The Activities of Antioxidant Nutrients in Human Plasma Depend on the Localization of Attacking Radical Species[1]

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ABSTRACT The oxidation of endogenous antioxidant nutrients in human plasma was determined to examine their activities against free radicals generated in the aqueous and lipid compartments of plasma. Free radicals were induced at a constant rate in the aqueous compartment by the hydrophilic radical generator, 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH; 10–20 mmol/L) and in the lipid compartment by the lipophilic radical generator, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN; 1–2 mmol/L). The depletion of endogenous plasma antioxidant nutrients (lutein, cryptoxanthin, β-carotene, lycopene, α-tocopherol, ascorbic acid, uric acid) was determined after incubation with either AAPH or MeO-AMVN at 37°C using HPLC. The oxidation of the aqueous and lipid compartments of plasma was selectively monitored by a fluorimetric method using either the hydrophilic probe, 2′,7′-dichlorodihydrofluorescein (DCFH) or the lipophilic probe, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591). When plasma was incubated with AAPH, the rates of consumption of the antioxidant nutrients were as follows: ascorbic acid > α-tocopherol > uric acid > lycopene > lutein > cryptoxanthin > β-carotene. When plasma was incubated with MeO-AMVN, α-tocopherol and carotenoids were depleted at similar rates and ahead of the major water-soluble antioxidants. Our study indicates that the antioxidant nutrients present in both the lipid and aqueous compartments can remove free radicals generated in plasma, and their activity depends on the localization of the attacking radical species. J. Nutr. 133: 2688–2691, 2003.

KEY WORDS: • carotenoids • ascorbic acid • α-tocopherol • oxidizability • azo-initiator

Free radicals are generated continuously in the body due to both normal metabolism and disease (1). When an imbalance occurs between oxidants and antioxidants in favor of the oxidants, excess reactive oxygen species are formed; these may contribute to the aging process as well as to chronic diseases such as cancer and coronary heart disease (2–4). Numerous epidemiologic studies have indicated that diets high in fruits and vegetables play a role in reducing the risk of several chronic diseases (5,6). It is possible that antioxidant nutrients in the fruits and vegetables can prevent certain damage from harmful free radicals that are produced in the body. However, it remains controversial whether the consumption of high levels of dietary antioxidants can significantly increase the antioxidant capacity of humans (7–10).

Currently available biomarkers of plasma antioxidant capacity, such as the radical trapping antioxidant parameter assay (11) and the oxygen radical absorbance capacity (ORAC)[4] assay (12), use hydrophilic radical generators, which produce radicals only in the aqueous compartment of plasma. However, plasma is made up of both aqueous and lipid compartments, and because antioxidants are either water soluble or lipid soluble, both the lipid and aqueous compartments should be monitored when assaying for the true total antioxidant capacity of plasma and for studying the influence that an individual nutrient or combinations of nutrients might have. Since Niki first reviewed the use of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) as the source of water- and lipid-soluble peroxyl radicals, respectively (13), the importance of lipophilicity vs. hydrophilicity in antioxidants and free radical–generating systems for determining antioxidant capacity has been addressed by others (13,14). We previously addressed the advantages of using MeO-AMVN (2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) instead of AMVN as a lipophilic radical generator (15). In the present study, we determined the consumption of antioxidant nutrients in both the aqueous and lipid compartments of human plasma when the free radicals were generated by either hydrophilic or lipophilic radical generators.

MATERIALS AND METHODS

Chemicals. All-*trans*-β-carotene (type II) and lycopene were purchased from Sigma Chemical (St. Louis, MO). Lutein was pur-

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[4] Abbreviations used: AAPH, 2,2′-azobis-(2-amidinopropane)dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile; BODIPY 581/591, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid; DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, DCFH diacetate; MeO-AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); ORAC, oxygen radical absorbance capacity.

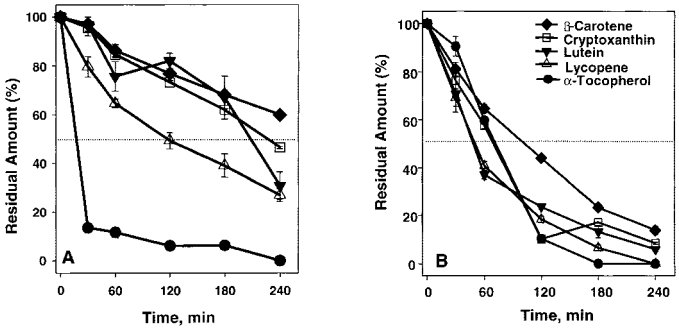

FIGURE 1 Effect of 10 mmol/L 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH), a hydrophilic radical generator (*A*), and 1 mmol/L 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), a lipophilic radical generator (*B*), on the oxidation of endogenous fat-soluble antioxidant nutrients in human plasma (1:5 with PBS). The baseline concentrations of lutein, cryptoxanthin, all-*trans* β-carotene, lycopene and α-tocopherol were 0.75, 1.91, 3.89, 2.43 and 46.16 μmol/L, respectively. Values are means ± SEM, $n = 3$.

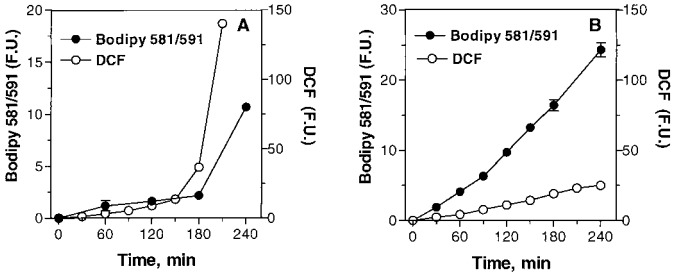

FIGURE 3 Oxidation of human plasma (1:5 with PBS) hydrophilic compartment monitored by oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) to DCF (λex 502nm, λem 520nm) and the lipophilic compartment monitored by oxidation of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591) (λex 500nm, λem 520nm) in the presence of 20 mmol/L 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH) and 2 mmol/L of 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN). Values are means ± SEM, $n = 3$.

chased from Kemin Industries (Des Moines, IA). Cryptoxanthin and echinenone were gifts from Hoffmann-La Roche (Nutley, NJ). The fatty acid analog 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). The radical initiators, AAPH, and MeO-AMVN were gifts from Wako Chemicals (Richmond, VA). All HPLC solvents were obtained from J. T. Baker Chemical (Philipsburg, NJ) and were filtered through a 0.45-μm membrane filter before use.

Plasma oxidation induced by hydrophilic or lipophilic radical initiators and monitored by hydrophilic or lipophilic probes. Fasting blood samples were collected in evacuated containers with 0.1% EDTA and kept on ice. Samples were protected from light and centrifuged for 20 min (800 × *g*, 4°C) within 30 min of collection. Aliquots of plasma were stored at −70°C until analyzed except for ascorbic acid analysis. As reported previously (15), plasma/PBS (1:5, v/v) was incubated at 37°C with either AAPH or MeO-AMVN, and the same level of free radical flux was achieved by adjusting the concentration of the two azo-initiators. The oxidation of the aqueous compartment of plasma was measured by monitoring the 2-electron oxidation of DCFH. The wavelength was set at 502 nm (slit 5 nm) and at 520 nm (slit 5 nm) for excitation (λex) and emission (λem) respectively. BODIPY 581/591 was incorporated into the lipid compartment of plasma at a final concentration of 2 μmol/L. The oxidation of the lipid compartment of plasma was monitored by the green fluorescent oxidation product of BODIPY 581/591 (λex = 500, λem = 520 nm). The fluorescence measurements were carried out using a Perkin Elmer spectrofluorometer (model 650–10s).

Plasma analysis for antioxidant nutrients. Plasma/PBS (1:5, v/v) was incubated at 37°C for up to 4 h in the presence or absence of AAPH (10 or 20 mmol/L) or MeO-AMVN (1 or 2 mmol/L). Carotenoids and α-tocopherol in plasma were determined at 30 min, and 1, 2, 3 and 4 h using an HPLC system with a C30 carotenoid column (3 μm, 150 × 4.6 mm, YMC, Wilmington, NC) as described previously (16). Ascorbic acid and uric acid were measured at 5, 15 and 30 min, and at 1, 2, 3 and 4 h by HPLC using an electrochemical detector (Bioanalytical System, N. Lafayette, IN) as described by Behren et al. (17) with minor modifications.

Confirmation of Bodipy581/591 incorporation into the lipid compartment of plasma. A density gradient ultracentrifugation procedure was utilized to separate plasma lipoprotein fractions as reported with minor modifications (18). Plasma samples (2 mL) were incubated at 37°C for 10 min in the presence and absence of 200 μL of BODIPY 581/591 (20 μmol/L) before the lipoprotein fractionation. Lipoprotein bands were detected in the density gradient by staining

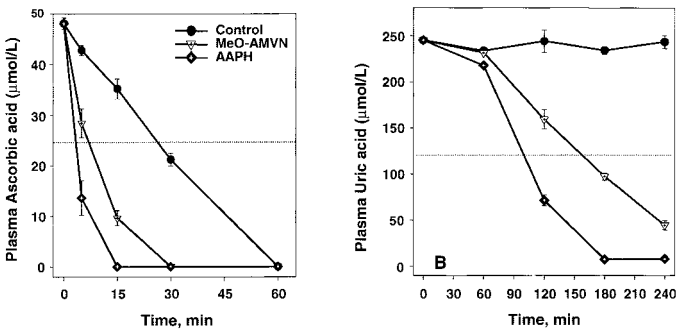

FIGURE 2 Effect of 20 mmol/L 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH) and 2 mmol/L 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) on the oxidation of endogenous ascorbic acid (*A*) and uric acid (*B*) in human plasma (1:5 with PBS). Values are means ± SEM, $n = 3$.

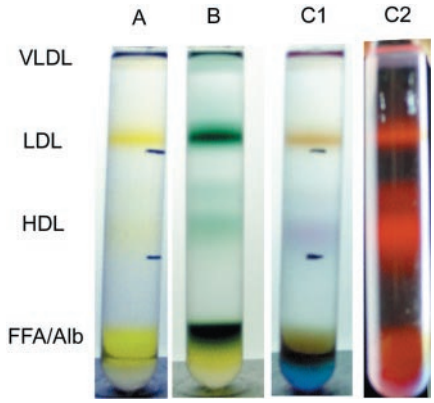

FIGURE 4 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591) incorporation into lipoproteins of human plasma. After density gradient ultracentrifugation of 2 mL human plasma, the lipoproteins, which appear yellow due to the presence of carotenoids (Tube A), were further identified by staining with 200 μL of 0.1% Sudan black solution (Tube B). When incubated with 200 μL of 20 μmol/L BODIPY 581/591 for 10 min at 37°C, the lipoproteins acquired a slight bluish tinge (Tube C1). When Tube C1 was illuminated in the dark at right angles, the typical red fluorescence of BODIPY 581/591 was associated with the lipoprotein fractions (Tube C2).

the plasma with 200 μL of Sudan black (0.1 g Sudan Black in 100 mL ethylene glycol) before ultracentrifugation for 21 h (acceleration rate of 4 and no brake) at 274,000 × *g* at 20°C using a Beckman L8–70M ultracentrifuge with SW 41 Ti rotor.

RESULTS

Consumption of plasma antioxidant nutrients induced by AAPH and MeO-AMVN. When the radicals were generated in the aqueous compartment of plasma by 10 mmol/L AAPH, α-tocopherol was the first fat-soluble antioxidant nutrient to be consumed (**Fig. 1***A*). Almost 80% of α-tocopherol was consumed within 30 min. The consumption of carotenoids was much slower than that of α-tocopherol. Lycopene was more rapidly oxidized than β-carotene by AAPH. On the other hand, when the radicals were generated in the lipid compartment of plasma by MeO-AMVN, α-tocopherol, lycopene and lutein were consumed at approximately the same rate, whereas β-carotene was oxidized more slowly (Fig. 1*B*). The order of oxidation of carotenoids by MeO-AMVN was similar to that when the radicals were generated from AAPH.

The reduced form of ascorbic acid was completely depleted within 60 min of our control incubation at 37°C under aerobic conditions (**Fig. 2***A*). The reduced form of ascorbic acid was completely consumed within 15 min by 20 mmol/L AAPH and within 30 min by 2 mmol/L MeO-AMVN. Uric acid was stable under aerobic conditions for the 4-h period of the experiment (Fig. 2*B*). Only 3% of uric acid remained at 3 h when incubated with 20 mmol/L of AAPH, whereas >40% of the uric acid remained after 3 h of incubation with 2 mmol/L MeO-AMVN (Fig. 2*B*). The half-life for uric acid was 100 min in the presence of 20 mmol/L AAPH and 160 min in the presence of 2 mmol/L MeO-AMVN.

Measurement of oxidation in the aqueous and lipid compartments of plasma. When the radicals were generated in the aqueous compartment of plasma by 20 mmol/L AAPH, DCFH oxidation, which reflects aqueous compartment oxidation, began accelerating between 150 and 180 min, whereas the BODIPY 581/591 oxidation accelerated at 180 min (**Fig. 3***A*). On the other hand, when the radicals were generated in the lipid compartment of plasma by 2 mmol/L MeO-AMVN, BODIPY 581/591 oxidation began accelerating between 90 and 120 min, whereas the rate of DCFH oxidation remained linear during 4 h of incubation (Fig. 3*B*).

BODIPY 581/591 was incorporated into the individual lipoprotein fractions, VLDL, LDL and HDL, as well as into the fractions containing the fatty acid/albumin complex (**Fig. 4**).

DISCUSSION

To optimize antioxidant defenses in the body, it is necessary to understand the true antioxidant potentials of dietary antioxidants and the interactions among the antioxidant nutrients. Several methods to measure "total" antioxidant capacity have been developed. However, conventional methods use hydrophilic radical generators and measure primarily the antioxidant capacity in the aqueous compartment of plasma. Consequently, water-soluble antioxidants such as ascorbic acid, uric acid and protein thiols mainly influence these assays, whereas the fat-soluble antioxidants such as carotenoids and tocopherols play only a minor role (19,20). Therefore, it is not surprising that most of the methods used to measure purported "total antioxidant capacity" of plasma are not affected by variations in the levels of the fat-soluble antioxidants such as carotenoids (9,10).

As shown in this study, AAPH generates hydrophilic peroxyl radicals, which first oxidize the aqueous compartment (monitored by DCFH oxidation) and subsequently induce lipid peroxidation (monitored by BODIPY 581/591 oxidation). Under these conditions, the first line of defense against oxidative damage is the water-soluble antioxidants such as ascorbic and uric acids, as well as α-tocopherol, which is located in the interface between the aqueous and lipid compartments at the surface of lipoproteins. It is clear that the fat-soluble antioxidants located in the core of lipoproteins (e.g., carotenoids) act as a second line of defense when plasma is treated with AAPH. However, all of the measured carotenoids reacted with the radicals, indicating an antioxidant role for these carotenoids in the circulation. Lycopene was the most rapidly depleted carotenoid in plasma in our experiments (Fig. 1*A*), in accordance with other reports in solvent systems (21,22), oil (23) or in an aqueous model system (24). It should not be overlooked that the antioxidant efficacies of carotenoids in solvent systems may differ from those found in membrane systems, as suggested by Woodall et al. (25).

Massaeli and colleagues (13) noted that the lipophilicity of antioxidants is an important factor for their antioxidant capabilities in lipoproteins. In their experiment, free radicals were generated with the lipophilic radical initiator, AMVN, in isolated LDL, and lipid peroxidation was measured by malondialdehyde formation. In our experiments, MeO-AMVN, which has a considerably higher rate of radical production [14.2 $(\mu mol/L) \cdot s^{-1}$ at 37°C in micelles] than AMVN (26), was used as a lipophilic radical generator in whole plasma. AMVN requires either high temperature (e.g., 50°C) for several hours (25) or a longer period of incubation (e.g., up to 24 h) at 37°C (27) to induce and sustain lipid peroxidation in phosphatidylcholine liposomes because the rates of peroxyl radical formation of 5 mM of AMVN are 4.58×10^{-3} and 1.8 $(\mu mol/L) \cdot s^{-1}$ at 37°C and 50°C, respectively. We confirmed that BODIPY 581/591, which has a high quantum yield and readily enters membranes (28), was incorporated into each lipoprotein fraction in plasma.

When the radicals were generated in the lipid compartment, α-tocopherol and the carotenoids acted together as the first line of defense against oxidative damage, followed by the oxidation of uric acid. In contrast to uric acid, ascorbic acid was rapidly consumed in the presence of MeO-AMVN, suggesting an active interaction between ascorbic acid and α-tocopherol, as reported earlier (29,30). Interactions between the antioxidant nutrients, such as the quenching of β-carotene radicals by vitamin C (31,32) and by α-tocopherol (33), and the recycling of α-tocopherol by green tea polyphenols (34) have also been reported. Therefore, it is conceivable that the antioxidant activity of each compartment can be greatly increased through interactions among water-soluble and fat-soluble antioxidant nutrients.

Several recent studies have paid attention to the antioxidant capacity in the lipid compartment of plasma. Mayer and colleagues (14) introduced a method to use selective fluorescence probes to determine antioxidant capacity in the aqueous and lipid phases of serum. Also, a modified ORAC assay was reported to analyze fat-soluble antioxidants by using randomly methylated β-cyclodextrin as a solubility enhancer, and hydrophilic fluorescein as the fluorescence probe (35).

Our study indicates that antioxidant nutrients present in both the aqueous and lipid compartments of plasma are capable of removing free radicals that depend on radical initiator solubility, and the possibility of further cross-talk between aqueous and lipid compartments antioxidants. The beneficial effect of a high intake of fruits and vegetables on the risk of degenerative/chronic diseases may not rely on the effect of a

single antioxidant but rather on a concerted action of several antioxidant nutrients

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