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Advantages and limitation of BODIPY as a probe for the evaluation of lipid peroxidation and its inhibition by antioxidants in plasma

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Abstract—Oxidative stress and the role of antioxidants are currently one of the most important subjects in the field of life science. In the present study, we assessed the oxidation of plasma lipids induced by free radicals and its inhibition by antioxidants with a fluorescence probe BODIPY. Vitamin E and C-depleted plasma was used to evaluate the inherent action of several antioxidants. BODIPY reacted with free radicals in plasma to emit fluorescence (ex. 510 nm, em. 520 nm), which was suppressed by the antioxidants in a concentration-dependent manner. However, the suppression of fluorescence emission by antioxidants did not always correlate quantitatively with the suppression of lipid peroxidation. For example, α-tocopherol suppressed BODIPY fluorescence but enhanced the peroxidation of plasma lipids in the absence of ascorbic acid. 2,2,5,7,8-Pentamethyl-6-chromanol, a vitamin E analogue without a phytyl side chain, almost completely suppressed both fluorescence emission and lipid peroxidation in the plasma. These results show that BODIPY can be used as a convenient probe for radical scavenging, but that care should be taken for the evaluation of antioxidant capacity.

Lipid peroxidation has been the subject of extensive studies,[1,2] and there is ample evidence showing the involvement of free radical-mediated lipid peroxidation in various disorders and diseases.[3] For example, the oxidation of low-density lipoprotein (LDL) has been accepted as an important initial event in the pathogenesis of atherosclerosis,[4] which leads to cerebral ischemia, stroke, and cardiovascular diseases. Small molecular weight antioxidant compounds such as tocopherols (vitamin E), ascorbic acid (vitamin C), and carotenoids play an important role as a part of physiological defense network. These antioxidants may prevent and suppress lipid peroxidation and related diseases; as a result, their role and action against lipid peroxidation and oxidative stress have gained much attention. Numerous studies have been carried out to evaluate the activity of various natural and synthetic antioxidants. It is well known that the antioxidant capacity is determined by many factors; these include not only the reactivity toward free radicals but also other factors such as interaction with other antioxidants and localization.[5] Simple model systems have often been employed to measure the activity of antioxidants. Although such studies are important and essential to understand the mechanisms and kinetics of antioxidant action, it should be well appreciated that the results of simple in vitro model experiments cannot be directly extrapolated to in vivo systems. For example, the antioxidant capacity of α-tocopherol, one of the important physiological lipophilic antioxidants, in solution and isolated LDL is considerably different from that in plasma.[5–7] Plasma is a more appropriate substrate than isolated LDL for the evaluation of antioxidant capacity in vivo, and the oxidation of plasma and its inhibition have been extensively studied.[6,8,9]

Fluorogenic probes have been applied to measure oxidative events and antioxidant action in vitro and in vivo. Fluorometric assays are based on the use of compounds that result in an increase or decrease in fluorescence intensity following oxidation. Boron dipyrromethane difluoride, particularly 4,4-difluoro-5-(4-phenyl-1, 3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-undecanoic acid (BODIPY), has been used to monitor oxidation in living cells.[10–12] This compound has also been applied for the analyses of lipid peroxidation and its inhibition by antioxidants in solution,[13] liposomal membranes,[13,14] cells,[15,16] and plasma.[17–19] The products obtained from BODIPY during lipid peroxidation have also been identified.[20]

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In the present study, we examined the advantages and limitations of BODIPY as a probe for the evaluation of lipid peroxidation and its inhibition by antioxidants in plasma. Vitamin E-depleted plasma was prepared from heterozygous α-tocopherol transfer protein knockout (α-TTP$^{(+/-)}$) mice which were obtained by crossing wild type and α-TTP$^{(-/-)}$ (B6.129S7 α-TTP^{tm1Csk}) mice[21] and fed with a vitamin E-deficient diet (EDD, Funabashi Nojyo, Chiba, Japan).

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology (AIST).

When required, the plasma was dialyzed using a dialysis membrane for 18 h at 4 °C in saline to eliminate ascorbic acid and other water-soluble small molecular weight antioxidants. Natural α-tocopherol (αT), γ-tocopherol (γT), and 2,2,5,7,8-pentamethyl-6-chromanol (PMC) were kindly supplied by Eisai Co. (Tokyo, Japan). BODIPY (in acetonitrile (190 mM as final concentration)), αT, γT, and PMC (in methanol (250 mM as final concentration)) or ascorbic acid (in PBS) were added to the plasma and incubated for 5 min at 37 °C before oxidation. It is not easy to properly incorporate lipid soluble antioxidants into lipid compartment of plasma. However, they are incorporated into lipoproteins much more easily than into cells and the effect of incubation time on the apparent antioxidant activity against plasma oxidation was found to be small. The oxidation of plasma diluted with PBS was carried out at 37 °C under air. The oxidation was initiated by the addition of either MeO-AMVN or AAPH dissolved in acetonitrile (190 mM as final concentration) or PBS, respectively. The increase in the fluorescence emission from BODIPY was measured in a 1-cm quartz cuvette at 37 °C. Fluorescence measurements were performed using a Shimadzu RF 5300PC spectrofluorophotometer equipped with temperature-controlled cell holders.[13] The excitation and emission were set at 510 and 520 nm, respectively. The consumption of antioxidants and formation of lipid peroxidation products were measured as reported previously.[9,13]

It is not difficult to evaluate the activities of radical-scavenging antioxidants such as vitamin E, vitamin C, carotenoids, and polyphenolic compounds in organic or aqueous solutions; however, this is difficult in biological fluids and tissues.[5,7,22] One of the important factors that determine the antioxidant efficacy is the heterogeneity of biological systems. Both hydrophilic and lipophilic antioxidants that may act individually, cooperatively, and synergistically with each other exist. Plasma has often been used as a substrate for oxidation and for evaluation of antioxidant activity. One of the popular and convenient methods involves measuring the UV absorption during the oxidation of plasma due to the formation of a conjugated diene from polyunsaturated fatty acids. However, a major drawback of this method is that the plasma has to be diluted extensively, usually 100-fold dilution, due to its own absorption in the UV region. This inevitably lowers the concentrations of lipids and antioxidants; as a result, the absorption increase and the lag phase produced by oxidation are considerably small. The low concentrations of lipids, of the order of 0.1 mM, make it difficult to add a lipophilic antioxidant to mimic physiological conditions, that is, to maintain the molar ratio of lipids to antioxidant at approximately 1000:1. Furthermore, it should be noted that only a small amount of lipophilic initiator may be used for distribution in the lipophilic compartment. Thus, it is difficult to evaluate quantitatively the efficacy of antioxidants against plasma oxidation by using this method.

In the present study, the formation of lipid peroxidation products and disappearance of antioxidants were followed. First, the oxidation of plasma obtained from normal rats was studied in the presence and absence of ascorbic acid. The oxidation was initiated by a radical initiator MeO-AMVN. As shown in Figure 1, neither

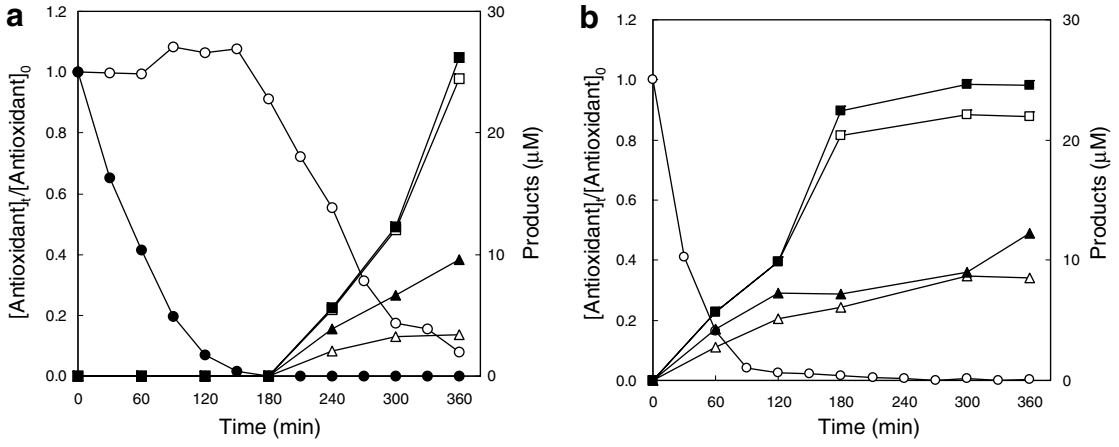

Figure 1. (a) Oxidation of rat plasma in PBS (1/1 by volume) at 37 °C under air. (b) Rat plasma was dialyzed and then oxidized in PBS (1/1 by volume). Oxidation was induced by MeO-AMVN (0.50 mM) and the consumption of α-tocopherol (αT: ○) and ascorbic acid (C: ●) and the formation of lipid peroxidation products were followed. ■: cholesteryl ester (CE) hydroperoxide plus hydroxide; □: CE hydroperoxide; ▲: phosphatidylcholine (PC) hydroperoxide plus hydroxide; △: PC hydroperoxide. Experiments were carried out twice independently and substantially the same results were obtained.

lipid peroxidation nor α-tocopherol consumption occurred to an appreciable extent in the presence of ascorbic acid. However, following the complete depletion of ascorbic acid, the consumption of αT and formation of lipid peroxidation products were observed. This result is in agreement with the results of a previous study on the oxidation of human plasma.[6] The oxidation of plasma was performed several times and substantially the same results were obtained. A typical example is shown in this paper. A greater number of oxidation products were obtained from cholesteryl ester (CE) than from phosphatidylcholine (PC). It is known that both CE and PC form hydroperoxides as primary products of free radical-mediated chain oxidation. Almost all CE oxidation products were found to be hydroperoxides, whereas those from PC comprised an almost equal amount of hydroxides and hydroperoxides (Fig. 1). This result is in agreement with a previous report in which it was stated that PC hydroperoxides are reduced by plasma glutathione peroxidase and selenoprotein P at a faster rate than CE hydroperoxides.[23] Few oxidation products from free cholesterol were found under the present experimental conditions employed (data not shown). The oxidation of rat plasma was also studied in the absence of ascorbic acid, which was eliminated by dialysis. The results shown in Figure 1b clearly indicate that in the absence of ascorbic acid, the consumption of αT and formation of lipid hydroperoxides occurred rapidly without any lag phase.

A previous study[13] showed that BODIPY reacts with peroxyl radicals at a rate constant of $6.0 \times 10^3\ M^{-1}\ s^{-1}$ to yield strong fluorescence (ex. 510 nm, em. 520 nm), and it may serve as a probe to assess lipid peroxidation and its inhibition. In the present study, BODIPY was added to plasma, and the oxidation was then induced by a free radical initiator. The examples of fluorescence emission from BODIPY during the plasma oxidation are shown in Figures 2 and 3. It was observed that αT suppressed fluorescence emission in a concentration-dependent manner (Fig. 2). However, as shown in Figure 3, the suppression of fluorescence emission and lipid peroxidation by αT was not complete. On the other hand, the combination of αT and ascorbic acid completely inhibited lipid peroxidation.

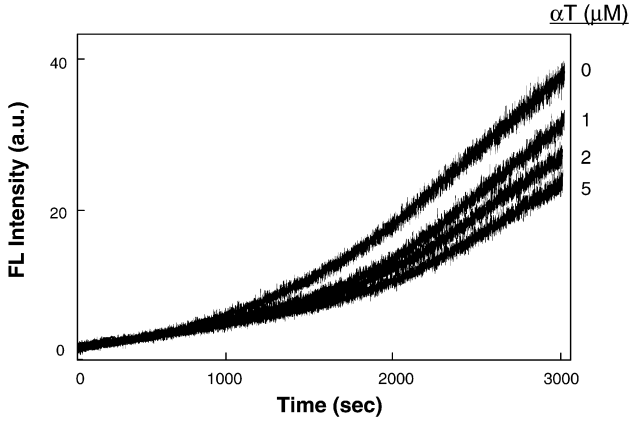

Figure 2. Effect of α-tocopherol on the fluorescence emission in the oxidation of vitamin E-depleted plasma (diluted 10 vol% by PBS (pH 7.4)) obtained from α-TTP$^{(+/-)}$ mice induced by 10 mM AAPH at 37 °C under air. BODIPY (30 μM) and α-tocopherol (concentrations are shown in the figure) were added to the solution before oxidation. Experiments were repeated several times independently and substantially the same results were obtained. A Typical data are shown in the figure.

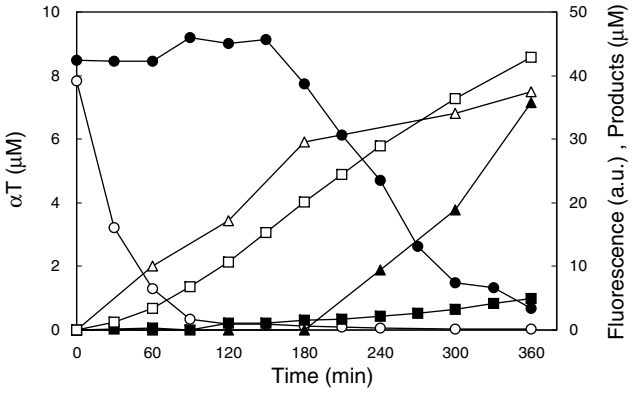

Figure 3. Fluorescence emission from BODIPY (final concentration 10 μM) (□, ■), formation of total lipid peroxidation products (△, ▲), and consumption of α-tocopherol (αT: ○, ●) in the oxidation of rat plasma (diluted 50 vol% by PBS (pH 7.4)) induced by MeO-AMVN (0.50 mM) at 37 °C under air. The solid and open marks represent the presence and absence of ascorbic acid, respectively. Experiments were carried out twice independently and substantially the same results were obtained.

Table 1 shows the effects of αT and ascorbic acid on fluorescence emission in the oxidation of plasma induced by either a lipophilic or hydrophilic radical initiator. Both αT and ascorbic acid suppressed fluorescence emission, and their combination was very effective against the oxidation induced by either the lipophilic or hydrophilic radical initiator. Additionally, the results show that ascorbic acid spared αT.

The correlation between the inhibition of fluorescence emission and that of lipid peroxidation by antioxidants

Table 1. Fluorescence emission in the oxidation of plasma[a]

No.	αT[b] (μM)	Asc[b] (μM)	AAPH (mM)	MeO-AMVN (mM)	FL[c] (a.u.)
1	0.0 (0.0)	0.0 (0.0)		0.50	17.1
2	0.0 (0.0)	11.1 (5.9)		0.50	1.7
3	0.0 (0.0)	0.0 (0.0)	10		8.1
4	0.0 (0.0)	11.1 (6.2)	10		0.2
5	1.44 (0.01)	0.0 (0.0)		0.50	14.1
6	1.44 (0.76)	11.1 (6.5)		0.50	1.8
7	1.44 (0.10)	0.0 (0.0)	10		5.1
8	1.44 (1.59)	11.1 (5.3)	10		0.3

[a] Plasma (diluted 50 vol% by PBS (pH 7.4)) prepared from α-TTP$^{(+/-)}$ mice (No. 1–4) and wild mice (No. 5–8) fed with vitamin E null diet and control diet containing 0.002 wt% α-tocopherol (αT), respectively, were oxidized at 37 °C in air. When necessary, ascorbic acid (Asc) was removed by dialysis (No. 1, 3, 5, and 7). Either AAPH or MeO-AMVN was used as a radical initiator. BODIPY (10 mM) was added before oxidation. Experiments were carried out twice and mean values are presented.

[b] The numbers in parentheses are the concentrations αT and Asc after 60 min oxidation.

[c] The fluorescence intensity at 60 min is shown in arbitrary unit.

was studied in the presence and absence of either or both αT and ascorbic acid. Vitamin E-depleted plasma was obtained from α-TTP$^{(+/-)}$ mice fed with a vitamin E-deficient diet. It was confirmed that plasma contained only a very low level of αT; this enabled us to examine the specific activity of different antioxidants in the absence of αT. The extent of lipid peroxidation was assessed by the amount of CE hydroperoxides formed in the plasma. The results are summarized in Table 2. Only hydrophilic radical initiator was used for evaluation of different antioxidant activity, since it was difficult to obtain ample plasma from α-TTP$^{(+/-)}$ mice and substantially the same results were obtained with either hydrophilic or lipophilic radical initiator. The following points should be noted: (1) ascorbic acid effectively suppressed fluorescence emission; however, this was not observed with either αT or γT; (2) neither αT nor γT alone could suppress lipid peroxidation; on the contrary, they accelerated it; (3) ascorbic acid alone did not completely suppress lipid peroxidation; however, it was completely inhibited by a combination of ascorbic acid together with either αT or γT; and (4) PMC, an analogue of αT without a phytyl side chain, efficiently inhibited both lipid peroxidation and fluorescence emission even in the absence of ascorbic acid. The above results show that the extent of inhibition of fluorescence emission by antioxidants does not consistently agree with that of lipid peroxidation by antioxidants. BODIPY acts as a radical scavenger and may be used to evaluate the efficacy of radical scavenging by antioxidants in plasma. However, the suppression of fluorescence emission does not always correlate quantitatively with the efficacy of inhibition of lipid peroxidation.

BODIPY is a sensitive fluorescence probe with a high quantum yield, and it readily enters membranes and lipoproteins in the plasma.[12,19] BODIPY has been used for the evaluation of antioxidant capacity against plasma oxidation.[13,17–19] It was found to be less reactive toward peroxyl radicals than tocopherol and ascorbic acid; however, it is more reactive than polyunsaturated fatty acids.[13] In fact, the results of this study show that BODIPY can be used as a probe for evaluating the reactivity of antioxidants toward peroxyl radicals. However, as shown above, it did not correlate quantitatively with the efficacy of inhibition of lipid peroxidation. This is primarily because αT enhances the oxidation of lipoproteins in the absence of ascorbic acid by phase-transfer and chain-transfer mechanisms, as first reported by Stocker et al.[24,25] Ubiquinol, a reduced form of coenzyme Q, is also capable of reducing α-tocopheroxyl radical to inhibit the prooxidant action of αT; however, the concentration of ubiquinol in plasma is too low to exert a significant effect. Thus, when plasma was dialyzed and ascorbic acid was eliminated, αT suppressed BODIPY fluorescence emission but enhanced lipid peroxidation. Under these conditions, the antioxidant efficacy cannot be assessed by using BODIPY fluorescence. This is the same in the oxidation of isolated LDL.

Table 2. Cholesteryl ester hydroperoxies and hydroxides (CEO(O)H) and fluorescence intensity observed in the oxidation of plasma (diluted 50 vol% by PBS (pH 7.4)) prepared from α-TTP$^{(+/-)}$ mice induced by AAPH (10 mM) in the presence of antioxidant for 60 min at 37 °C in air

αT[a] (μM)	γT[a] (μM)	PMC[a] (μM)	Asc[a] (μM)	CEO(O)H (μM)	Fluorescence[b] (a.u.)
0	0	0	0	0.84	6.78
0	0	0	10 (0)	0.31	3.09
0	0	0	50 (20)	0.26	0.01
5.0 (1.1)	0	0	0	2.62	4.18
10 (3.3)	0	0	0	2.96	3.65
5.0 (3.0)	0	0	50 (23)	0.0	0.55
10 (8.7)	0	0	50 (20)	0.0	0.70
0	10 (4.9)	0	0	1.40	10.5
0	10 (6.2)	0	50 (26)	0.0	0.13
0	0	10 (1.9)	0	0.0	0.77
0	0	10 (4.4)	10 (0)	0.0	0.87

Experiments were carried out twice and mean values are presented.
[a] Initial concentrations. The numbers in parentheses are the concentrations at 60 min. T, tocopherol; PMC, 2,2,5,7,8-pentamethyl-6-chromanol; Asc, ascorbic acid.
[b] Fluorescence intensity form BODIPY at 60 min in arbitrary unit.

In conclusion, the following points should be noted. BODIPY is a useful fluorogenic probe and may be used for the evaluation of the reactivity of antioxidants toward free radicals in biological fluids. However, the extent of suppression of fluorescence emission and that of lipid peroxidation by antioxidants may not always be consistent. This implies the importance of the synergistic interaction between hydrophilic/lipophilic antioxidants in the defense mechanisms against oxidative stress in humans. Furthermore, as recently indicated,[7] the determination of antioxidant capacity in the absence of either of the groups, such as isolated LDL and dialyzed plasma, may not have biological relevance.

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