GS) as indicated. Formation of conjugated dienes was measured by monitoring the absorbance increase at 234 nm. (B) SIN-1 (0.5 mM)-mediated oxidative modification of LDL (50 μg/ml) in the presence and absence of genistein (as indicated) was assessed by monitoring conjugated diene formation. Aliquots from reaction mixtures of SIN-1 and genistein were taken at the indicated times and the isoflavone extracted and measured by HPLC (panel C, points are averages of two determinations). Similar to the effects in a copper oxidation system, genistein is not significantly modified over times at which it exhibits an antioxidant effect.

dent manner by forming the oxidant peroxynitrite ($ONOO^-$) [27]. As with copper-dependent oxidation, genistein inhibited Mb-dependent LDL oxidation in a concentration-dependent manner (Fig. 3A). The result of exposing LDL to SIN-1 with and without genistein is shown in Fig. 3B. In the absence of genistein, addition of SIN-1 promotes immediate oxidation of lipids with no lag phase. This is consistent with the inability of endogenous antioxidants in LDL to significantly inhibit oxidation by $ONOO^-$ [45]. Genistein inhibits this oxidative process (Fig. 3B), and similar to copper-dependent oxidation, was not consumed over the time period during which antioxidant effects were observed (Fig. 3C). A potential mechanism for an antioxidant effect in this system is the scavenging of $ONOO^-$ that could then yield oxidation and/or nitration product(s) of genistein as previously reported [46]. However, analysis by HPLC and mass spectrometry showed no evidence of oxidation or nitration of the polyphenolic compound after exposure to SIN-1 (not shown). These data show that genistein can inhibit both metal-dependent and independent mechanisms of lipid peroxidation and that antioxidant mechanisms must be sought that do not involve metal chelation.

Reduction of lipid hydroperoxides

A common mechanism, independent of peroxyl radical scavenging, that may account for inhibition of both copper- and metMb-dependent oxidation by genistein is the reduction of lipid peroxides in the LDL particle. To test for this possibility, the lipid hydroperoxide, 13-S-hydroperoxy-9,11-*cis*,*trans*-octadecadienoic acid (150 μM), was incubated either alone, with genistein (30 μM), or with the glutathione peroxidase mimetic compound, ebselen (30 μM) at 37°C in PBS. Lipid hydroperoxide (LOOH) concentrations were measured using the FOX assay after 1 h and 21 h. The decomposition of LOOH alone was minimal (2.5%) after 1 h and this increased to between 15 and 20% at 21 h. In the presence of ebselen, LOOH concentrations decreased rapidly by 66.5% at 1 h and 75% after 21 h. Interestingly, genistein had no effect on LOOH decomposition, with only minimal loss observed after 1 h, 0.04% $\pm$ 3% (mean $\pm$ SEM) and 22.8% $\pm$ 5.9% (mean $\pm$ SEM). These data indicate that the antioxidant effects of genistein are not mediated by reduction of LOOH.

Inhibition of lipid peroxidation: synergism with ascorbate

A number of phenolic compounds exhibit a synergistic interaction with other antioxidants such as ascorbate and this has recently been shown with the isoflavones genistein, daidzein, and equol [24]. By analogy to other peroxyl radical scavenging antioxidants (e.g., α-tocopherol or probucol) this presumably arises from the reduction of the phenoxyl radical that is formed as a consequence of the reaction with a peroxyl radical back to the phenolic antioxidant. Increased antioxidant potency of the combination of antioxidants is generally interpreted as maintaining the antioxidant concentration at a steady state as long as the reductant is available [47].

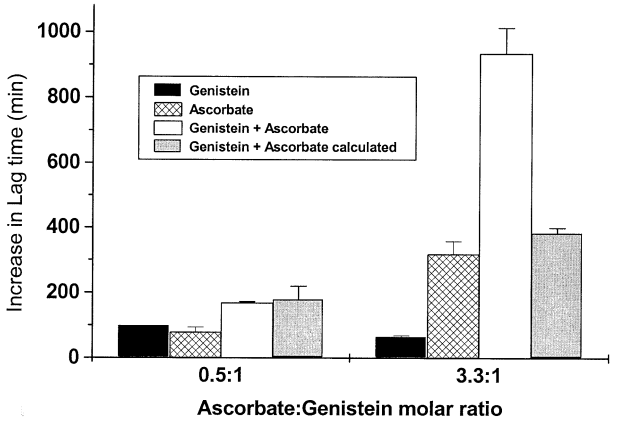

Fig. 4. Effect of ascorbate on the antioxidant effects of genistein towards copper-mediated oxidative modification of LDL. Oxidation of LDL (75 μg/ml) was initiated by copper (5 μM) alone or in the presence of genistein (black bars), ascorbate (hatched bars), or genistein + ascorbate (white bars). Formation of conjugated dienes was monitored continuously and lag times calculated. The data shown represent the increase in lag times relative to copper alone incubations. The grey bars represent the calculated increase in lag times if ascorbate and genistein were acting independently, i.e., an additive inhibition. Concentrations of ascorbate and genistein were 16 and 32 μM, respectively, in the 0.5:1 molar ratio and 50 and 15 μM, respectively, in the 3.3:1 molar ratio. All experiments were performed in PBS at 37°C. Data represents mean ± SEM (n = 3) using two different LDL preparations.

Ascorbic acid also inhibits so-called tocopherol-mediated peroxidation, a process in which the tocopheroxyl radical can initiate lipid peroxidation [48,49]. This occurs under conditions of low peroxyl radical flux. To test for the possibility that reactions between genistein and ascorbate lead to a synergistic interaction that protects against oxidative damage to lipids in LDL, the increases in lag times were measured in the presence of genistein alone, ascorbate alone, or in combination. Figure 4 shows the results of this experiment under conditions where the molar ratio of ascorbate:genistein was 0.5 or 3.3. As expected, the addition of ascorbate alone increases the lag phase in a concentration-dependent manner, due to the recycling of the endogenous α-tocopherol radical in the LDL particle (Fig. 4). If ascorbate and genistein were acting independently, an additive antioxidant effect would be anticipated. This was calculated under the two experimental conditions and compared to the observed increase in lag times when both antioxidants were present. Consistent with a recent study, when ascorbate was present in excess of genistein, a synergistic interaction, analogous to other phenolic antioxidants was seen [24]. Interestingly however, when ascorbate was present at concentrations lower than genistein, only an additive effect on inhibition of lipid peroxidation was observed (Fig. 4). Furthermore, under conditions where a synergistic inhibition was observed, no loss of genistein was observed using HPLC and LC-MS (not shown). This is in marked contrast to the mechanisms by which classical peroxyl radical scavenging phenolics interact with ascorbate, in which upon loss of ascorbate, the polyphenol is also consumed.

Scavenging of lipid peroxyl radicals by genistein

The data presented above show that genistein is not consumed although it clearly inhibits lipid oxidation in LDL and liposomes stimulated by diverse oxidation mechanisms. This is not consistent with the mechanism for other chain-breaking peroxyl radical scavengers (e.g., BHT, probucol) in which the phenoxyl radical reacts with another peroxyl radical resulting in consumption of the compound [47]. However, under conditions where ascorbate is present at a molar concentration higher than genistein, a synergistic inhibition of lipid oxidation is observed. This observation is consistent with the hypothesis that genistein reacts with peroxyl radicals generating a phenoxyl radical that can then be re-reduced by ascorbate.

To gain further insights into the antioxidant mechanisms of isoflavones therefore, the ability of isoflavones to scavenge peroxyl radicals in a transition metal-independent lipid peroxidation assay was measured [50]. Estradiol and BHT were used for comparative purposes (Fig. 1). Lipid peroxidation was initiated in liposomes by the addition of the azo-initiator AAPH, and was followed by measuring the rates of oxygen consumption. AAPH-derived peroxyl radicals abstract a hydrogen atom from a polyunsaturated fatty acid (LH). The resultant alkyl radical reacts with oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). Hence, the consumption of oxygen can be used to monitor the reaction. The peroxyl radicals propagate the oxidation process by abstracting a hydrogen atom from another polyunsaturated fatty acid. In this system therefore, a peroxyl radical scavenger will decrease the rate of oxygen consumption, the magnitude of this effect being proportional to the antioxidant efficiency. The decrease in the rate of oxygen uptake after addition of antioxidant was then measured and normalized with respect to the maximal (i.e., uninhibited) rate of oxygen consumption. Figure 5A shows a typical trace from the oxygen electrode with the rates of oxygen consumption in the presence of increasing concentrations of genistein. Addition of genistein decreases the rate of oxygen consumption, indicating that it reacts with peroxyl radicals (Fig. 5A).

To determine the efficacy of the compounds as antioxidants, the rates of oxygen consumption measured after addition of each compound were normalized with respect to the maximal rate of oxygen uptake (AAPH plus PC liposomes alone) and plotted against concentration of the compounds (Fig. 5B). In this representation,

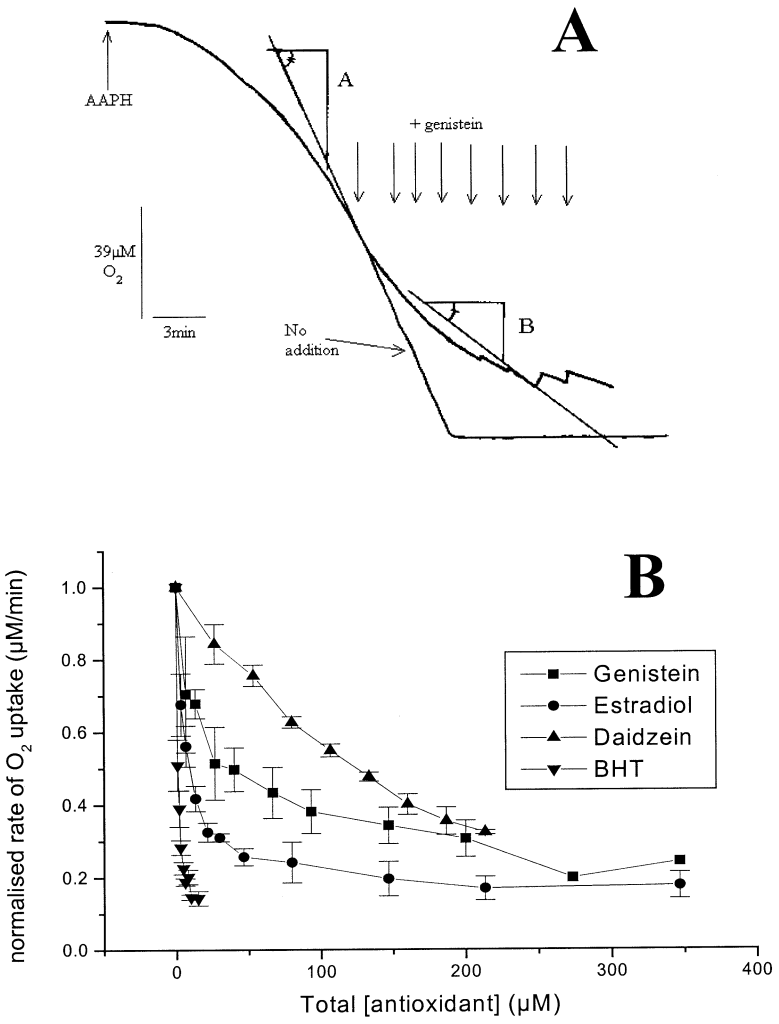

Fig. 5. Effect of genistein on AAPH initiated lipid peroxidation. Oxidation of liposomes was initiated using the peroxyl radical generating system, AAPH, and the reaction followed by monitoring oxygen consumption. (A) After a small lag phase, oxidation continues at a maximal rate until oxygen is completely consumed. Addition of genistein (as indicated) decreases the rate of oxygen uptake in a concentration-dependent manner. These rates were measured as shown, and normalized with respect to the maximal rate. This was repeated with three different liposome preparations, and the effects of daidzein, 17β-estradiol, and BHT were also examined. Experiments were conducted at 37°C, in 50 mM sodium phosphate buffer, pH 7.4. (B) After subtraction of the background rate (due to the oxygen electrode), the rates were normalized with respect to the maximal rates of oxygen uptake and are shown plotted as a function of the concentration of the added compound. All experiments were conducted at 37°C, in 50 mM sodium phosphate, pH 7.4. Values are means ± standard error (n = 3–4).

the greater the left shift in the inhibition curve the more potent the compound is as a peroxyl radical scavenger. It is clear that all the compounds tested are capable of inhibiting lipid peroxidation. However, the relative efficiencies vary considerably, with the IC_{50} ranging from $\approx$1 μM, BHT; $\approx$ 10 μM, estradiol; $\approx$40 μM, genistein; $\approx$125 μM, daidzein.

In a further series of experiments, the fate of the genistein during AAPH-dependent liposome oxidation was determined. Under similar conditions as described in Fig. 5, genistein was incubated with liposomes with and without AAPH or AAPH alone and then the samples extracted and analyzed by HPLC (Fig. 6). Analysis for genistein after oxidation of liposomes had exceeded 70% based upon oxygen consumption measurements, indicated that there was no change in isoflavone concentrations compared to buffer controls. In contrast however, addition of AAPH to genistein alone resulted in substantial loss of the isoflavone. These data are consistent with a recent study characterizing the specific oxidation products formed from the reaction between genistein and AMVN [51]. However, co-incubation of genistein with liposomes undergoing AAPH-dependent oxidation, which resulted in an inhibition of oxygen consumption (approximately 25–30%), did not result in significant consumption of the isoflavone (Fig. 6). Similar effects

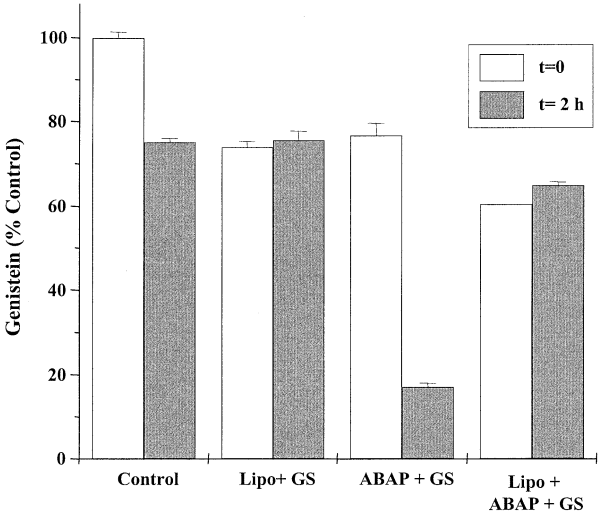

Fig. 6. Effect of oxidation by lipid and nonlipid derived peroxyl radicals on genistein. Quantification of genistein (10 μM) when reacted with 50 mM NaPi with 500 μM DTPA (control), liposomes (10 mg/ml) in 50 mM NaPi with 500 μM DTPA, AAPH (22 mM), or liposomes with AAPH. Liposomes were allowed to equilibrate to 37°C in shaking water bath for 10 min, followed by the addition of AAPH. After 30 min exposure to AAPH, genistein was added to the reaction mixture. One ml of the reaction mixture was immediately removed (t = 0) and 50 μM BHT was added and samples were put on ice. After 2 h incubation with genistein, 1 ml of the reaction was removed and 50 μM BHT added. Samples were extracted with ether and dried under air. Dried residues were resuspended in 150 μl of 80% MeOH, 75 μl was injected on a reverse-phase HPLC.

were observed if linoleic acid was substituted for liposomes. Linoleic acid (20 mM) prevented AAPH mediated loss of genistein by 50%.

These data suggest that polyunsaturated fatty acids prevent reaction between AAPH-derived peroxyl radicals and genistein and can be explained by (i) competition between liposomes and genistein for peroxyl radicals and/or (ii) reactions of genistein-derived phenoxyl radicals with lipids to regenerate the native isoflavone and form a lipid-derived alkyl radical.

Oxidation and formation of isoflavone-derived free radicals

Scavenging of reactive species is an important mechanism by which isoflavones could protect against oxidative damage to lipids. This possibility is demonstrated by the consumption of genistein when incubated with AAPH (Fig. 6). To test the capacity for the isoflavones to be oxidized by reactive species, direct measurement of compound-derived free radicals was undertaken by EPR spectroscopy. Peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) were selected [46,52–54] as potent oxidants of biological relevance that can mediate both 1- or 2-electron oxidation reactions, and are capable of generating a radical from the phytoestrogens over a time scale that allows detection by EPR [55]. In the first series of experiments, no radical species was detected from the reaction of $ONOO^-$ with genistein (Fig. 7A). However, it is known that for the detection of phenoxyl radicals in structures containing adjacent hydroxyl and ketone functional groups that divalent cations are needed to stabilize

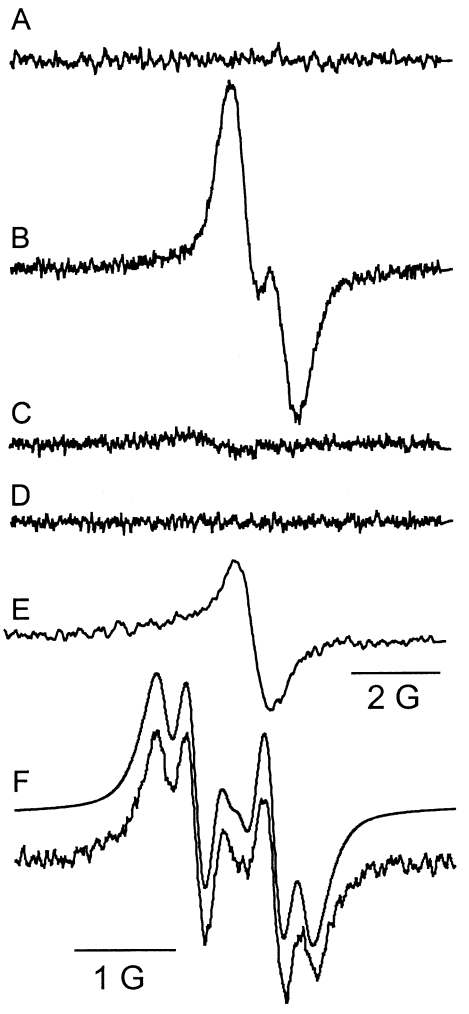

Fig. 7. EPR spectra of phytoestrogen radicals after oxidation with peroxynitrite and HOCl. Methanolic solutions (88% methanol, 12% aqueous) of genistein (A, B, E, F), biochanin A (C), or daidzein (D), each at 1.2 mM, were rapidly mixed with either $ONOO^-$ (1 mM, A–D, F) or HOCl (10 mM) (E). Magnesium chloride (120 mM) was also present in the experiments corresponding to traces B–F. The samples were placed in a flat cell and examined by EPR. Spectrometer conditions for A–E, Scan time: 1 min, scan width 10 G, modulation amplitude 0.5 G, microwave power, 5 mW, time constant 0.128 s. Spectrometer conditions for F, Scan time: 1 min, scan width 5 G, modulation amplitude 0.2 G, microwave power, 5 mW, time constant 0.128 s. All spectra are the accumulation of 10 scans. The noise-free line in spectrum F was simulated assuming three proton hyperfine splittings of $a^H = 0.79$, 0.32, and 0.29 G. A- Genistein + $ONOO^-$; B- Genistein + $ONOO^-$ + Mg^{2+}; C- Biochanin A + $ONOO^-$ + Mg^{2+}; D- Daidzein + $ONOO^-$ + Mg^{2+}; E-Genistein + HOCl + Mg^{2+}; F-Genistein + $ONOO^-$ + Mg^{2+}.

the radical [55]. Therefore, $ONOO^-$ was added to genistein in the presence of magnesium ions (Mg^{2+}). Figure 7B shows that in the presence of Mg^{2+} a radical could now be detected indicating that genistein was oxidized to form free radical products. The stabilizing effect of Mg^{2+} is consistent with the radical being centered around a delocalized enol structure:

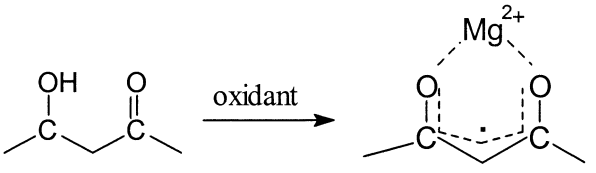

Figure 7C and 7D compares the EPR signals of the corresponding free radicals obtained after addition of $ONOO^-$, in the presence of Mg^{2+}, to biochanin A and daidzein, respectively. Similar to genistein, biochanin A also contains a hydroxy-keto group, whereas daidzein lacks the 5-hydroxy group of the A ring (Fig. 1). The signal from biochanin A was significantly smaller and less persistent than genistein (Fig. 7C). This could either be because biochanin A is more resistant to oxidation, or that its phenoxyl radical is less stable. In contrast, daidzein gave no radical signal, again either due to resistance to oxidation or instability of the radical. To gain further insights into the structure of the genistein-derived free radical species, the spectrum was collected at higher resolution using a modulation amplitude of 0.2 G. This high-resolution spectrum (Fig. 7F) clearly shows hyperfine interactions and was successfully simulated assuming contributions from three inequivalent protons (a^H = 0.79, 0.32, and 0.29 G). The more detailed structure of the free radical however, remains to be determined. Interestingly, when HOCl was used to oxidize genistein, a narrower signal than that observed using $ONOO^-$ was found with little or no hyperfine structure that could be resolved (Fig. 7E). This is consistent with the ring proton being replaced by a diamagnetic chlorine atom as shown previously [55]. These data demonstrate that 1-electron oxidation of isoflavones can occur forming the corresponding phenoxyl radicals. However, it should be stated that the yield of isoflavone radicals was relatively low indicating that these species are either labile and/or difficult to form.

SUMMARY

From the data presented here, it is clear that genistein can inhibit lipid oxidation in simple lipid systems (liposomes) and more complex lipoproteins. Furthermore, this inhibition is independent of the oxidizing system and occurs in both metal-dependent and independent processes. The most likely mechanism, therefore, to account for these antioxidant properties is scavenging of lipid peroxyl radicals, and this was also demonstrated supporting the concept that hydrogen atom donation reactions can occur with this compound, albeit at a low rate in simple oxidation systems. In support of this concept and by analogy to other phenolic peroxyl radical scavengers, synergistic interactions with ascorbate may be explained by regeneration of the native isoflavone by reduction of the isoflavone-derived phenoxyl radical. In addition, direct reactions with HOCl and $ONOO^-$ involving isoflavone-radical intermediates have been demonstrated by EPR spectrometry for the first time. However, these studies reveal an unanticipated aspect of the antioxidant mechanisms of the isoflavones and potentially other polyphenolics, specifically that these compounds are not consumed or oxidized during the oxidation process. This is inconsistent with classical chain terminating peroxyl radical scavenging antioxidants. To rationalize these observations we suggest that polyphenols exhibit antioxidant effects via a mechanism that is analogous to tocopherol-mediated peroxidation. In this mechanism, polyphenols react with lipid peroxyl radicals, forming the corresponding hydroperoxide and polyphenol radical. Whereas, this radical would react with a second lipid peroxyl radical in the case of a classical antioxidant such as BHT, in the case of polyphenols we propose that the radical reacts with an unsaturated fatty acid to initiate lipid peroxidation. This "polyphenol-mediated peroxidation" therefore decreases the antioxidant efficacy of the compound and is shown schematically in Fig. 8, whereby genistein (Gen) reacts with peroxyl radicals ($ROO^\bullet$) to form the corresponding phenoxyl radical (Reaction 1, Fig. 8). This species in turn abstracts a hydrogen atom from a polyunsaturated fatty acid (LH), which regenerates genistein and forms a carbon-centered radical ($L^\bullet$) that reacts with oxygen to form another lipid peroxyl radical (Reaction 2, Fig. 8). We hypothesize that polyunsaturated fatty acids reduce the phenoxyl radical, thereby regenerating genistein, and that this process competes kinetically with the termination reactions that occur with peroxyl radicals (Reactions 2 and 3, Fig. 8). However, an antioxidant effect is still evident and can be explained by the slower rate at which the isoflavone radical initiates oxidation events compared to a lipid peroxyl radical. This would account for the lack of isoflavone consumption during the time over which antioxidant effects are observed

The additive effect of ascorbate at low concentrations and synergism at high concentrations can also be explained by this scheme. In this case, for a synergistic effect in inhibiting lipid oxidation to occur, ascorbate has

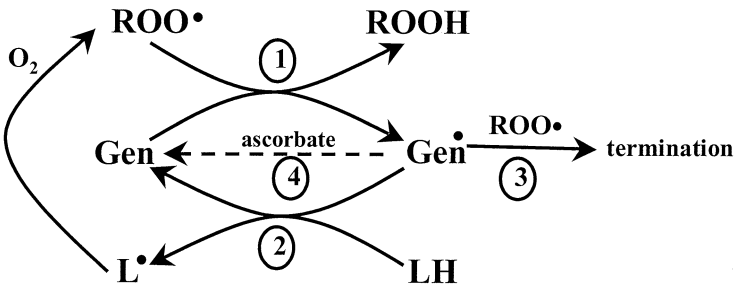

Fig. 8. Proposed scheme by which genistein inhibits lipid peroxidation. Reaction of genistein (Gen) with peroxyl radicals (ROO$^{\bullet}$) generates the corresponding phenoxyl radical (Gen$^{\bullet}$) and hydroperoxide (ROOH) (Reaction 1). To account for the inhibition of lipid oxidation and the lack of loss of Gen we propose that Gen$^{\bullet}$ is reduced by reaction with polyunsaturated fatty acids (LH) to form Gen and a carbon centered lipid radical (L$^{\bullet}$) that in turn reacts with oxygen (O_2) to re-form ROO$^{\bullet}$ (Reaction 2). This process competes with termination reactions between Gen$^{\bullet}$ and ROO$^{\bullet}$ that would result in consumption of the isoflavone (Reaction 3). The effects of ascorbate will depend on the competition of its reaction with Gen$^{\bullet}$ with LH. When ascorbate concentrations are relatively higher such that Reaction 4 is favored over Reaction 2, a synergistic inhibition of oxidation will be observed.

to compete with the polyunsaturated lipids for reaction with the phytoestrogen-derived phenoxyl radical. Our data indicate that this can occur at higher ascorbate concentrations (Fig. 4). The pro-oxidant reaction of the genistein-derived free radical would then prevent the antioxidant effects that arise from the initial scavenging of peroxyl radicals. In this scheme, the genistein molecule is not consumed since the phenoxyl radicals are regenerated.

Since AAPH alone resulted in consumption of genistein, it is most likely that it is the unsaturated fatty acid that is the reductant with the concomitant production of an alkyl radical that then contributes to lipid peroxidation (Fig. 8). Although this scheme is analogous to that described for α-tocopherol-mediated peroxidation, some important differences exist. Notably, α-tocopherol is consumed during tocopherol-mediated peroxidation, whereas, no loss of genistein was observed in the studies reported herein. A possible explanation for this observation is the requirement for low radical fluxes, for tocopherol-mediated peroxidation to occur. In the experiments performed with genistein, high rates of lipid oxidation and hence high rates of radical flux are present. Thus genistein is not oxidized over the time during which oxidative damage to LDL lipids is occurring. Several factors will determine the extent of PMP and include the relative concentrations of isoflavones and unsaturated fatty acids, the flux of peroxyl radicals, and presence of other reductants that may regenerate the genistein-derived free radical. Inhibition of the pro-oxidant effect of the genistein-derived phenoxyl radical would then greatly enhance the antioxidant capacity of these compounds. Recycling by ascorbate represents such a mechanism.

Acknowledgements — This work was supported in part by grants from the American Institute for Cancer Research (91B47R), the National Cancer Institute (5R01 CA-61668), the United Soybean Board (7312), and the NIH Office of Dietary Supplements and the National Center for Complementary and Alternative Medicine (P50 AT00477-01), National Institutes of Health grant RO1ES/HL10167. R.P.P. is a Parker B. Francis Fellow in Pulmonary Research and acknowledges support from AHA Southeastern Affiliate (0060328B). This work was also supported by National Institute of Health grant RR01008 from Research Resources.

REFERENCES

[1] Berliner, J. A.; Navab, M.; Fogelman, A. M.; Frank, J. S.; Demer, L. L.; Edwards, P. A.; Watson, A. D.; Lusis, A. J. Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* **91:**2488–2496; 1995.

[2] Berliner, J. A.; Heinecke, J. W. The role of oxidized lipoproteins in atherogenesis. *Free Radic. Biol. Med.* **20:**707–727; 1996.

[3] Parthasarathy, S.; Santanam, N. Mechanisms of oxidation, antioxidants, and atherosclerosis. *Curr. Opin. Lipidol.* **5:**371–375; 1994.

[4] Parthasarathy, S.; Santanam, N.; Auge, N. Oxidized low-density lipoprotein, a two-faced Janus in coronary artery disease? *Biochem. Pharmacol.* **56:**279–284; 1998.

[5] Patel, R. P.; Moellering, D.; Murphy-Ullrich, J.; Jo, H.; Beckman, J. S.; Darley-Usmar, V. M. Cell signaling by reactive nitrogen and oxygen species in atherosclerosis. *Free Radic. Biol. Med.* **28:** 1780–1794; 2000.

[6] Ross, R. Atherosclerosis—an inflammatory disease. *N. Engl. J. Med.* **340:**115–126; 1999.

[7] Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:**915–924; 1989.

[8] Barnes, S. Evolution of the health benefits of soy isoflavones. *Proc. Soc. Exp. Biol. Med.* **217:**386–392; 1998.

[9] Carr, A. C.; Zhu, B. Z.; Frei, B. Potential antiatherogenic mechanisms of ascorbate (Vitamin C) and alpha-tocopherol (Vitamin E). *Circ. Res.* **87:**349–354; 2000.

[10] Giugliano, D. Dietary antioxidants for cardiovascular prevention. *Nutr. Metab. Cardiovasc. Dis.* **10:**38–44; 2000.

[11] Wiseman, H.; O'Reilly, J. D.; Adlercreutz, H.; Mallet, A. I.; Bowey, E. A.; Rowland, I. R.; Sanders, T. A. Isoflavone phytoestrogens consumed in soy decrease F(2)-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans. *Am. J. Clin. Nutr.* **72:**395–400; 2000.

[12] Dieber-Rotheneder, M.; Puhl, H.; Waeg, G.; Striegl, G.; Esterbauer, H. Effect of oral supplementation with D-alpha-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. *J. Lipid Res.* **32:**1325–1332; 1991.

[13] Esterbauer, H.; Puhl, H.; Dieber-Rotheneder, M.; Waeg, G.; Rabl,

H. Effect of antioxidants on oxidative modification of LDL. *Ann. Med.* **23:**573–581; 1991.

[14] Anthony, M. S.; Clarkson, T. B.; Bullock, B. C.; Wagner, J. D. Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. *Arterioscler. Thromb. Vasc. Biol.* **17:**2524–2531; 1997.

[15] Anthony, M. S.; Clarkson, T. B.; Williams, J. K. Effects of soy isoflavones on atherosclerosis: potential mechanisms. *Am. J. Clin. Nutr.* **68:**1390S–1393S; 1998.

[16] Hayek, T.; Fuhrman, B.; Vaya, J.; Rosenblat, M.; Belinky, P.; Coleman, R.; Elis, A.; Aviram, M. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler. Thromb. Vasc. Biol.* **17:**2744–2752; 1997.

[17] Kapiotis, S.; Hermann, M.; Held, I.; Seelos, C.; Ehringer, H.; Gmeiner, B. M. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. *Arterioscler. Thromb. Vasc. Biol.* **17:**2868–2874; 1997.

[18] Kirk, E. A.; Sutherland, P.; Wang, S. A.; Chait, A.; LeBoeuf, R. C. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. *J. Nutr.* **128:**954–959; 1998.

[19] Leighton, F.; Cuevas, A.; Guasch, V.; Perez, D. D.; Strobel, P.; San Martin, A.; Urzua, U.; Diez, M. S.; Foncea, R.; Castillo, O.; Mizon, C.; Espinoza, M. A.; Urquiaga, I.; Rozowski, J.; Maiz, A.; Germain, A. Plasma polyphenols and antioxidants, oxidative DNA damage and endothelial function in a diet and wine intervention study in humans. *Drugs Exp. Clin. Res.* **25:**133–141; 1999.

[20] Puddey, I. B.; Croft, K. D. Alcohol, stroke and coronary heart disease. Are there anti-oxidants and pro-oxidants in alcoholic beverages that might influence the development of atherosclerotic cardiovascular disease? *Neuroepidemiology* **18:**292–302; 1999.

[21] Tikkanen, M. J.; Adlercreutz, H. Dietary soy-derived isoflavone phytoestrogens. Could they have a role in coronary heart disease prevention? *Biochem. Pharmacol.* **60:**1–5; 2000.

[22] Tikkanen, M. J.; Wahala, K.; Ojala, S.; Vihma, V.; Adlercreutz, H. Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. *Proc. Natl. Acad. Sci. USA* **95:**3106–3110; 1998.

[23] Yamakoshi, J.; Piskula, M. K.; Izumi, T.; Tobe, K.; Saito, M.; Kataoka, S.; Obata, A.; Kikuchi, M. Isoflavone aglycone-rich extract without soy protein attenuates atherosclerosis development in cholesterol-fed rabbits. *J. Nutr.* **130:**1887–1893; 2000.

[24] Hwang, J.; Sevanian, A.; Hodis, H. N.; Ursini, F. Synergistic inhibition of LDL oxidation by phytoestrogens and ascorbic acid. *Free Radic. Biol. Med.* **29:**79–89; 2000.

[25] Kerry, N.; Abbey, M. The isoflavone genistein inhibits copper and peroxyl radical mediated low density lipoprotein oxidation in vitro. *Atherosclerosis* **140:**341–347; 1998.

[26] Ruiz-Larrea, M. B.; Mohan, A. R.; Paganga, G.; Miller, N. J.; Bolwell, G. P.; Rice-Evans, C. A. Antioxidant activity of phytoestrogenic isoflavones. *Free Radic. Res.* **26:**63–70; 1997.

[27] Hogg, N.; Darley-Usmar, V. M.; Wilson, M. T.; Moncada, S. The oxidation of alpha-tocopherol in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Lett.* **326:**199–203; 1993.

[28] Hogg, N.; Rice-Evans, C.; Darley-Usmar, V.; Wilson, M. T.; Paganga, G.; Bourne, L. The role of lipid hydroperoxides in the myoglobin-dependent oxidation of LDL. *Arch. Biochem. Biophys.* **314:**39–44; 1994.

[29] Kalyanaraman, B.; Darley-Usmar, V.; Struck, A.; Hogg, N.; Parthasarathy, S. Role of apolipoprotein B-derived radical and alpha-tocopheroxyl radical in peroxidase-dependent oxidation of low density lipoprotein. *J. Lipid Res.* **36:**1037–1045; 1995.

[30] O'Leary, V. J.; Darley-Usmar, V. M.; Russell, L. J.; Stone, D. Pro-oxidant effects of lipoxygenase-derived peroxides on the copper-initiated oxidation of low-density lipoprotein. *Biochem. J.* **282:**631–634; 1992.

[31] Patel, R. P.; Diczfalusy, U.; Dzeletovic, S.; Wilson, M. T.; Darley-Usmar, V. M. Formation of oxysterols during oxidation of low density lipoprotein by peroxynitrite, myoglobin, and copper. *J. Lipid Res.* **37:**2361–2371; 1996.

[32] Patel, R. P.; Darley-Usmar, V. M. Molecular mechanisms of the copper dependent oxidation of low-density lipoprotein. *Free Radic. Res.* **30:**1–9; 1999.

[33] Maggi, E.; Marchesi, E.; Ravetta, V.; Falaschi, F.; Finardi, G.; Bellomo, G. Low-density lipoprotein oxidation in essential hypertension. *J. Hypertens.* **11:**1103–1111; 1993.

[34] Regnstrom, J.; Nilsson, J.; Tornvall, P.; Landou, C.; Hamsten, A. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* **339:**1183–1186; 1992.

[35] Tsai, E. C.; Hirsch, I. B.; Brunzell, J. D.; Chait, A. Reduced plasma peroxyl radical trapping capacity and increased susceptibility of LDL to oxidation in poorly controlled IDDM. *Diabetes* **43:**1010–1014; 1994.

[36] Esterbauer, H.; Striegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic. Res. Commun.* **6:**67–75; 1989.

[37] Peterson, G.; Barnes, S. Genistein inhibition of the growth of human breast cancer cells: independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Commun.* **179:**661–667; 1991.

[38] Darley-Usmar, V. M.; Hogg, N.; O'Leary, V. J.; Wilson, M. T.; Moncada, S. The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radic. Res. Commun.* **17:**9–20; 1992.

[39] Chung, B. H.; Wilkinson, T.; Geer, J. C.; Segrest, J. P. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:**284–291; 1980.

[40] Jiang, Z. Y.; Hunt, J. V.; Wolff, S. P. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* **202:**384–389; 1992.

[41] Patel, R. P.; Darley-Usmar, V. M. Using peroxynitrite as oxidant with low-density lipoprotein. *Methods Enzymol.* **269:**375–384; 1996.

[42] Duling, D. R. Simulation of multiple isotropic spin-trap EPR spectra. *J. Magn. Reson. B* **104:**105–110; 1994.

[43] Patel, R. P.; Svistunenko, D.; Wilson, M. T.; Darley-Usmar, V. M. Reduction of Cu(II) by lipid hydroperoxides: implications for the copper-dependent oxidation of low-density lipoprotein. *Biochem. J.* **322:**425–433; 1997.

[44] Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids [published erratum appears in Free Radic. Biol. Med. 1996;21:417]. *Free Radic. Biol. Med.* **20:**933–956; 1996.

[45] Hogg, N.; Darley-Usmar, V. M.; Graham, A.; Moncada, S. Peroxynitrite and atherosclerosis. *Biochem. Soc. Trans.* **21:**358–362; 1993.

[46] Boersma, B. J.; Patel, R. P.; Kirk, M.; Jackson, P. L.; Muccio, D.; Darley-Usmar, V. M.; Barnes, S. Chlorination and nitration of soy isoflavones. *Arch. Biochem. Biophys.* **368:**265–275; 1999.

[47] Kalyanaraman, B.; Darley-Usmar, V. M.; Wood, J.; Joseph, J.; Parthasarathy, S. Synergistic interaction between the probucol phenoxyl radical and ascorbic acid in inhibiting the oxidation of low density lipoprotein. *J. Biol. Chem.* **267:**6789–6795; 1992.

[48] Bowry, V. W.; Ingold, K. U.; Stocker, R. Vitamin E in human low-density lipoprotein. When and how this antioxidant becomes a pro-oxidant. *Biochem. J.* **288:**341–344; 1992.

[49] Upston, J. M.; Terentis, A. C.; Stocker, R. Tocopherol-mediated peroxidation of lipoproteins: implications for vitamin E as a potential antiatherogenic supplement. *FASEB J.* **13:**977–994; 1999.

[50] Darley-Usmar, V. M.; Hersey, A.; Garland, L. G. A method for the comparative assessment of antioxidants as peroxyl radical scavengers. *Biochem. Pharmacol.* **38:**1465–1469; 1989.

[51] Arora, A.; Valcic, S.; Cornejo, S.; Nair, M. G.; Timmermann, B. N.; Liebler, D. C. Reactions of genistein with alkylperoxyl radicals. *Chem. Res. Toxicol.* **13:**638–645; 2000.

[52] Beckman, J. S.; Chen, J.; Ischiropoulos, H.; Crow, J. P. Oxidative chemistry of peroxynitrite. *Methods Enzymol.* **233:**229–240; 1994.

[53] Chapman, A. L.; Senthilmohan, R.; Winterbourn, C. C.; Kettle,

A. J. Comparison of mono- and dichlorinated tyrosines with carbonyls for detection of hypochlorous acid modified proteins. *Arch. Biochem. Biophys.* **377:**95–100; 2000.

[54] Eiserich, J. P.; Cross, C. E.; Jones, A. D.; Halliwell, B.; van der Vliet, A. Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. A novel mechanism for nitric oxide-mediated protein modification. *J. Biol. Chem.* **271:** 19199–19208; 1996.

[55] Kalyanaraman, B.; Sealy, R. C.; Liehr, J. G. Characterization of semiquinone free radicals formed from stilbene catechol estrogens. An ESR spin stabilization and spin trapping study. *J. Biol. Chem.* **264:**11014–11019; 1989.

ABBREVIATIONS

AAPH—2,2′-azobis-amidino-propane hydrochloride
AMVN—2,2′-azobis(2,4-dimethylvaleronitrile)
BHT—butylated hydroxy toluene
DTPA—Diethylenetriaminepenta-acetic acid
EDTA—ethylenediaminetetraacetic acid
EPR—Electron paramagnetic resonance
FOX—ferrous oxidation by xylenol orange
LDL—low-density lipoprotein
LH—polyunsaturated fatty acid
$LOO^{\bullet}$—lipid peroxyl radical
LOOH—lipid hydroperoxide
NaPi—sodium phosphate buffer
PBS—phosphate-buffered saline
PMP—polyphenol-mediated peroxidation
REM—Relative electrophoretic mobility
SIN-1—3-morpholinosydnonimine-N-ethylcarbamide