Biomarkers for Antioxidant Defense and Oxidative Damage:
Principles and Practical Applications

Chapter 1
Antioxidant Activity and Oxidative Stress: An Overview

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INTRODUCTION

Oxidative stress is involved in the process of aging (Kregel and Zhang 2007) and various chronic diseases such as atherosclerosis (Fearon and Faux 2009), diabetes (Ceriello and Motz 2004), and eye disease (Li et al. 2009a), whereas fruit and vegetable diets rich in antioxidants such as polyphenols, vitamin C, and carotenoids are correlated with a reduced risk of such chronic diseases (Christen et al. 2008, Dauchet et al. 2006, Dherani et al. 2008). An excessive amount of reactive oxygen/nitrogen species (ROS/RNS) leading to an imbalance between antioxidants and oxidants can cause oxidative damage in vulnerable targets such as unsaturated fatty acyl chains in membranes, thiol groups in proteins, and nucleic acid bases in DNA (Ceconi et al. 2003). Such a state of "oxidative stress" is thought to contribute to the pathogenesis of a number of human diseases (Thannickal and Fanburg 2000).

Sensitive and specific biomarkers for antioxidant status/oxidative stress are essential to better understand the role of antioxidants and oxidative stress in human health and diseases, thereby maintaining health and establishing effective defense strategies against oxidative stress. Several assays to measure "total" antioxidant capacity of biological systems have been developed to investigate the involvement of oxidative stress in pathological conditions or to evaluate the functional bioavailability of dietary antioxidants. Conventional assays to determine antioxidant capacity primarily measure 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 fat-soluble antioxidants such as tocopherols and carotenoids show little influence over the many results. However, there are new approaches to define the total antioxidant capacity of plasma, which reflect the antioxidant network between water- and fat-soluble antioxidants. Revelation of the mechanism of action of antioxidants and their true antioxidant potential can lead to identifying proper strategies to optimize the antioxidant defense systems in the body.

Methodological aspects of various antioxidant capacity assays have been extensively discussed recently (Magalhaes et al. 2008). This chapter focuses on important antioxidants in biological systems, factors affecting bioavailability of antioxidants and, therefore, antioxidant capacity, and basic principles of various biomarkers for antioxidant capacity and their applications.

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OXIDATIVE STRESS AND ANTIOXIDANTS IN A BIOLOGICAL SYSTEM

ROS are continuously generated by normal metabolism in the body (Gate et al. 1999) and these ROS are necessary to maintain biological homeostasis through various functions such as vasoregulation and various cellular signal transduction (Hensley and Floyd 2002). However, overproduction of these ROS can also cause damage to the macromolecules necessary for cell structure and function.

Cellular production of ROS such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and alkoxyl radical ($RO^{\bullet}$) occurs from both enzymatic and non-enzymatic reactions. Mitochondria appear to be the most important subcellular site of ROS production, in particular of $O_2^{\bullet -}$ and H_2O_2 in mammalian organs. The electron transfer system of the mitochondrial inner membrane is a major source of superoxide production when molecular oxygen is reduced by a single electron. Superoxide can then dismutate to form hydrogen peroxide (H_2O_2), and then can further react to form the hydroxyl radical ($HO^{\bullet}$) and ultimately water.

In addition to intracellular membrane-associated oxidases, soluble enzymes such as xanthine oxidase, aldehyde oxidase, dihydroorotate dehydrogenase, flavoprotein dehydrogenase, and tryptophan dioxygenase can generate ROS during catalytic cycling. Auto-oxidation of small molecules such as dopamine, adrenaline (epinephrine), flavins, and quinols can be an important source of intracellular ROS production as well. In most cases, the direct product of such auto-oxidation reactions is the superoxide anion (Thannickal and Fanburg 2000).

Any compound that can inhibit oxidation of external oxidants is considered to be an antioxidant. This is a relatively simple definition but, at times, it becomes very difficult to evaluate whether a compound actually has an antioxidant action, particularly *in vivo*.

It is still not clear what kinds of ROS play a role in the pathogenesis of human disease and where the major sites of ROS action occur. There is, however, convincing evidence that lipid peroxidation is related to human pathology, such as in atherosclerosis (Valkonen and Kuusi 1997). The actions of antioxidants in biological systems depend on the nature of oxidants or ROS imposed on the systems, and the activities and amounts of antioxidants present and their cooperative/synergistic interactions in these systems.

Numerous epidemiological studies have indicated that diets rich in fruits and vegetables are correlated with a reduced risk of chronic diseases (Czernichow et al. 2009, Hung et al. 2004, Liu et al. 2001, Liu et al. 2000). It is probable that antioxidants, present in the fruits and vegetables such as polyphenols, carotenoids, and vitamin C, prevent damage from harmful reactive oxygen species, which either are continuously produced in the body during normal cellular functioning or are derived from exogenous sources (Gate et al. 1999). The possible protective effect of antioxidants in fruits and vegetables against ROS has led people to consume antioxidant supplements such as β-carotene, α-tocopherol, and/or multivitamins. It is not surprising to note that more than 11% of US adults age 20 years or older consume at least 400 IU of vitamin E per day from supplements (Ford et al. 2005). However, intervention studies have failed to show a consistent beneficial effect of antioxidant supplements such as vitamin E (Lee et al. 2005) or β-carotene (Baron et al. 2003, Omenn et al. 1996) against chronic diseases. How can we explain these apparent contradictory results between observational studies and intervention trials?

It is interesting to note that although seven and a half years of supplementation with a combination of antioxidants (vitamin C, β-carotene, zinc, and selenium) did not affect the risk of metabolic syndrome, baseline concentrations of serum vitamin C and β-carotene were negatively associated with metabolic syndrome in a generally well-nourished population (Czernichow et al. 2009). It is probable that the generally well-nourished population maintains optimal ranges of antioxidants through a balanced dietary fruit and vegetable intake. However, high doses of a single or limited mixture of antioxidant supplements may not affect the already saturated *in vivo* antioxidant network, but rather could result in an imbalance in the antioxidant

network and could possibly even act as pro-oxidants. A recent prospective study showing an inverse association of baseline plasma antioxidant concentrations with the risk of heart disease and cancer also supports the beneficial effect of a balanced antioxidant status, which can be attained by eating diets high in fruits and vegetables (Buijsse et al. 2005).

MARKERS OF ANTIOXIDANT CAPACITY IN A BIOLOGICAL SYSTEM

Several human studies have failed to show a direct correlation between the physiologic consumption of dietary fat-soluble antioxidants and subsequent changes in antioxidant capacity (Castenmiller et al. 1999, Pellegrini et al. 2000). For example, it has even been suggested that carotenoids may not act as antioxidants *in vivo* (Rice-Evans et al. 1997). These suggestions derive from the lack of proper analytical methods for measuring antioxidant capacity. Inasmuch as conventional methods, such as total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC), etc., use primarily hydrophilic radical generators and measure primarily antioxidant capacity in the aqueous compartment of plasma, they are unable to determine the antioxidant capacity of the lipid compartment (Cao et al. 1993, Lussignoli et al. 1999). Therefore, it is not surprising that most of the methods used to measure purported "total antioxidant capacity" of plasma are not affected by lipophilic antioxidants, such as carotenoids (Cao et al. 1998b, Castenmiller et al. 1999, Pellegrini et al. 2000).

This can be explained by the fact that plasma carotenoids, which are deeply embedded in the core of lipoproteins, are not available for reaction with aqueous radical species or ferric complexes used in these assays. In addition, an assay to measure total antioxidant capacity in a biological sample such as plasma must consider the heterogeneity of the sample, which consists of both hydrophilic and lipophilic compartments that contain water-soluble and fat-soluble antioxidants, respectively. Possible cooperative/synergistic interactions among antioxidants in biological samples should not be overlooked.

Azo initiators are a class of radical inducers (which contain the –N=N– group) widely used in experiments *in vitro* to generate radical species. The azo initiators decompose at a temperature-controlled rate to give carbon-centered radicals, which react rapidly with O_2 to yield the peroxyl radical ($ROO^{\bullet}$).

$$R-N=N-R \rightarrow N_2 + 2R^{\bullet}$$
$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

Peroxyl radicals derived from azo initiators can induce the lipid peroxidation cascade and can also damage proteins. Depending on the lipophilicity of the azo initiators [2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) is water soluble whereas 2,2′-azobis(2,4-dimethylvaleronitrile (AMVN) and ,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) are lipophilic], the peroxyl radicals are generated in the aqueous or lipid phase of the sample, respectively. The choice of the site of radical generation is of great importance, because the activities of antioxidants present in both the lipid and aqueous compartments depend on the localization of the attacking radical species (Yeum et al. 2003).

Table 1.1 shows the currently available assays to determine antioxidant capacity in hydrophilic and lipophilic environments in biological samples such as plasma. When used alone, those assays (Cao et al. 1993, Valkonen and Kuusi 1997) that use hydrophilic radical initiators and probes are insufficient for determining the antioxidant activity of carotenoids, which are deeply embedded in the lipoprotein core of biological samples. There have been attempts to determine the activity of fat-soluble antioxidants by measuring the antioxidant activity of lipid extracts dissolved in an organic solvent (Prior et al. 2003). This approach, however, cannot appreciate the possible interactions between the fat-soluble and water-soluble antioxidants. The alternative approach of producing radicals in the lipid compartment of whole plasma and monitoring lipid peroxidation by a lipophilic probe (Aldini et al. 2001) allows measurement

Table 1.1. Assays to determine antioxidant capacity in biological systems.

Assay	Radical inducer	Oxidizable substrate (probe)	Wavelength	Calculation	Reference
Plasma susceptibility against exogenous pro-oxidant induced oxidation (hydrophilic assay)					
TRAP	AAPH	DCFH	λex = 480, λem = 526	Lag time	Valkonen and Kuusi 1997
		R-Phycoerythrin	λex = 495, λem = 595		Ghiselli et al. 1995
ORAC	AAPH	R-Phycoerythrin	λex = 495, λem = 595	AUC	Cao et al. 1995
Crocin bleaching	ABAP		445 nm	Absorbance	Tubaro et al. 1998 Kampa et al. 2002
Plasma quenching ability of stable/pre-formed radicals (hydrophilic assay)					
TEAC	ABTS+•		734 nm	Absorbance	Miller et al. 1993 Re et al. 1999
FRAP	Fe^{3+}		593 nm	Absorbance	Benzie and Strain 1996
Plasma susceptibility against exogenous pro-oxidant induced oxidation (lipophilic assay)					
Lipophilic ORAC	AAPH	Fluorescein	λex = 485, λem = 520	AUC	Prior et al. 2003
Lipophilic antioxidant activity	AAPH	DPHPC	λex = 354, λem = 430	Lag time	Mayer et al. 2001
TAP	MeO-AMVN	BODIPY 581/591	λex = 500, λem = 520	AUC	Aldini et al. 2001

TRAP: Total radical-trapping antioxidant parameter.
ORAC: Oxygen radical-absorbing capacity.
TEAC: Trolox equivalent antioxidant capacity.
FRAP: Ferric-reducing ability of plasma.
TAP: Total antioxidant performance.
AAPH, ABAP: 2,2′-Azobis-(2-amidinopropane)dihydrochloride.
ABTS: 2,2′-Azinobis(3-ethylbenzothiazoline 6-sulphonate).
AUC: Area under the curve.
MeO-AMVN: 2,2′-Azobis(4-methoxy-2,4-dimethylvaleronitrile).
DCFH: 2′,7′-Dichlorodihydrofluorescein.
DPHPC: 1-Palmitoyl-2-((2-(4-(6-phenyl-trans-1,3,5-hexatrienyl)phenyl)ethyl)-carbonyl-sn-glycero-3-phosphocholine.
BODIPY 581/591: 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid.
Modified from Yeum et al. 2009b.

of the actual "total" antioxidant activity including possible interactions among antioxidants located in the hydrophilic and lipophilic compartments, because the interference of large amounts of protein (e.g. albumin) in the hydrophilic compartment can be overcome by this approach.

HYDROPHILIC ANTIOXIDANT CAPACITY ASSAYS

There are mainly two hydrophilic approaches to determine the antioxidant capacity in plasma. The first approach measures the antioxidant capacity in plasma using hydrophilic assays in the presence of oxidants that act as pro-oxidants. These assays determine the susceptibility of plasma against oxidation induced by added pro-oxidants (radical inducers) and monitored by an exogenous oxidizable substrate (probe). The oxidation of the probe is theoretically inhibited by the antioxidants present in plasma during the induction period. The TRAP and ORAC assays are presently the most widely used methods for measuring antioxidant capacity in biological systems such as serum and tissues. Dichlorofluorescein-diacetate, phycoerythrin (R-Pe), and crocin-based assays also are included in this category of assays. Specifically, plasma or serum, when challenged with a hydrophilic radical inducer such as 2,2′-azobis(2,4-amidinopropane) dihydrochloride (AAPH), can be monitored by a hydrophilic oxidizable substrate such as 2′,7′-dichlorodihydrofluorescein (DCFH) (Valkonen and Kuusi 1997), crocin (Kampa et al. 2002, Tubaro et al. 1998), or R-Pe (Cao and Prior 1999). Antioxidant capacity can be expressed in various ways such as lag phase, area under the curve, or competition kinetics.

AAPH is a hydrophilic azo-compound that spontaneously decomposes at 37°C with a known rate constant ($R_i = 1.36 \times 10^{-6}$ [AAPH] mol/liter/sec), giving rise to carbon-centered radicals that then react with oxygen, yielding the corresponding peroxyl radicals. DCFH, which can be oxidized to highly fluorescent (Exc 480 nm, Em 526 nm) dichlorofluorescein by peroxyl radicals, is used as an oxidizable substrate in the TRAP assay (Valkonen and Kuusi 1997). R-Pe is a protein isolated from *Corallina officinalis*, and is used as the oxidizable substrate in the TRAP (Ghiselli et al. 1995) and ORAC (Cao and Prior 1999) assays. R-Pe is a fluorescent protein that emits in the visible region (Exc 495 nm, Em 595 nm) and is characterized by fluorescence quenching upon reaction with peroxyl radicals. Crocin, isolated from saffron and characterized by a polyene chain with a high extinction coefficient, has been used as an oxidizable substrate in the assay developed by Tubaro (Tubaro et al. 1998) and then automated by Kampa (Kampa et al. 2002) in the crocin bleaching assay. The reaction of crocin with peroxyl radical leads to a loss of the double bond conjugation and hence to bleaching that can be readily monitored at 445 nm.

The second approach to measure antioxidant capacity in plasma using a hydrophilic assay is to quench a stable and pre-formed radical that does not act as a pro-oxidant. The trolox equivalent antioxidant capacity (TEAC) assay, which was reported by Miller et al. (1993), determines the antioxidant capacity of plasma by measuring the ability of plasma to quench the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS). The quenching reaction is monitored by measuring the decay of the radical cation at 734 nm. The ferric reducing ability of plasma (FRAP) assay has received a great deal of attention because of its quick and simple methodology (Benzie and Strain 1996). The FRAP assay measures the reduction of the ferric ion to ferrous ion at low pH, which causes a colored ferrous-tripyridyltriazine complex to form. FRAP values can be obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing the ferrous ion in a known concentration.

LIPOPHILIC ANTIOXIDANT CAPACITY ASSAYS

Two decades ago, Niki (1990) introduced AAPH and AMVN as the sources of water- and lipid-soluble peroxyl radicals respectively. As shown in the work of Massaeli et al. (1999), where preincubation of LDL with fat-soluble antioxidants increased the protective effect

against free radicals while preincubation with water-soluble antioxidants did not show any effect, the importance of lipophilicity vs. hydrophilicity in antioxidants and free radical generating systems for determining antioxidant capacity has been recognized. It has also been demonstrated (Yeum et al. 2003) that the activities of antioxidants present in both the lipid and aqueous compartments depend on the localization of the attacking radical species.

In an effort to understand the biological significance of lipophilic antioxidants, several recent studies have paid attention to the antioxidant capacity in the lipid compartment of plasma. Mayer et al. (2001) proposed a continuous spectroscopic method using selective fluorescence markers to monitor the aqueous and lipid phases in human serum. In particular, diphenylhexatriene-labeled proprionic acid was used as an appropriate probe for the aqueous phase because it preferentially binds to albumin, while diphenylhexatriene-labeled phosphatidylcholine, which incorporates into lipoproteins, monitors the lipid compartment oxidizability. AAPH was selected as the radical inducer for both compartments.

By using this method, the authors reported that supplementation of human serum with quercetin, rutin, vitamins E and C, or total apple phenolics *in vitro* led to a decrease in oxidizability depending on the oxidation marker and the hydrophobicity of the antioxidant. That is, fat-soluble antioxidants such as quercetin and vitamin E showed higher protective effects against lipoprotein oxidation, whereas water-soluble lutin and vitamin C more efficiently protected the aqueous phase.

An improved TEAC assay has been reported by Re et al. (1999). By using a pre-formed radical mono-cation of ABTS and an appropriate solvent system, the assay is applicable to both hydrophilic and lipophilic systems. The ORAC assay has also been expanded to reflect lipophilic antioxidants by using randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer, AAPH as a radical initiator, and fluorescein as an oxidizable substrate (Huang et al. 2002). Recently, this updated ORAC assay was applied to human plasma (Prior et al. 2003) and the authors reported that lipophilic antioxidants represent less than 30% of the total antioxidant capacity of the protein-free plasma. For the lipophilic ORAC assay, lipophilic antioxidants were extracted by hexane, dried, and resuspended in 7% RMCD solution (50% acetone/50% water, v/v). However, this assay, which partitioned hydrophilic and lipophilic antioxidants, may not be relevant to a true biological system in which active communication occurs among hydrophilic and lipophilic antioxidants.

Aldini et al. (2001) reported a method that measures antioxidant capacity in both the hydrophilic and lipophilic compartments of plasma and allows for interaction between the antioxidants in the two compartments. A lipophilic radical generator coupled with a selective fluorescent probe capable of detecting lipid peroxidation was used to measure the lipid compartment. 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN), which decomposes at 37°C, was selected as a lipid-soluble radical inducer, and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (BODIPY581/591) was used as a selective lipophilic oxidizable substrate (Drummen et al. 2002, Pap et al. 1999). The significantly higher rate constant of MeO-AMVN as compared to that of AMVN allows it to easily achieve lipid peroxidation in a biological system (Aldini et al. 2001). An oxidation-sensitive fluorescent probe, BODIPY 581/591, which has a high quantum yield and readily enters membranes (Drummen et al. 2002), provided the sensitive and selective measurement of oxidation in the lipid compartment of plasma. The selective incorporation of BODIPY 581/591 into the individual lipoprotein fractions, VLDL, LDL, and HDL, of human plasma has been further confirmed (Yeum et al. 2003). A significant correlation ($p < 0.0001$) between plasma carotenoid concentration and antioxidant capacity determined by this assay was found in subjects who participated in a dietary intervention trial with high fruit and vegetable diets (Yeum et al. 2005).

It is interesting to note that a high amount of single antioxidant (>15 mg of α-tocopherol) has been reported to be required to show a difference in antioxidant capacity, whereas less than a half serving of fruits and vegetables resulted in significant difference in antioxidant capacity

in a recently reported cross-sectional study (Talegawkar et al. 2009). This observation supports the importance of synergistic action among the numerous antioxidants found in foods vs. a single antioxidant supplement. Another notable improvement of this assay is that it requires a much lower dilution of plasma (5 to 10 × dilution) as compared to those of previously reported assays, which require 100 ×, 150 ×, and 250 × dilutions for the FRAP (Benzie and Strain 1996), ORAC (Cao et al. 1995), and TRAP (Ghiselli et al. 1995) assays, respectively. One of the drawbacks of conventional assays to measure antioxidant capacity has been the high dilution of plasma resulting in very low concentrations of antioxidants in the reaction mixtures.

APPLICATION OF HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT CAPACITY ASSAYS

When hydrophilic assays are applied, the majority of the antioxidant capacity of plasma can be accounted for by protein (10% to 28%), uric acid (7% to 60%), and ascorbic acid (2% to 27%), whereas the effect of vitamin E (<10%) is minimal (Benzie and Strain 1996, Cao and Prior 1998, Tubaro et al. 1998, Valkonen and Kuusi 1997, Wayner et al. 1987) as shown in Table 1.2.

As discussed previously, these assays mainly measure the antioxidant capacity of the aqueous compartment, because the radicals produced in the hydrophilic compartment and probes are also located in the hydrophilic compartment oxidized by aqueous peroxyl radicals. α-Tocopherol (vitamin E), which has its chroman head group oriented toward the lipoprotein membrane, may participate somewhat in the antioxidant action through interaction with water-soluble antioxidants such as ascorbic acid. However, it is clear that carotenoids, which are deeply embedded in the lipid core, cannot participate in the antioxidant effect under these experimental

Table 1.2. Estimated percent contribution of plasma antioxidants in various antioxidant capacity assays.

		Estimated percent contribution					
			ORAC[3]				
Plasma antioxidant	Plasma normal range (μmol)	TRAP[1,2]	Total	PCA*	TEAC[3]	FRAP[4]	Crocin[5]
Water-soluble antioxidant							
Protein	800–1,000	21–24	28	0	28	10	21
Uric acid	150–450	58	7	39	19	60	51
Ascorbic acid	30–150	9–14	<2	7	3	15	27
Fat-soluble antioxidant							
Tocopherols	20–50	7–9	<1	—	<2	5	—
Carotenoids	0.5–3	—	—	—	—	—	—

*Serum non-protein fractions extracted with perchloric acid (PCA).
[1]Valkonen and Kuusi 1997.
[2]Wayner et al. 1987.
[3]Cao et al. 1995.
[4]Benzie and Strain 1996.
[5]Tubaro et al. 1998.
Modified from Yeum et al. 2004.

conditions. The lack of contribution of fat-soluble antioxidants can also be ascribed to the relatively lower amount of fat-soluble antioxidants than water-soluble antioxidants in plasma, although it should be recognized that the antioxidant activity of fat-soluble antioxidants can be greatly enhanced by synergistic interactions with water-soluble and other fat-soluble antioxidants.

Thus, foods such as green tea (Benzie and Szeto 1999, Serafini et al. 1996), cocoa (Rein et al. 2000), red wine (Serafini et al. 1998, Tubaro et al. 1998), coffee (Natella et al. 2002), and strawberries (Cao et al. 1998a) that contain considerable amounts of water-soluble polyphenols significantly increase plasma antioxidant capacity as determined by hydrophilic antioxidant capacity assays. It is interesting to note that several studies have pointed out that the increase in plasma antioxidant capacity observed after the consumption of flavonoid-rich foods such as wine was due to a plasma uric acid increase not caused by the flavonoids (Caccetta et al. 2000, Day and Stansbie 1995, Lotito and Frei 2006).

On the other hand, diets rich in carotenoids (e.g. lycopene or β-carotene) do not affect antioxidant capacity as measured by the hydrophilic TRAP, FRAP, or ORAC assays (Bohm and Bitsch 1999, Bub et al. 2000, Pellegrini et al. 2000). In spite of the consistent failure to show the modification of antioxidant capacity by consumption of a high carotenoid diet (Pellegrini et al. 2000) or supplementation with carotenoid in humans (Li et al. 2009b), it is noteworthy that there is considerable and consistent evidence from experiments *in vitro* for antioxidant actions of carotenoids (Miller et al. 1996, Palozza and Krinsky 1992a), including their geometrical isomers (Bohm et al. 2002), tested in solvent systems *in vitro*.

BIOLOGICAL SIGNIFICANCE OF ANTIOXIDANT INTERACTIONS

The actions of antioxidants in biological systems such as plasma depend on (1) the nature of oxidants or ROS imposed on the biological systems, (2) the activities and amounts of antioxidants, and (3) their cooperative/synergistic interactions. It is still not clear what kinds of ROS play a role in human pathologies and where the major sites of ROS action occurs. In an attempt to gain a better understanding of the biological actions of antioxidants, the activity of single antioxidants or various combinations of antioxidants have been studied over the last decade (Burke et al. 2001, Mortensen and Skibsted 1997, Palozza and Krinsky 1992b). Most of these studies were carried out in homogeneous solvent systems (i.e. either aqueous or lipid) (Niki et al. 1984) or artificial membranes (liposomes, micelles) in buffer solutions (Fukuzawa et al. 1997, Woodall et al. 1995), or by using isolated LDLs (Carroll et al. 2000), cells (Palozza et al. 2004), and tissue preparations (Palozza and Krinsky 1992b). However, these types of model systems are far different from an actual biological system such as human serum/plasma, in that plasma is a heterogeneous entity consisting of hydrophilic and lipophilic compartments and contains high concentrations of other components such as protein (~600 μmol/L).

Interactions of different antioxidants in plasma have also been studied extensively over the past decade. In particular, work has focused on both the interactions between hydrophilic and lipophilic antioxidants, such as ascorbic acid and α-tocopherol (Niki et al. 1995), or carotenoids and ascorbic acid (Burke et al. 2001), and between lipophilic antioxidants (carotenoids and α-tocopherol) (Mortensen and Skibsted 1997, Palozza and Krinsky 1992b). The combination of α-tocopherol and β-carotene has been reported to act cooperatively as well to slow down MDA formation initiated by the aqueous peroxyl radical, AAPH, in a liver microsomal membrane preparation (Palozza and Krinsky 1992b). β-Carotene added to preformed lipid bilayers produced much less of an antioxidant effect than β-carotene incorporated in the liposomes during bilayer formation (Liebler et al. 1997). It is possible that α-tocopherol reduces β-carotene peroxyl radicals (LOO-β-C-OO$^{\bullet}$) as well as β-carotene radical cations (β-C$^{\bullet+}$), as has been shown in a homogeneous solution (Mortensen and Skibsted 1997). In addition, β-carotene may recycle α-tocopherol from the α-tocopheroxyl radical (α-TO$^{\bullet}$) through electron transfer

(Bohm et al. 1997), although this possible mechanism of action should be further studied, because the reduction potential of β-carotene is reported to be lower than that of α-tocopherol (Buettner 1993, Edge et al. 2000). In addition, a synergistic antioxidant activity of lycopene in combination with vitamin E in a liposome system has been reported (Shi et al. 2004).

It has been reported that β-carotene, which is located in the lipophilic core of the membrane bilayer, can directly interact with water-soluble antioxidants. Because β-carotene can be converted into β-carotene peroxyl radical cations by scavenging radical species in a heterogeneous micellar environment (Hill et al. 1995), the more polar β-carotene radical cation ($\beta\text{-}C^{\bullet+}$) can be reoriented toward the hydrophilic compartment, allowing ascorbic acid to repair the β-carotene radical (El-Agamey et al. 2004). Other work (Burke et al. 2001) has also shown an interaction between β-carotene radical cations and ascorbic acid. Ascorbic acid can also spare uric acid, and uric acid not only scavenges radicals but can also stabilize ascorbic acid by iron chelation (Sevanian et al. 1991). It has been reported that the major green tea polyphenols, (-)-epigallocatechin-(3)-gallate (EGCG) located in the aqueous phase, also can recycle α-tocopherol through an H-transfer mechanism (Aldini et al. 2003).

Daily supplementation with moderate doses of combined antioxidants (100 mg vitamin C, 100 mg vitamin E, 6 mg β-carotene, and 50 μg selenium) has been reported to increase plasma antioxidant capacity and decrease chromosome aberrations significantly in lymphocytes (Volkovova et al. 2005). On the other hand, a meta-analysis of randomized trials with antioxidant supplements suggested that high doses of β-carotene (Vivekananthan et al. 2003) or α-tocopherol (Miller et al. 2005) led to significant increases in mortality due to all causes and no effect against coronary heart disease risk (Eidelman et al. 2004, Knekt et al. 2004). It is likely that physiological doses of a combination of water-soluble and fat-soluble antioxidants, which can be successfully obtained by adequate fruit and vegetable intake, are required to establish an effective antioxidant network *in vivo*.

It should be also recognized that even though various combinations of "two" antioxidants in physiologic concentrations showed additive/synergistic interactions within and between the hydrophilic and lipophilic compartments *in vitro* (Niki 1987, Yeum et al. 2009), the much more complex *in vivo* system, in which many different antioxidants such as uric acid and protein already exist, is generally maintained in homeostasis. Thus, the potency of the entire antioxidant network is not subject to swift modifications through supplementation of a single antioxidant or their combinations when given in physiologic doses to healthy people.

BIOLOGICAL RELEVANCE OF ANTIOXIDANT SUPPLEMENTATION

It has been believed that dietary supplementation with antioxidants can be a part of a protective strategy to minimize the oxidative damage in vulnerable populations, such as the elderly. It should be pointed out that the metabolism and functions of antioxidants *in vivo* and *in vitro* may not be the same. For example, antioxidant nutrients can interact with each other during gastrointestinal absorption and metabolism (Kostic et al. 1995, Paetau et al. 1997, van den Berg and van Vliet 1998, White et al. 1994). Although epidemiological evidence continues to accumulate showing that diets high in fruits and vegetables are associated with a reduced risk of chronic diseases such as cardiovascular disease (Gaziano et al. 1995, Hu 2003, Hung et al. 2004, Mayne 2003, Osganian et al. 2003), several attempts to alter overall antioxidant activity by supplementing antioxidant nutrients or implementing dietary modification in healthy subjects (Castenmiller et al. 1999, Jacob et al. 2003, Pellegrini et al. 2000) have not been successful. Considering that the biological antioxidant network in healthy subjects already contains adequate amounts of water- and fat-soluble antioxidants working in an interactive manner, further increases of single or small combinations of antioxidants within a physiologic range might not affect the overall *in vivo* antioxidant network (Czernichow et al. 2009, Li et al. 2009b). It should also be appreciated that synergistic interactions with respect to antioxidant

activity as well as biological functions can occur not only among well recognized antioxidants (e.g. vitamin C, vitamin E) but also between flavonoids such as soy and green tea (Bertipaglia de Santana et al. 2008), and between micromineral and phytochemicals such as selenium and sulforaphane (Campbell et al. 2007).

As reported by Valtuena et al. (2008), food selection based on "total antioxidant capacity" values for foods can modify antioxidant intake without altering markers of oxidative stress or total antioxidant activity in plasma. Thus, antioxidant supplementation may alter other markers of biological function such as systemic inflammation and liver function without any changes in various markers of antioxidant capacity or lipid peroxidation (Valtuena et al. 2008).

ANTIOXIDANTS AND GENE INTERACTIONS

Considering the fact that the total antioxidant capacity of plasma is the susceptibility of the biological system against exogenous free radicals, endogenous antioxidants and lipid profiles may be the primary factors in determining the reduction potential of the plasma. In addition, enzymatic antioxidant defense systems as well as genetic variance should not be overlooked (Figure 1.1).

Although vitamin E supplementation has been recommended to prevent cardiovascular diseases (Jialal and Devaraj 2000, Lonn et al. 2005, Vivekananthan et al. 2003), a meta-analysis conducted by Miller et al. (2005) indicated that a high dose of vitamin E (>400 IU) was associated with a higher incidence of all causes of mortality. The study by Milman et al. (2008) provides a clue for this discrepancy: diabetic patients with haptoglobin 2–2 genotype, who were supplemented with 400 IU of vitamin E, showed a protective effect against cardiovascular events. It is probable that high-dose antioxidant administration may benefit individuals who are under high oxidative stress because haptoglobin 2 allele protein products are inferior antioxidants as compared to that of Haptoglobin 1 (Asleh et al. 2005, Bamm et al. 2004, Melamed-Frank et al. 2001). Furthermore, a recent study by Cahill et al. (2009) indicated that glutathione S-transferease genotypes can influence the association between dietary vitamin C and serum ascorbic acid, which in turn could affect the antioxidant capacity. Finally, it has been reported that dietary antioxidant supplementation (blueberry and apple juice mixture) improves the

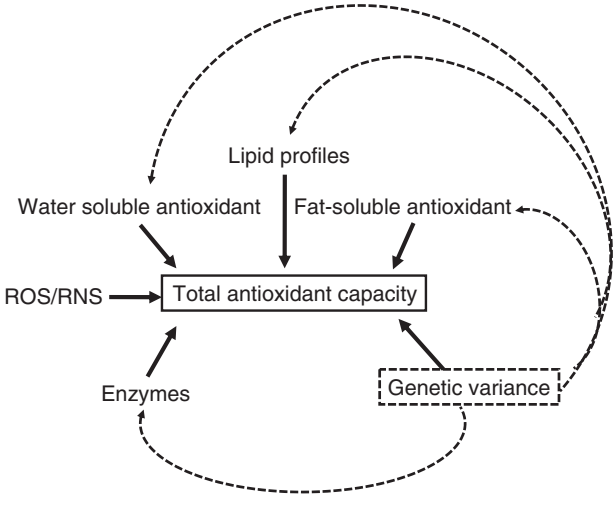

Figure 1.1. Postulated factors affecting total antioxidant capacity. ROS/RNS, reactive oxygen species/reactive nitrogen species.

nucleotide excision repair capacity in individuals carrying multiple low-activity alleles, indicating that polymorphisms in a certain gene (*XPA* G23A) can predict the value of dietary antioxidants for the nucleotide excision repair capacity (Langie et al. 2009). Therefore, the bioavailability of antioxidants, the blood response to supplementation with antioxidants, and the oxidative stress associated genomic stability can all be affected by the genetic variance of individuals.

SUMMARY

Evidence has accumulated that high fruit and vegetable intakes are associated with lower risk of chronic diseases such as cardiovascular diseases, and eye diseases such as cataract. It is possible that antioxidants such as polyphenols, vitamin C, and carotenoids in fruits and vegetables can prevent or reduce the damage from excessive amounts of free radicals that are produced in the body. However, intervention studies have failed to show a consistent beneficial effect of high doses of antioxidant supplementation against chronic diseases. One possible explanation for these apparently contradictory results between observational studies and intervention trials is that the antioxidant system *in vivo*, which is finely balanced, requires the right amount, possibly an optimal range, of both hydrophilic and lipophilic antioxidants to work properly. The optimal ranges of antioxidants might be achieved best by a balanced dietary fruit and vegetable intake, but not by a high dose of only one or a limited mixture of antioxidant supplements, which could cause an imbalance of the antioxidant machinery leading in some cases to a pro-oxidant effect. In addition, other phytochemicals abundant in fruits and vegetable may not only exert unique biological functions, but may also interact synergistically with well recognized antioxidants to promote antioxidant effects. Furthermore, genetic variances, which have been reported to affect the bioavailibility of antioxidants such as vitamin C (Cahill et al. 2009) and vitamin E (Milman et al. 2008) and the response to the dietary antioxidant supplementation (Langie et al. 2009) may affect overall antioxidant capacity in humans.

Various biomarkers to determine the antioxidant capacity in a biological system have been developed and advanced. However it seems that there is not yet one system that predicts health outcomes, due to the various factors affecting the antioxidant capacity in a biological system such as interactions of antioxidants, genetic variance, and the origin of reactive oxygen species.

Therefore, an important future direction of research would be to elucidate how best to improve our body defense systems against oxidative damage, which in turn might reduce the risk of chronic diseases, by means of dietary modification rather than by taking large amounts of antioxidant supplements. The advances in developing proper markers to evaluate the overall antioxidant network including both water- and fat-soluble antioxidants and their interactions in a biological system would support such efforts. Finally, continuous effort to understand gene-nutrient interactions may provide a clue to inconsistent results of various intervention studies with antioxidants to prevent or delay the process of aging or certain chronic diseases.

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