JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Assessment of Radical Scavenging Capacity and Lipid Peroxidation Inhibiting Capacity of Antioxidant

ETSUO NIKI,* YO OMATA, AKIKO FUKUHARA, YOSHIRO SAITO, AND YASUKAZU YOSHIDA

National Institute of Advanced Industrial Science and Technology, Human Stress Signal Research Center, 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan

The role of radical scavenging antioxidants against oxidative stress has received much attention, and the antioxidant capacity has been assessed by various methods. Among them, a method that measures the effect of antioxidant on decay of the probe is one of the most widely used methods. The present study was performed to compare the two methods to assess the antioxidant capacity, one to follow the decay of the probe and the other to measure lipid peroxidation products in human plasma. It was shown that the method following probe decay was suitable for assessment of radical scavenging capacity of antioxidant, but not for the capacity to inhibit lipid peroxidation in plasma. This is true whether a hydrophilic or lipophilic probe is used. Such different results arise from the fact that the efficacy of inhibition of lipid peroxidation by antioxidants depends on the fate of antioxidant-derived radical and interaction between antioxidants as well as the capacity of free radical scavenging. Thus, the capacity of antioxidants for inhibition of lipid peroxidation should be assessed from the effect on the extent of oxidation, not from the effect on probe decay.

KEYWORDS: Antioxidant capacity; free radical; lipid peroxidation; ORAC method

INTRODUCTION

Free radicals and active oxygen and nitrogen species oxidize biological molecules such as lipids, proteins, DNA, and sugars to induce their modification, which may eventually result in various disorders and diseases (*1*). During the course of evolution, aerobic organisms have developed a fine defense network system against oxidative stress, in which various enzymes, proteins, and small molecules with different functions play an important protective role against oxidative stress in vivo. The radical scavenging antioxidants, which are denoted simply antioxidants in this paper, play an important role in the defense network in vivo and protect biological molecules from oxidative modification and damage induced by free radicals.

The assessment of antioxidant capacity in vivo and in vitro for biological samples, foods, beverages, nutraceuticals, and dietary supplements has been the subject of extensive studies and argument (*2*−*7*). Various methods have been developed and applied. Of these, the prevailing methods such as oxygen radical absorbance capacity (ORAC) (*8*) and total radical trapping antioxidant parameter (TRAP) (*9*) apply probes as reference compounds, and the antioxidant capacity is assessed by the extent of suppression of consumption of the probe, measured by spectrophotometry or fluorescence. Various probes such as fluorescein, ABTS, pyrogallol red, phycoerythrin, crocin, and pyranine have been used as reference compounds (*10*). The use of azo initiator is most convenient to generate free radicals at a known, constant, and controlled rate, which enables quantitative assessment. Both lag phase and rate of probe decay are measured. More recently, the effect of antioxidant has been evaluated by measuring the area under the curve (AUC) of the probe decay as compared to that of the blank in which no antioxidant is present (*5, 6*).

Lipid peroxidation is accepted as being involved in the pathogenesis of various disorders and diseases, and its inhibition is one of the important roles for antioxidants, because lipid peroxidation induces disruption of membrane organization, causing changes in fluidity and permeability, inhibition of metabolic processes, and alteration of ion transport; furthermore, lipid hydroperoxides and their secondary reaction products such as aldehydes are cytotoxic, pro-inflammatory, and capable of modifying proteins and DNA bases.

It is an important issue to examine the correlation between the capacity of antioxidant for scavenging free radicals and that for inhibition of lipid peroxidation. The objective of the present study was to compare the capacity of several antioxidants for scavenging free radicals as assessed by the effect on the probe decay and that for inhibition of lipid peroxidation as assessed by the formation of lipid peroxidation products in the oxidation of human plasma. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt) was used as a probe (*11*−*15*). Although pyranine has been used less frequently than other probes such as fluorescein, phycoerythrin, and crocin, it is a suitable probe because it reacts with free radicals at an appreciable rate and

* Author to whom correspondence should be addressed (telephone 81-72-751-9991; fax 81-72-751-9964; e-mail etsuo-niki@aist.go.jp).

10.1021/jf800605x CCC: $40.75
Published on Web 08/22/2008

yet more slowly than many biological antioxidants. This is advantageous for obtaining a clear lag phase. Furthermore, its decay can be measured easily by following its maximum absorption at 454 nm or fluorescence intensity (excitation at 460 nm and emission at 510 nm) (*11*−*15*).

MATERIALS AND METHODS

Materials. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) used as a hydrophilic radical generator was obtained from Wako Pure Chemical Industries (Osaka, Japan). The natural form of *RRR*-α-tocopherol was kindly provided by Eisai Co. Ltd. (Tokyo, Japan). Trolox, 2,2,5,7,8-pentamethyl-6-chromanol (PMC), and pyranine were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan) and used as received. A synthetic antioxidant, 2,3-dihydro-5-hydroxy-4,6-di-*tert*-butyl-2,2-dipentylbenzofuran (BO-653), was obtained from Chugai Pharmaceutical Co. Ltd. Other chemicals were of the highest quality available commercially.

Blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes from a healthy volunteer after overnight fasting. Plasma was obtained by centrifugation at 1580*g* for 10 min at 4 °C. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the local ethics committees of the National Institute of Advanced Industrial Science and Technology. The volunteer gave written informed consent after a complete explanation of the purpose of this study.

Methods. The consumption of pyranine was followed in PBS with or without plasma by a decrease in absorbance at 454 nm with a UV−visible light spectrophotometer (Shimadzu UV-2450) equipped with a thermostated cell maintained at 37 °C (*15*). The reaction was initiated by the addition of AAPH (final concentration being 50 mM). The lag phase was obtained graphically by extrapolating the slope of maximal probe decay to intersect with the slope of minimal probe decay at the initial stage. The area under the curve (AUC) was measured as reported in the literature (*5, 13*). Briefly, the ratio of absorbance at time t to that at the start, $(Abs)_t/(Abs)_0$, was plotted as a function of time. Integration of AUC was performed up to a time such that the above absorbance ratio reached a plateau.

Plasma was dialyzed using dialysis membrane (Seamless Cellulose Tubing, UC20-32-100, Viskase Co. Inc., Osceola, AR) for 18 h at 4 °C in saline to remove vitamin C and other water-soluble antioxidants. It was confirmed that ascorbic acid and uric acid were removed from plasma, whereas tocopherols were not by this dialysis (*16, 17*). The lipophilic antioxidants were added as a methanol solution to the plasma and incubated for 10 min at 37 °C before oxidation. The amount of methanol was 1% or less by volume of the total solution. A water-soluble antioxidant was added as aqueous solution to the reaction mixture. The oxidation of plasma (10 vol %) was initiated by the addition of AAPH (final concentration = 50 mM) and carried out at 37 °C under air. The mixture of the oxidized samples was extracted with chloroform/methanol (2:1 by volume) by twice the volume as the sample, and the chloroform layer was analyzed for hydroperoxides and hydroxides of cholesteryl ester and phosphatidylcholine and oxycholesterols as reported previously (*16, 17*). Briefly, cholesteryl ester hydroperoxide (CEOOH) and CE hydroxide (CEOH) were measured with an HPLC by spectrophotometric detector at 234 nm. An ODS column (Wakosil-II 5C18RS, 5 μm, 250 mm × 4.6 mm) was used, and acetonitrile/isopropyl alcohol/water (44:54:2 by volume) was eluted at 1 mL/min. In some cases, four regio- and stereoisomers of hydroxyoctadecadienoic acids derived from linoleates were identified and quantified by GC-MS (*18, 19*).

Reproducibility. The experiments were repeated several times, at least three times for each set of experiments. The absolute rate of oxidation varied with plasma samples, but the pattern of the oxidation and its inhibition by antioxidants were essentially the same and satisfactorily (±10%) reproducible.

RESULTS

Pyranine was consumed at a constant rate in the presence of AAPH, and the addition of human plasma suppressed the consumption of pyranine and produced a clear lag phase, which was dependent on the amount of plasma added (**Figure 1**). On the other hand, when plasma dialyzed beforehand was added, pyranine was consumed more slowly without a distinct lag phase (**Figure 1**). The dialysis of plasma removes small hydrophilic antioxidants such as ascorbic acid and uric acid, but not lipophilic antioxidants such as vitamin E (*16, 17*). It is clear from **Figure 1** that the removal of ascorbic acid and uric acid by dialysis increased the rate of pyranine consumption and eliminated the distinct lag phase.

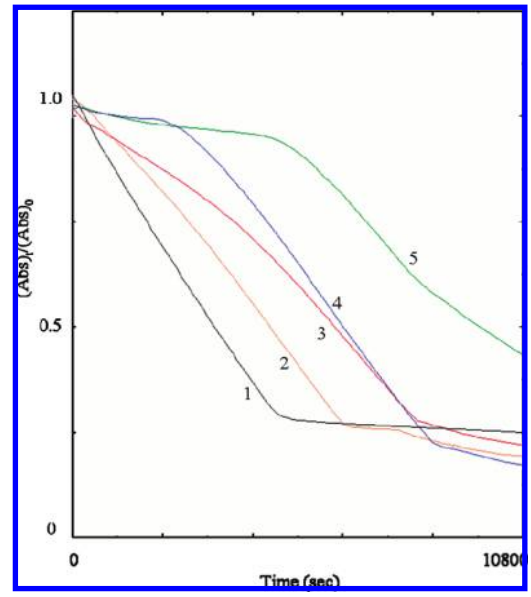

Figure 1. Effects of intact and dialyzed human plasma on the consumption of pyranine induced by 50 mM AAPH at 37 °C. Lines: 1, without plasma; 2 and 3, with dialyzed plasma, 5 and 10 vol %, respectively; 4 and 5, with 5 and 10 vol % intact plasma, respectively.

The antioxidant added to the intact plasma solution suppresses the consumption of pyranine to prolong the lag phase. Examples are shown in **Figure 2**. It can be seen that ascorbic acid and uric acid inhibited pyranine consumption efficiently, and after the lag phase, pyranine was consumed at a rate similar to that in the absence of added plasma or antioxidant. The addition of uric acid increased the lag phase, the length of which was dependent directly on uric acid concentration added (**Figure 3**). Similar results were observed for ascorbic acid (data not shown). On the other hand, lipophilic α-tocopherol and BO-653 did not inhibit the consumption of pyranine as efficiently as ascorbic acid or uric acid (**Figure 2A**). In contrast to α-tocopherol, Trolox and PMC suppressed pyranine consumption almost completely and produced a clear lag phase (**Figure 2B**). It may be noteworthy that the peroxyl radicals were formed at a rate of 65 nM/s under the experimental conditions employed here (*20*), suggesting that pyranine (initial concentration being 50 μM) scavenges only a small fraction of peroxyl radicals formed from AAPH.

In addition to the lag phase, the area under curve (AUC) was measured as described in the literature (*5, 13*) from the difference between the integrated areas under the absorption decay curves in the presence of antioxidant and that without antioxidant, net AUC = AUC (with antioxidant) − AUC (without antioxidant). The AUC produced by uric acid was directly proportional to the amount of uric acid added and the lag phase (**Figure 4**).

To search for the correlation between the radical scavenging capacity assessed by a method applying a probe decay and the

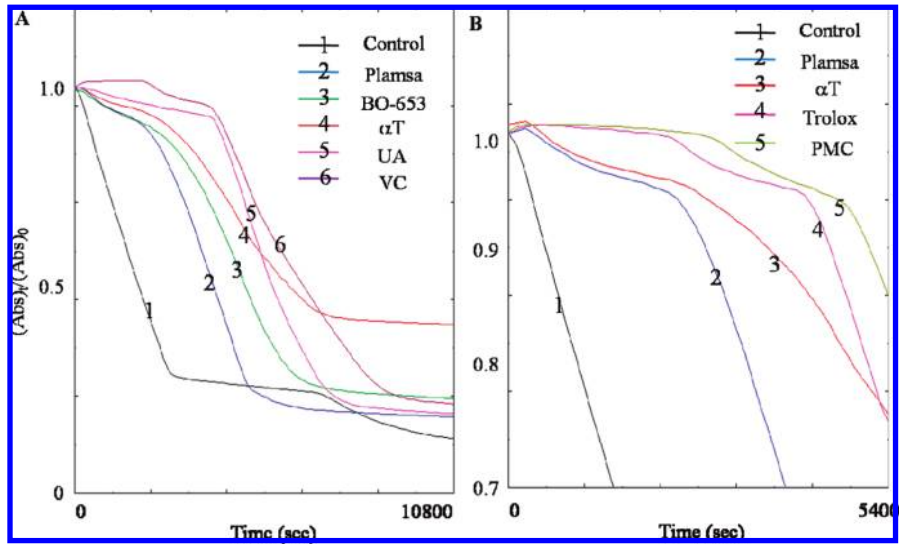

Figure 2. Effects of plasma and antioxidant on the consumption of pyranine. The consumption of pyranine induced by free radicals generated from AAPH at 37 °C was followed from the absorption at 454 nm and the decrease in the ratio of absorbance at time *t* to that at time 0, $(Abs)_t/(Abs)_0$. The initial concentrations of pyranine, plasma, and AAPH were 50 μM, 10 vol %, and 50 mM, respectively. The antioxidants (50 μM) were added to the solution containing 10 vol % plasma. (**A**) VC, ascorbic acid; UA, uric acid; αT, α-tocopherol. (**B**) Results for Trolox and PMC at the early stage are shown in comparison with α-tocopherol. The control reactions were performed without plasma.

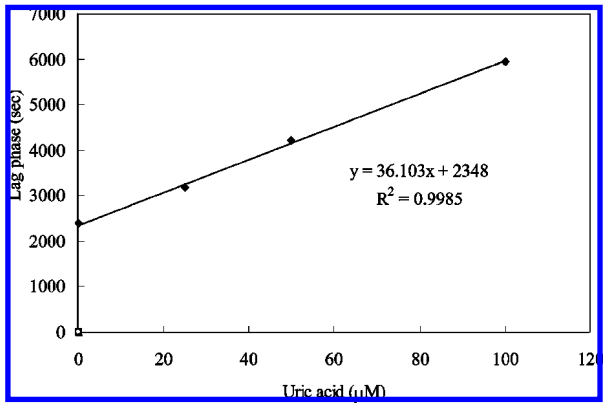

Figure 3. Effects of uric acid on the lag phase. The experimental conditions are the same as those in **Figure 1** except uric acid concentration.

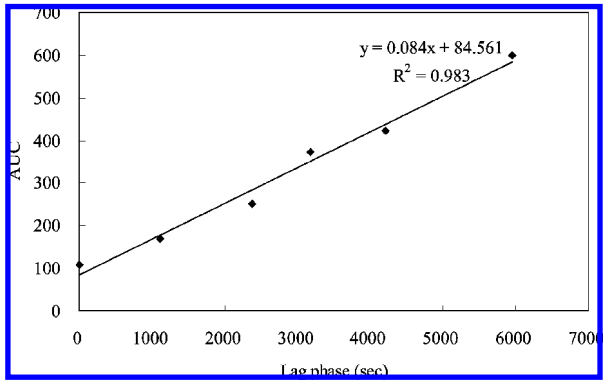

Figure 4. Correlation between area under curve (AUC) and lag phase observed in the consumption of pyranine induced by AAPH in the presence of different concentrations of uric acid. The experimental conditions are the same as those in **Figure 1** except uric acid concentration.

capacity for inhibition of lipid peroxidation, the effect of antioxidant on the formation of cholesteryl ester hydroperoxides (CEOOH) and hydroxides (CEOH) was measured in the oxidation of dialyzed human plasma. The oxidation of plasma induced by free radicals has been studied extensively by many groups including ours, and we have shown previously that the free radical mediated oxidation of human plasma gives CEOOH as a major product with smaller amounts of CEOH and phosphatidylcholine hydro(pero)xides (PCOOH and PCOH) (*16, 17*). Few products are formed from cholesterol. PCOOH is readily reduced by plasma glutathione peroxidase (eGPx or GPx3) and selenoprotein P to give the corresponding hydroxides PCOH, whereas CEOOH is not reduced as readily as PCOOH (*21*). Thus, the extent of lipid peroxidation in plasma can be assessed from the formation of cholesteryl ester hydro(pero)xides, CEO(O)H.

The oxidation of human plasma dialyzed beforehand (10 vol % in PBS) was carried out in the absence and presence of added antioxidant (25 μM) at 37 °C for 30 min in the presence of AAPH. Because the human plasma contained about 25 μM α-tocopherol (*19*), the reaction mixture contained about 2.5 μM endogenous α-tocopherol. The effects of ascorbic acid, uric acid, α-tocopherol, Trolox, PMC, and BO-653 added exogenously on the formation of CEO(O)H were measured. The reaction time was determined to be 30 min, because most of the added antioxidants were depleted in 30 min (data not shown). As mentioned above, the free radicals were formed under the present reaction conditions at a rate of 65 nM/s from 50 mM AAPH (*20*), which suggests that about 60 μM antioxidant is consumed in 30 min if each molecule of antioxidant scavenges 2 molecules of free radicals such as tocopherol, 65 (nM/s) × 1800 s/2 = 60 μM. The results of the effect of antioxidants on lipid peroxidation are summarized in **Table 1**. The effect of antioxidants on pyranine decay is also included in **Table 1** for comparison.

Uric acid inhibited pyranine consumption efficiently, but it inhibited lipid peroxidation much less efficiently than PMC or BO-653, the ratio of CEO(O)H formed in the presence of added antioxidant to that without antioxidant being 0.78 for uric acid versus 0.32 and 0.23 for PMC and BO-653, respectively. The effects of vitamin E homologues are interesting. Water-soluble Trolox inhibited both pyranine consumption and lipid peroxidation. α-Tocopherol suppressed neither pyranine consumption nor lipid peroxidation efficiently, whereas PMC inhibited both. The amount of CEO(O)H in the presence of 50 μM α-tocopherol

Table 1. Capacity of Antioxidant for Radical Scavenging and Inhibition of Lipid Peroxidation in Human Plasma at 37 °C

antioxidant	$AUC/(AUC)_{none}$[a]	$CE(O)OH/(CE(O)OH)_{none}$[b]
none	(1)	(1)
α-tocopherol	1.41	0.99
PMC	nd	0.32
Trolox	1.69	0.50
ascorbic acid	1.32	0.69
uric acid	1.68	0.78
BO-653	1.30	0.23

[a] Ratio of net area under curve in the presence of antioxidant (AUC) to that in the absence of antioxidant $(AUC)_{none}$. The experimental conditions are the same as those in **Figure 2**. The values are the average of several experiments. nd, not determined. [b] Ratio of cholesteryl ester hydro(pero)xides formed in the oxidation of dialyzed human plasma induced by 50 mM AAPH in the presence of 25 μM antioxidant in 30 min to that without antioxidant. The values are the average of several experiments.

was higher than that with 25 μM α-tocopherol, 39.4 versus 15.6 μM, respectively. The apparent prooxidant effect of α-tocopherol in the absence of ascorbic acid is in agreement with the results reported previously for the oxidation of isolated low-density lipoprotein (LDL) (*22, 23*). A synthetic lipophilic antioxidant BO-653 did not inhibit pyranine consumption as efficiently as uric acid and ascorbic acid, but it inhibited lipid peroxidation more efficiently than uric acid and ascorbic acid. BO-653 suppressed pyranine consumption less efficiently but suppressed lipid peroxidation more efficiently than α-tocopherol. These results show that the radical scavenging capacity of antioxidants as assessed by a decay of probe does not correlate well with the efficacy of inhibition of lipid peroxidation in plasma.

DISCUSSION

The assessment of capacity and action of pure compounds and the mixtures contained in foods, beverages, supplements, and drugs as antioxidant has been the subject of extensive studies (*2*−*7, 24*−*27*), but the argument is in some cases in confusion. One of the reasons for such confusion may be ascribed to inconsistent terminology. Admittedly, the word "antioxidant" means different things to different people (*28*). The term "antioxidant capacity" means in some cases the capacity of antioxidant compounds to scavenge free radicals or in other cases the capacity of antioxidative action to inhibit oxidative modification of lipids, proteins, and DNA, sometimes called antioxidation. In this paper, these two "antioxidant capacities" were compared by two methods, one by measuring the effect of antioxidant on the decay of probe and the other by measuring the effect on the formation of lipid peroxidation products in the oxidation of human plasma.

The results of the present study show that relative activities of antioxidants as assessed by the capacity for free radical scavenging and that from inhibition of lipid peroxidation do not agree. In the present study, hydrophilic pyranine scavenges aqueous peroxyl radicals generated from water-soluble AAPH. Hydrophilic antioxidants such as uric acid and ascorbic acid scavenge aqueous free radicals more quickly than pyranine and suppress the consumption of pyranine efficiently. On the other hand, lipophilic antioxidants such as α-tocopherol and BO-653 incorporated and retained in lipoprotein particles in plasma, although chemically more reactive toward free radicals than uric acid, cannot compete well with pyranine in aqueous phase for scavenging aqueous free radicals. Thus, the use of hydrophilic probe and free radicals results in an estimation of high capacity of hydrophilic antioxidants such as uric acid and protein thiols (*29, 30*), whereas the lipophilic antioxidants are assessed to be less potent. PMC, although lipophilic, is not retained firmly in the lipoprotein particles, and it is capable of moving rapidly within and between lipoproteins (*31, 32*) and suppressing pyranine consumption very efficiently. PMC has been shown also to suppress lipid peroxidation in LDL particles more efficiently than α-tocopherol (*31*−*33*). Hydrophilic Trolox suppresses pyranine consumption easily. However, Trolox did not inhibit lipid peroxidation from taking place within lipoprotein particles as efficiently as PMC and BO-653 (**Table 1**). It may be added that Trolox is less potent than α-tocopherol in suppressing AAPH-induced hemolysis of erythrocytes because it is not capable of breaking chain propagation in the erythrocyte membranes (*32*).

To assess the capacity of lipophilic antioxidants, lipophilic radical source and/or probes have been used (*34*−*38*). Huang et al. (*34*) employed randomly methylated β-cyclodextrin as an enhancer for lipophilic antioxidants for ORAC assay and obtained the ORAC value of 0.5 for α-tocopherol relative to Trolox. AAPH was used as a radical source. Yeum, Aldini, and their colleagues applied both hydrophilic and lipophilic radical source, AAPH and MeO-AMVN, and also the fluorescent probes 2′,7′-dichlorodihydrofluorescein (DCFH) and 4,4-difluoro-5-(4-phenyl-1.3-butadienyl)-4-bora-3a,4a-diaza-*S*-indacene-3-undecanoic acid (BODIPY), respectively, to distinguish the hydrophilic and lipophilic components of the total antioxidant capacity of plasma (*35*−*38*). BODIPY was applied also for liposome (*39*) to assess the capacity of lipophilic antioxidant. We have also applied BODIPY to plasma oxidation (*23, 40*). BODIPY has been found to be useful for evaluating the radical scavenging capacity of the antioxidant present in a lipophilic domain, but its capacity did not agree with the capacity for inhibition of lipid peroxidation (*14*).

The different relative activities of the antioxidants assessed by the two methods are explained by considering the factors that determine the efficacy of inhibition of lipid peroxidation. The capacity of free radical scavenging is determined primarily by the chemical reactivity toward free radicals and concentration of antioxidants. This capacity can be assessed by following the decay of probes as shown in many previous studies (*5, 6*). The efficacy for inhibition of lipid peroxidation depends not only on the capacity of free radical scavenging but also on the fate of antioxidant-derived radical, interaction with other antioxidants, localization, and mobility (*3, 32*). It has been shown previously that α-tocopheroxyl radical may propagate the chain oxidation of lipids in LDL particles by a tocopherol-mediated peroxidation (TMP) mechanism, but that ascorbic acid inhibits this TMP by reducing the α-tocopheroxyl radical (*41*). Although the reduction of oxygen radicals in lipoprotein particles by ascorbic acid becomes less efficient as the radicals go deeper into the particles (*31*), it has been shown that ascorbic acid reduces α-tocopheroxyl radicals in the lipoprotein particles efficiently (*42*). Thus, α-tocopherol may act as a prooxidant, rather than an antioxidant, in the oxidation of isolated LDL or dialyzed plasma in the absence of ascorbic acid, but it can inhibit the lipid peroxidation of plasma efficiently in the presence of ascorbic acid (*16, 17, 41, 43*). In fact, little formation of CEO(O)H and PCO(O)H was observed in the oxidation of plasma during the presence of ascorbic acid and α-tocopherol (*17*). On the other hand, uric acid is not capable of reducing α-tocopheroxyl radical, nor does it scavenge free radicals localized within lipoprotein particles (*42*). Therefore, uric acid is not an efficient antioxidant against lipid peroxidation, whether

hydrophilic or lipophilic azo initiator is used, although it is assessed to exert high radical scavenging capacity by a method of hydrophilic probe decay. It has been reported in studies applying probe decay that uric acid plays a major role in scavenging free radicals in plasma. Wayner et al. found by TRAP method that uric acid and plasma proteins accounted for major peroxyl radical scavenging activity in human plasma samples using AAPH as a radical source (*29*). Yeum et al. also showed that the majority of antioxidant capacity of plasma was accounted for by protein (10−28%), uric acid (7−58%), and ascorbic acid (3−27%), whereas the effect of vitamin E (<10%) was small (*38*). These results again show that the free radical scavenging capacity and efficacy for inhibition of lipid peroxidation do not always agree.

In conclusion, this study shows that the capacity of antioxidants for inhibition of lipid peroxidation should be assessed from the extent of lipid peroxidation, because the lipid peroxidation inhibiting capacity is determined not only by the free radical scavenging capacity but also by the actions of antioxidant-derived radicals and interactions between antioxidants, which are not measured by a probe method. It should be noted that, as often pointed out, the capacity of antioxidants for inhibition of lipid peroxidation in vivo is determined not only by the factors discussed in this paper but also by the concentration at the microenvironment, that is, bioavailability, and the form of antioxidant, that is, free form or conjugated metabolites such as glucuronide. The biological efficacy of antioxidants in vivo should be assessed from the biomarkers of lipid peroxidation such as isoprostanes (*44, 45*), hydroxyoctadecadienoic acid (HODE) (*18, 46*), and oxysterols (*47*) in animal and human studies. The information and understanding obtained from the basic in vitro studies are important for proper interpretation of the capacity and action of antioxidants in vivo.

LITERATURE CITED

(1) Halliwell, B.; Gutteridge, J. M. C. *Free Radical Biology and Medicine*, 3rd ed.; Oxford University Press: Oxford, U.K., 1999.

(2) Cao, G.; Prior, R. L. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.* **1998**, *44*, 1309–1315.

(3) Niki, E.; Noguchi, N. Evaluation of antioxidant capacity. What capacity is being measured by which method? *IUBMB Life* **2000**, *50*, 323–329.

(4) Ghiselli, A.; Serafini, M.; Natella, F.; Scaccini, C. Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radical Biol. Med.* **2000**, *29*, 1106–1114.

(5) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

(6) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(7) Magalhaes, L. M.; Segundo, M. A.; Reis, S.; Lima, J. L. F. C. Methodological aspects about in vitro evaluation of antioxidant properties. *Anal. Chim. Acta* **2008**, *613*, 1–19.

(8) Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* **1993**, *14*, 303–311.

(9) Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Locke, S. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. *FEBS Lett.* **1985**, *187*, 33–37.

(10) Balavoine, G. G. A.; Geletii, Y. V. Peroxynitrite scavenging by different antioxidants. Part 1: convenient assay. *Nitric Oxide Biol. Chem.* **1999**, *3*, 40–54.

(11) Pino, E.; Campos, A. M.; Lissi, E. 8-Hydroxy-1,3,6-pyrene trisulfonic acid (pyranine) bleaching by 2,2′-azobis(2-amidinopropane) derived peroxyl radicals. *Int. J. Chem. Kinet.* **2003**, *35*, 525–531.

(12) Campos, A. M.; Sotomayor, C. P.; Pino, E.; Lissi, E. A pyranine based procedure for evaluation of the total antioxidant potential (TRAP) of polyphenols. A comparison with closely related methodologies. *Biol. Res.* **2004**, *37*, 287–292.

(13) Lopez-Alarcon, C.; Aspee, A.; Henriquez, C.; Campos, A. M.; Lissi, E. A. Interaction and reactivity of urocanic acid towards peroxyl radicals. *Redox Rep.* **2005**, *10*, 227–234.

(14) Niki, E.; Fukuhara, A.; Omata, Y.; Saito, Y.; Yoshida, Y. Antioxidant capacity of BO-653, 2,3-dihydro-5-hydroxy-4, 6-di-*tert*-butyl-2,2-dipentylbenzofuran, and uric acid as evaluated by ORAC method and inhibition of lipid peroxidation. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2464–2466.

(15) Omata, Y.; Saito, Y.; Yoshida, Y.; Niki, E. A simple assessment of radical scavenging capacity of beverages. *J. Agric. Food Chem.* **2008**, *56*, 3386–3390.

(16) Itoh, N.; Yoshida, Y.; Hayakawa, M.; Noguchi, N.; Kodama, T.; Cynshi, O.; Niki, E. Inhibition of plasma lipid peroxidation by anti-atherogenic antioxidant BO-653, 2,3-dihydro-5-hydroxy-4,6-di-*tert*-butyl-2,2-dipentylbenzofuran. *Biochem. Pharmacol.* **2004**, *68*, 813–818.

(17) Yoshida, Y.; Ito, N.; Shimakawa, Niki, E. Susceptibility of plasma lipids to peroxidation. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 747–753.

(18) Yoshida, Y.; Hayakawa, M.; Niki, E. Total hydroxyoctadecadienoic acid as a marker for lipid peroxidation in vivo. *BioFactors* **2005**, *24*, 7–15.

(19) Yoshida, Y.; Saito, Y.; Hayakawa, M.; Habuchi, Y.; Imai, Y.; Sawai, Y.; Niki, E. Levels of lipid peroxidation in human plasma and erythrocytes: comparison between fatty acids and cholesterol. *Lipids* **2007**, *42*, 439–449.

(20) Niki, E. Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.* **1990**, *186*, 100–108.

(21) Takebe, G.; Yarimizu, J.; Saito, Y.; Hayashi, T.; Nakamura, H.; Yodoi, J.; Nagasawa, S.; Takahashi, K. A comparative study on the hydroperoxide and thiol specificity of the glutathione peroxidase family and selenoprotein P. *J. Biol. Chem.* **2002**, *277*, 41254–41258.

(22) Waldeck, A. R.; Stocker, R. Radical-initiated lipid peroxidation in low density lipoproteins: insights obtained from kinetic modeling. *Chem. Res. Toxicol.* **1996**, *9*, 954–964.

(23) Itoh, N.; Cao, J.-F.; Chen, Z.-H.; Yoshida, Y.; Niki, E. Advantages and limitation of BODIPY as a probe for the evaluation of lipid peroxidation and its inhibition by antioxidants in plasma. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2059–2063.

(24) Aruoma, O. I. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.* **2003**, *523−524*, 9–20.

(25) Scalzo, J.; Mezzetti, B.; Battino, M. Total antioxidant capacity evaluation: critical steps for assaying berry antioxidant features. *BioFactors* **2005**, *23*, 221–227.

(26) Lotito, S. B.; Frei, B. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon. *Free Radical Biol. Med.* **2006**, *41*, 1727–1746.

(27) Tubaro, F.; Ghiselli, A.; Rapuzzi, P.; Maiorino, M.; Ursini, F. Analysis of plasma antioxidant capacity by competition kinetics. *Free Radical Biol. Med.* **1998**, *24*, 1228–1234.

(28) Halliwell, B. Antioxidant characterization. Methodology and mechanism. *Biochem. Pharmacol.* **1995**, *49*, 1341–1348.

(29) Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochim. Biophys. Acta* **1987**, *924*, 408–419.

(30) Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E.; Ferro-Luzzi, A. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Radical Biol. Med.* **1995**, *18*, 29–36.

(31) Gotoh, N.; Noguchi, N.; Tsuchiya, J.; Morita, K.; Sakai, H.; Shimasaki, H.; Niki, E. Inhibition of oxidation of low density

lipoprotein by vitamin E and related compounds. *Free Radical Res.* **1996**, *24*, 123–134.
(32) Niki, E.; Noguchi, N. Dynamics of antioxidant action of vitamin E. *Acc. Chem. Res.* **2004**, *37*, 45–51.
(33) Bowry, V. W.; Ingold, K. U. The unexpected role of vitamin E (α-tocopherol) in the peroxidation of human low-density lipoprotein. *Acc. Chem. Res.* **1999**, *32*, 27–34.
(34) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002**, *50*, 1815–1821.
(35) Yeum, K.-J.; Russell, R. M.; Krinsky, N. I.; Aldini, G. Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. *Arch. Biochem. Biophys.* **2004**, *430*, 97–103.
(36) Aldini, G.; Yuem, K.-J.; Russell, R. M.; Krinsky, N. I. A method to measure the oxidizability of both the aqueous and lipid compartments of plasma. *Free Radical Biol. Med.* **2001**, *31*, 1043–1050.
(37) Yuem, K.-J.; Aldini, G.; Chung, H.−Y.; Krinsky, N. I.; Russell, R. M. The activities of antioxidant nutrients in human plasma depend on the localization of attacking radical species. *J. Nutr.* **2003**, *133*, 2688–2691.
(38) Beretta, G.; Aldini, G.; Facino, R. M.; Russell, R. M.; Krinsky, N. I.; Yeum, K.−J. Total antioxidant performance: a validated fluorescence assay for the measurement of plasma oxidizability. *Anal. Biochem.* **2006**, *354*, 290–298.
(39) Zhang, J.; Stanley, R. A.; Melton, L. D. Lipid peroxidation inhibition capacity assay for antioxidants based on liposomal membranes. *Mol. Nutr. Food Res.* **2006**, *50*, 714–724.
(40) Yoshida, Y.; Shimakawa, S.; Itoh, N.; Niki, E. Action of DCFH and BODIPY as a probe for radical oxidation in hydrophilic and lipophilic domain. *Free Radical Res.* **2003**, *37*, 861872.
(41) Thomas, S. R.; Stocker, R. Molecular action of vitamin E in lipoprotein oxidation: implications for atherosclerosis. *Free Radical Biol. Med.* **2000**, *28*, 1795–1805.
(42) Sato, K.; Niki, E.; Shimasaki, H. Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. *Arch. Biochem. Biophys.* **1990**, *279*, 402–405.
(43) Neuzil, J.; Thomas, S. R.; Stocker, R. Requirement for, promotion, or inhibition by α-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation. *Free Radical Biol. Med.* **1997**, *22*, 57–71.
(44) Milne, G. L.; Musiek, E. S.; Morrow, J. D. F2-isoprostanes a markers of oxidative stress in vivo: an overview. *Biomarkers* **2005**, *1*, S10−23.
(45) Montsuschi, P.; Barnes, P.; Roberts, L. J., 2nd. Insights into oxidative stress; the isoprostanes. *Curr. Med. Chem.* **2007**, *14*, 703–717.
(46) Yoshida, Y.; Hayakawa, M.; Habuchi, Y.; Itoh, N.; Niki, E. Evaluation of lipophilic antioxidant efficacy in vivo by the biomarkers hydroxyoctadecadienoic acid and isoprostane. *Lipids* **2007**, *42*, 463–472.
(47) Arca, M.; Natoli, S.; Micheletta, F.; Riggi, S.; Di Angelantonio, E.; Montali, A.; Antonini, T. M.; Antonini, R.; Diczfalusy, U.; Iuliano, L. Increased plasma levels of oxysterols, in patients with familial combined hyperlipidemia: reduction during atorvastatin and fenofibrate therapy. *Free Radical Biol. Med.* **2007**, *42*, 698–705.

Received for review February 28, 2008. Revised manuscript received July 1, 2008. Accepted July 30, 2008.

JF800605X