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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

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Abstract—The role of radical-scavenging antioxidant against oxidative stress has received much attention. The antioxidant capacity has been assessed by various methods. Above all, oxygen radical absorbance capacity (ORAC) has been frequently employed [Prior et.al., *J. Agric. Food Chem.* **2005**, *53*, 4290]. In the present study, the antioxidant capacity of 2,3-dihydro-5-hydroxy-4,6-di-*tert*-butyl-2,2-dipentylbenzofuran (BO-653) and uric acid was assessed by ORAC method using pyranine as a reference probe and compared with that against lipid peroxidation of human plasma. It was found that BO-653 was assessed to be much less potent than uric acid by ORAC method, whereas BO-653 exerted much higher antioxidant activity than uric acid against plasma lipid peroxidation. The reason for such discrepancy is discussed. The results suggest that ORAC method is suitable for the assessment of free radical scavenging capacity, but not for the assessment of antioxidant capacity against lipid peroxidation in plasma.

The free radical-mediated lipid peroxidation in vivo has been implicated in the pathogenesis of various disorders and diseases.[1] Consequently, the role of antioxidants against lipid peroxidation has received much attention and the antioxidant capacity of many natural and synthetic compounds has been assessed by various methods.[2–6] Above all, the method called oxygen radical absorbance capacity (ORAC) is one of the most widely used methods.[3,6] In this ORAC assay, the effect of antioxidant compound on the consumption of reference probe induced by free radicals is measured. The antioxidant capacity is determined by a decreased rate and amount, which is measured from the 'area under the curve' (AUC).[6] This technique is applied for quantitation of both inhibition rate and inhibition time. Several kinds of reference probes have been applied[7] such as β-phycoerythrin,[3] fluorescein,[8] BODIPY,[9] and pyrogallol red.[10] The ORAC method has such advantages that it applies for antioxidants which exhibit distinct lag phase and also those samples which have no lag phase. Furthermore, it measures both the lag time and the rate and it can be used for samples containing multiple ingredients. In fact, a direct linear correlation between the net AUC and antioxidant concentration has been observed.[11] Thus, it may be said that ORAC is an appropriate method to measure the total amount and rate of oxygen radical scavenging by pure antioxidant and also samples containing several antioxidants.

One of the characteristics of lipid peroxidation in vivo is that it proceeds in heterogeneous system such as cellular membranes and lipoproteins. Both hydrophilic and lipophilic antioxidants exert their role at respective sites in vivo. This should be taken into consideration in evaluation of the antioxidant capacity.[12] In the present study, the antioxidant capacity of hydrophilic uric acid and lipophilic BO-653 was assessed by ORAC method and by the inhibition of lipid peroxidation in human plasma. BO-653 is a synthetic, lipophilic radical-scavenging antioxidant and it has been shown that it inhibits lipid peroxidation of low-density lipoprotein (LDL)[13] and plasma[14] as well as that in organic solution.[15]

In the present study, pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt, was used as a reference probe. Pyranine acts as a radical scavenger with moder-

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ate reactivity and its reaction with free radicals can be followed quantitatively by either visible light absorption or fluorescence spectroscopy. Pyranine has been used as a probe to assess the antioxidant capacity.[16,17] A water-soluble azo compound 2,2′-azobis(amidinopropane) dihydrochloride, AAPH, was used as a radical initiator to generate free radicals at a constant rate.[18] The oxidation of plasma lipids was initiated by the addition of AAPH into phosphate buffered saline (pH 7.4) containing 10% human plasma with or without added antioxidant as described before.[14] Blood was collected from healthy subject in EDTA-containing tube after overnight fasting. Plasma was separated by centrifugation in 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 Committee of the National Institute of Advanced Industrial Science and Technology.

A consumption of pyranine at a constant rate was observed when AAPH was added to PBS solution containing 10 vol% of human plasma. In the absence of plasma, pyranine was consumed at a constant rate without any lag phase. The addition of human plasma produced lag phase, apparently because the endogenous antioxidants contained in plasma scavenged free radicals faster than pyranine. The addition of uric acid suppressed the consumption of pyranine and extended the lag phase in a concentration dependent manner (Fig. 1). On the other hand, BO-653 did not suppress the rate of pyranine consumption, nor did it extend lag phase as long as uric acid (Fig. 2). The rates of pyranine consumption during and after the lag phase were calculated from the slope in Figure 2 and summarized in Table 1. The rates of pyranine consumption after the lag phase were similar to that without plasma independent of the antioxidants added. The AUC was also measured and included in Table 1. Integration of the AUC was performed up to a time such that the absorbance at 454 nm reached a constant value of around 0.2 (Figs. 1 and 2). The net AUC produced by antioxidant was calculated from the difference AUC (with added antioxidant)—AUC (without added antioxidant, that is, with 10% plasma only).[6] The results in Table 1 show that uric acid is assessed to be more potent antioxidant than BO-653 as judged by ORAC method, both by rate and AUC.

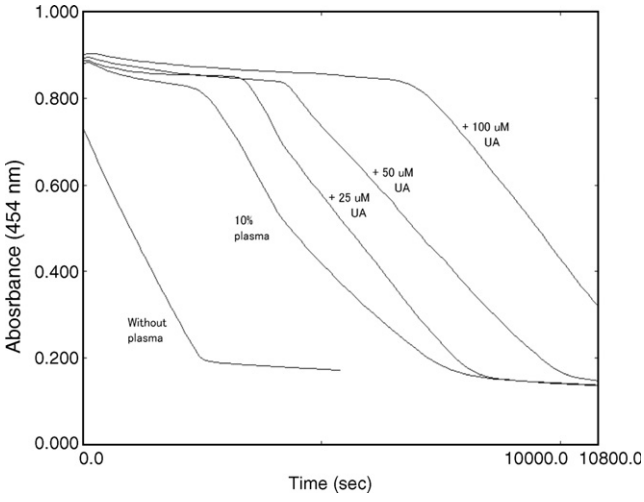

Figure 1. Effect of uric acid on the consumption of pyranine in the oxidation of human plasma. The oxidation of human plasma (10% in phosphate buffered saline) was induced by the addition of 50 mM AAPH in the presence of 50 μM pyranine and added uric acid at 37 °C. The numbers in the Figure show the concentration of uric acid added in μM.

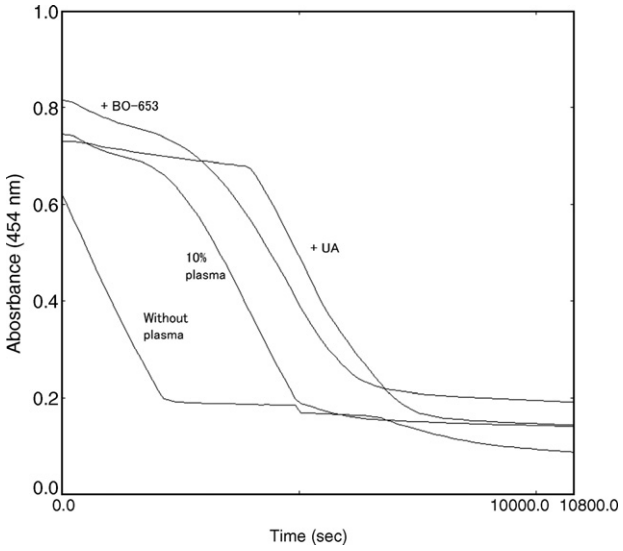

Figure 2. Effects of addition of uric acid or BO-653 (50 μM) on the consumption of pyranine in the oxidation of human plasma. The experimental conditions are the same as those in Figure 1.

The antioxidant effects of BO-653 and uric acid against lipid peroxidation in human plasma were measured. It has been well established that the free radical-mediated oxidation of plasma gives cholesteryl ester hydroperoxides (CEOOH) as a major product with minor amount of phosphatidylcholine hydroperoxide and hydroxide, PCOOH and PCOH, respectively.[14] PCOOH is reduced readily by extracellular glutathione peroxidase (GPx), whereas CEOOH is not. Therefore, the extent of plasma lipid peroxidation can be estimated by CEOOH. In the present study, the formation of CEOOH and CEOH was measured by HPLC-UV method using absorption at 234 nm due to conjugated diene formed.[19] The results are also included in Table 1. As reported previously,[14] it was confirmed that BO-653 inhibited the formation of CEO(O)H efficiently. On the contrary, uric acid was found to be ineffective in the suppression of CEO(O)H formation. Thus, in contrast to the ORAC method, BO-653 was more effective in suppressing the lipid peroxidation in plasma than uric acid.

That uric acid is assessed as a potent antioxidant by ORAC method, but it does not act as an efficient antioxidant against lipid peroxidation of plasma is conceivable. The lipid peroxidation in plasma proceeds substantially in the lipophilic domain within the lipoprotein particles. Pyranine is a hydrophilic probe which reacts with free radicals at moderate rate and uric acid can compete well with pyranine for scavenging peroxyl rad-

Table 1. Oxidation of human plasma in the presence of pyranine with and without added antioxidant[a]

Plasma (%)	Antioxidant[b]	Lag phase (s)	Rate 1[c]	Rate 2[c]	AUC[d] (relative)	$[CEOOH]I/[CEOOH]_o$[e]
0	None	0	—	1.54	0.23	—
10	None	1920	0.26	1.81	1.0	1.0
10	Uric acid	3960	0.13	1.84	1.52	0.78
10	BO-653	2760	0.29	1.58	1.23	0.23

[a] The oxidation was induced at 37 °C by the addition of 50 mM AAPH to the PBS containing 50 mM pyranine and 10% human plasma, unless otherwise stated. The consumption of pyranine was followed by absorption at 454 nm.
[b] When used, 25 mM in final reaction mixture.
[c] Rate of pyranine consumption during (rate 1) and after (rate 2) the lag phase, in arbitrary unit.
[d] The area under the curve (relative).
[e] The ratio of the cholesteryl ester hydroperoxide formed in 60 min in the presence of added antioxidant to that without added antioxidant.

icals formed in the aqueous region from AAPH. However, uric acid is not capable of scavenging free radicals localized in lipophilic domain, nor can it reduce α-tocopheroxyl radical present in lipoprotein particle. In fact, it was observed previously that both ascorbic acid and uric acid could spare α-tocopherol in the oxidation of human LDL initiated by aqueous radicals produced from AAPH, whereas uric acid, unlike ascorbic acid, did not spare α-tocopherol when the free radicals were generated within LDL particles from lipophilic radical initiator.[20] Uric acid has also been assessed as a major antioxidant in plasma by TRAP (total radical trapping antioxidant parameter) method.[21] Uric acid present in aqueous phase cannot scavenge radicals within lipophilic domain. It may be also added that the efficacy of scavenging radicals by ascorbic acid decreases as the radicals go deeper into the LDL particles from the surface.[22]

In conclusion, this study clearly shows that BO-653 acts as a potent antioxidant against lipid peroxidation in plasma, although it is assessed as a poor antioxidant by ORAC assay. On the contrary, uric acid is assessed as a potent antioxidant by ORAC assay, but actually it does not exert a potent antioxidant activity against plasma lipid peroxidation. These results show that ORAC assay is not always a reliable method for the antioxidant capacity evaluation.

References and notes

1. Halliwell, B.; Gutteridge, J. M. C. *Free Radical Biology and Medicine*, 3rd ed.; Oxford University Press: Oxford, 1999.
2. Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Locke, S. *FEBS Lett.* **1985**, *187*, 33.
3. Cao, G.; Alessio, H. M.; Cutler, R. G. *Free Rad. Biol. Med.* **1993**, *14*, 303.
4. Cao, G.; Prior, R. L. *Methods Enzymol.* **1999**, *299*, 50.
5. Ghiselli, A.; Serafini, M.; Natella, F.; Scaccini, C. *Free Rad. Biol. Med.* **2000**, *29*, 1106.
6. Prior, R. L.; Wu, X.; Schaich, K. *J. Agric. Food Chem.* **2005**, *53*, 4290.
7. Balavoine, G. G. A.; Geletii, Y. V. *Nitric Oxide* **1999**, *3*, 40.
8. Naguib, Y. M. A. *Anal. Biochem.* **2000**, *284*, 93.
9. Naguib, Y. M. A. *J. Agric. Food Chem.* **2000**, *48*, 1150.
10. Lopez-Alarcon, C.; Lissi, E. *Free Rad. Res.* **2006**, *40*, 979.
11. Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. *J. Agric. Food Chem.* **2002**, *50*, 1815.
12. Yeum, K.-J.; Russell, R. M.; Krinsky, N. I.; Aldini, G. *Arch. Biochem. Biophys.* **2004**, *430*, 97.
13. Noguchi, N.; Okimoto, Y.; Tsuchiya, J.; Cynshi, O.; Kodama, T.; Niki, E. *Arch. Biochem. Biophys.* **1997**, *347*, 141.
14. Itoh, N.; Yoshida, Y.; Hayakawa, M.; Noguchi, N.; Kodama, T.; Cynshi, O.; Niki, E. *Biochem. Pharmacol.* **2004**, *68*, 813.
15. Noguchi, N.; Iwaki, Y.; Takahashi, M.; Komuro, E.; Kato, Y.; Tamura, K.; Cynshi, O.; Kodama, T.; Niki, E. *Arch. Biochem. Biophys.* **1997**, *342*, 236.
16. Pino, E.; Campos, A. M.; Lissi, E. *Int. J. Chem. Kinetics* **2003**, *35*, 525.
17. Campos, A. M.; Sotomayor, C. P.; Pino, E.; Lissi, E. *Biol. Res.* **2004**, *37*, 287.
18. Niki, E. *Methods Enzymol.* **1990**, *186*, 100.
19. The accumulation of CE hydroperoxide (CEOOH) and CE hydroxide (CEOH) was also followed with an HPLC by spectrophotometric detector (SPD-10AV, Shimadzu, Japan) at 234 nm. ODS column (LC-18, 5 μm, 250 × 4.6 mm, Supelco, Japan) was used and acetonitrile/isopropyl alcohol/water (44/54/2 by volume) was eluted at 1 ml/min.
20. Sato, K.; Niki, E.; Shimasaki, H. *Arch. Biochem. Biophys.* **1990**, *279*, 402.
21. Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Barclay, L. R. C.; Locke, S. J. *Biochim. Biophys. Acta* **1987**, *924*, 408.
22. Gotoh, N.; Noguchi, N.; Tsuchiya, J.; Morita, K.; Sakai, M.; Shimasaki, H.; Niki, E. *Free Rad. Res.* **1996**, *24*, 123.