Subadditive Interactions between Antioxidants in the Protection against Lipid Peroxidation

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Synergistic interactions between antioxidants have been postulated but not proven. On the contrary, it has been reported that the antioxidant activity of mixtures of antioxidants can be lower than the sum of the antioxidant activities of individual components. We report that such a situation can be observed in 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-treated phosphatidylcholine liposomes in which lipid peroxidation was monitored by oxidation of 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (C_{11}-BODIPY$^{581/591}$). Glutathione, present inside liposomes, and hydrophobic antioxidants, present in the lipid bilayer, protected against lipid peroxidation, but their simultaneous action was lower than the sum of individual contributions. A possible explanation for this effect is proposed.

Key words: Lipid Peroxidation, Antioxidants, Liposomes, Glutathione

Introduction

The discovery of the ubiquitous presence of reactive oxygen species and their role in physiology and pathology gave rise to a broad interest in the mechanisms of action and efficiency of antioxidants. An important facet of antioxidant activity is the interaction between antioxidants in complex mixtures and in biological material. It has been postulated that antioxidants are more efficient in natural products than in synthetic formulas because of the possibility of synergistic cooperation between diverse antioxidant compounds present in natural products (Liu, 2003, 2004). However, experimental evidence for such synergy seems to be rather weak if not doubtful, while the antioxidant efficiency of a mixture of various fruit extracts is of course higher than that of the single components. It is not necessarily higher, or even lower, than the sum of these activities (Fig. 7 in Liu, 2004). Instead, subadditive interactions in the total antioxidant capacity assay have been reported: the antioxidant capacity of human blood plasma mixed with quercetin, rutin, catechin or 7-monohydroxyethylrutoside was lower than the sum of the antioxidant activities of both components. This effect was much lower in deproteinized plasma; so it was attributed to the interaction of the catechols with plasma proteins (Arts *et al.*, 2001). Studies of isolated proteins confirmed that interactions of flavonoids with albumin and also with β- and κ-casein may mask some 10–20% of the antioxidant activity of tea catechins (Arts *et al.*, 2002). The masking of the antioxidant activity of catechols has been ascribed to their association with proteins and suggested to contribute to effects of lowering the antioxidant capacity of tea when mixed with milk (Langley-Evans *et al.*, 2000) and to weak effects of antioxidant ingestion on the total antioxidant capacity (TAC) of blood plasma (Boyle *et al.*, 2000). Moreover, when studying the TAC of mixtures of antioxidants, we observed a much wider phenomenon of interaction between hydrophilic antioxidants, leading to subadditivity in the values of antioxidant activities.

An intriguing question is the interaction between hydrophilic antioxidants, present in the aqueous phase of cells and in extracellular fluids, and hydrophobic antioxidants, present mainly in cellular membranes. Synergistic effects in the interactions between hydrophobic and hydrophilic antioxidants have been reported (Kadoma *et al.*, 2006; Atsumi *et al.*, 1999). The interaction between vitamin E and vitamin C has been studied taking into account the sparing effect of vitamin C on

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vitamin E (Niki, 1987; Haramaki *et al*., 1998). Also the interaction between glutathione and hydrophobic antioxidants has been postulated (Haenen and Bast, 1983) as well as the enzyme-mediated regeneration of bilirubin by NADPH (Baranano *et al*., 2002). The aim of the present study was to investigate the interactions between hydrophilic and hydrophobic antioxidants in a simple and well-defined system of phospatidylcholine liposomes.

Materials and Methods

Materials

L-α-Phosphatidylcholine from egg yolk, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), α-tocopherol, butylated hydroxytoluene (BHT), glutathione, quercetin, melatonin, lipoic acid and coenzyme Q_{10} were purchased from Sigma-Aldrich (Poznan, Poland). Bilirubin was obtained from Fluka (Buchs, Switzerland). Menadione was purchased from International Enzymes Limited (Windsor, Berkshire, UK). 4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undeca-noic acid (C_{11}-BODIPY$^{581/591}$) was purchased fromMolecular Probes (Invitrogen Corporation, California, USA). All other chemicals were from POCh (Gliwice, Poland) and were of the highest grade available.

Preparation of large unilamellar vesicles (LUVs)

Large unilamellar liposomes were prepared using the Avanti Polar Lipids Mini-Extruder device. Phosphatidylcholine was dissolved in chloroform with known amounts of α-tocopherol, butylated hydroxytoluene, quercetin, melatonin, bilirubin, coenzyme Q_{10}, lipoic acid or menadione. The solvent was evaporated under argon leaving a thin lipid film inside the tube. The film was hydrated using Tris/HCl buffer (10 mM, pH 7.4) containing reduced glutathione solution. The hydration (30 min) was followed by vortex stirring (5 min) and three cycles of freezing and thawing. The suspensions of prepared multilamellar liposomes were passed eleven times through a polycarbonate membrane (pores of 0.1 μm) using a Mini-Extruder to form unilamellar vesicles. The final concentrations of the components in the samples (if present) were: 10 mM phosphatidylcholine, 20 μM α-tocopherol, 40 μM BHT, 20 μM quercetin, 50 μM melatonin, 20 μM bilirubin, 100 μM coenzyme Q_{10}, 200 μM lipoic acid, 20 μM menadione, 500 μM glutathione.

Sample dialysis

Prepared samples of ca. 800 μl were dialyzed against Tris/HCl buffer at 6 °C overnight in order to remove free glutathione outside the liposomes.

Measurement of lipid peroxidation

Lipid peroxidation was estimated on the basis of the decay of C_{11}-BODIPY$^{581/591}$ fluorescence (Zhang *et al*., 2006; Makrigiorgos, 1997). Samples were transferred to the test tubes and incubated with C_{11}-BODIPY$^{581/591}$ for 30 min on ice, in the darkness. Then the aliquots of samples were transferred to a 96-well plate and the peroxidation reaction was started by addition of AAPH solution. The plate was incubated at 37 °C in a Fluoroskan Ascent FL reader (Labsystems, Helsinki, Finland). The loss of fluorescence was monitored at λ_{ex} = 530 nm and λ_{em} = 590 nm for 120 min (5-min intervals). The final concentrations were: 1 μM C_{11}-BODIPY$^{581/591}$, 10 mM AAPH.

Data analysis

The results were presented as fluorescence intensity [a.u.] decrease versus time [min] curves. The area-under-curves (*AUC*) was calculated according to

$$AUC = (f_0/2 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + f_{20}/f_0 + \ldots + f_i/f_0) \cdot CT,$$

where f_0 is the initial fluorescence at time 0, f_i the fluorescence at time i, *CT* the cycle time in 5 min. The data was collected from two separate experiments and analyzed by Microsoft Excel software (mean ± standard deviation). Significant differences ($P \leq 0.05$) between means of the calculated *AUC* parameters were determined by t-Student's test, two-tailed ($df = 1$).

Results and Discussion

In our previous study (Blauz *et al*., 2008) we firmed previous observations that the total antioxidant activity of mixtures of hydrophilic antioxidants may be lower than the sum of the activities of individual compounds (Arts *et al*., 2001, 2002) and demonstrated that this phenomenon is not infrequent. In order to study interactions between hydrophilic and hydrophobic antioxidants, in the present study we chose a liposome system in which lipid peroxidation was induced by AAPH and the parameter studied was the decay of C_{11}-BODIPY$^{581/591}$

fluorescence. C_{11}-BODIPY$^{581/591}$ is a hydrophobic fluorescent probe which localizes in the liposome membrane. AAPH is an agent often used as a model for a free-radical-generating system; however, since it is hydrophilic, it generates free radicals in the aqueous phase, and the main way of action of hydrophilic antioxidants can be expected to consist in reactions with AAPH-derived radicals before they reach the liposome membrane. In order to minimize this effect, we employed a system in which hydrophilic antioxidants were contained inside unilamellar liposomes by exhaustive dialysis of liposomes formed in the presence of glutathione while hydrophobic antioxidants were accumulated in the liposomal membrane.

There are contradictory data in the literature on whether AAPH penetrates cellular membranes. Some authors claim that this compound does not easily cross the erythrocyte membrane (Glantzounis *et al.*, 2001) while others assume an easy membrane penetration by AAPH (Sato *et al.*, 1999). We decided therefore to check if external AAPH is able to oxidize compounds inside liposomes. We found that carboxyfluorescein contained inside liposomes was oxidized by AAPH added from outside at a rate similar to that of carboxyfluorescein dissolved in the solution (not shown). Therefore, the system used only partly eliminated the interaction of the hydrophilic antioxidant (glutathione) with AAPH-derived peroxyl radicals: only the peroxyl radicals formed outside liposomes were not intercepted by the hydrophilic antioxidant.

When estimated on the basis of augmentation of the area-under-curve value of C_{11}-BODIPY$^{581/591}$ fluorescence (Fig. 1), GSH provided protection of the fluorescent probe which, however, did not reach the level of statistical significance. From the hydrophobic compounds, statistically significant effects of protection were found for α-tocopherol, BHT and quercetin. Interestingly, no obvious synergistic effects were seen for GSH and hydrophobic antioxidants (Fig. 2).

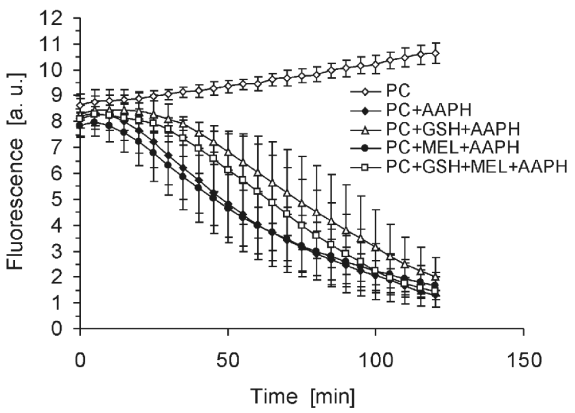

Fig. 1. Typical time course of AAPH-induced oxidation of C_{11}-BODIPY$^{581/591}$ in phosphatidylcholine liposomes. GSH, glutathione; MEL, melatonin; PC, phosphatidylcholine.

The following reactions can be expected to decrease the fluorescence of C_{11}-BODIPY$^{581/591}$ in the system studied:

$$\text{AAPH} \rightarrow 2\,\text{R}^{\bullet}, \quad (1)$$

$$\text{R}^{\bullet} + \text{O}_2 \rightarrow \text{ROO}^{\bullet}, \quad (2)$$

$$\text{ROO}^{\bullet} + \text{H-BODIPY} \rightarrow \text{ROOH} + \text{BODIPY}^{\bullet} \rightarrow \text{non-fluorescent products}, \quad (3)$$

$$\text{ROO}^{\bullet} + \text{LH} \rightarrow \text{ROOH} + \text{L}^{\bullet}, \quad (4)$$

$$\text{L}^{\bullet} + \text{O}_2 \rightarrow \text{LOO}^{\bullet}, \quad (5)$$

$$\text{LOO}^{\bullet} + \text{H-BODIPY} \rightarrow \text{LOOH} + \text{BODIPY}^{\bullet} \rightarrow \text{non-fluorescent products}, \quad (6)$$

where R are radical products of AAPH decomposition and LH is lipid.

Antioxidants (AH) can interfere with these reactions by scavenging free radicals:

$$\text{AH} + \text{R}^{\bullet} \rightarrow \text{A}^{\bullet} + \text{RH}, \quad (7)$$

$$\text{AH} + \text{ROO}^{\bullet} \rightarrow \text{A}^{\bullet} + \text{ROOH}, \quad (8)$$

$$\text{AH} + \text{L}^{\bullet} \rightarrow \text{A}^{\bullet} + \text{LH}, \quad (9)$$

$$\text{AH} + \text{LOO}^{\bullet} \rightarrow \text{A}^{\bullet} + \text{LOOH}. \quad (10)$$

Apparently, chemical repair of the radical form of BODIPY is also possible:

$$\text{BODIPY}^{\bullet} + \text{AH} \rightarrow \text{H-BODIPY} + \text{A}^{\bullet}. \quad (11)$$

The free radicals of antioxidants are less reactive than the radicals scavenged by these compounds. However, in some cases they can be expected to be able to induce free radical reactions, possibly including a free radical damage to BODIPY:

$$\text{A}^{\bullet} + \text{H-BODIPY} \rightarrow \text{AH} + \text{BODIPY}^{\bullet}. \quad (12)$$

The last reaction can be postulated taking into account the recent report by MacDonald *et al.* (2007) demonstrating that BODIPY is more sensitive to free radical oxidation than membrane lipids.

The protection of BODIPY by glutathione can be ascribed mainly to scavenging of AAPH-derived radicals inside the liposome before they reach the liposomal membrane, reactions (7) and (8), though some contribution of reactions (9)–(11) cannot be excluded (provided the radical centres are available

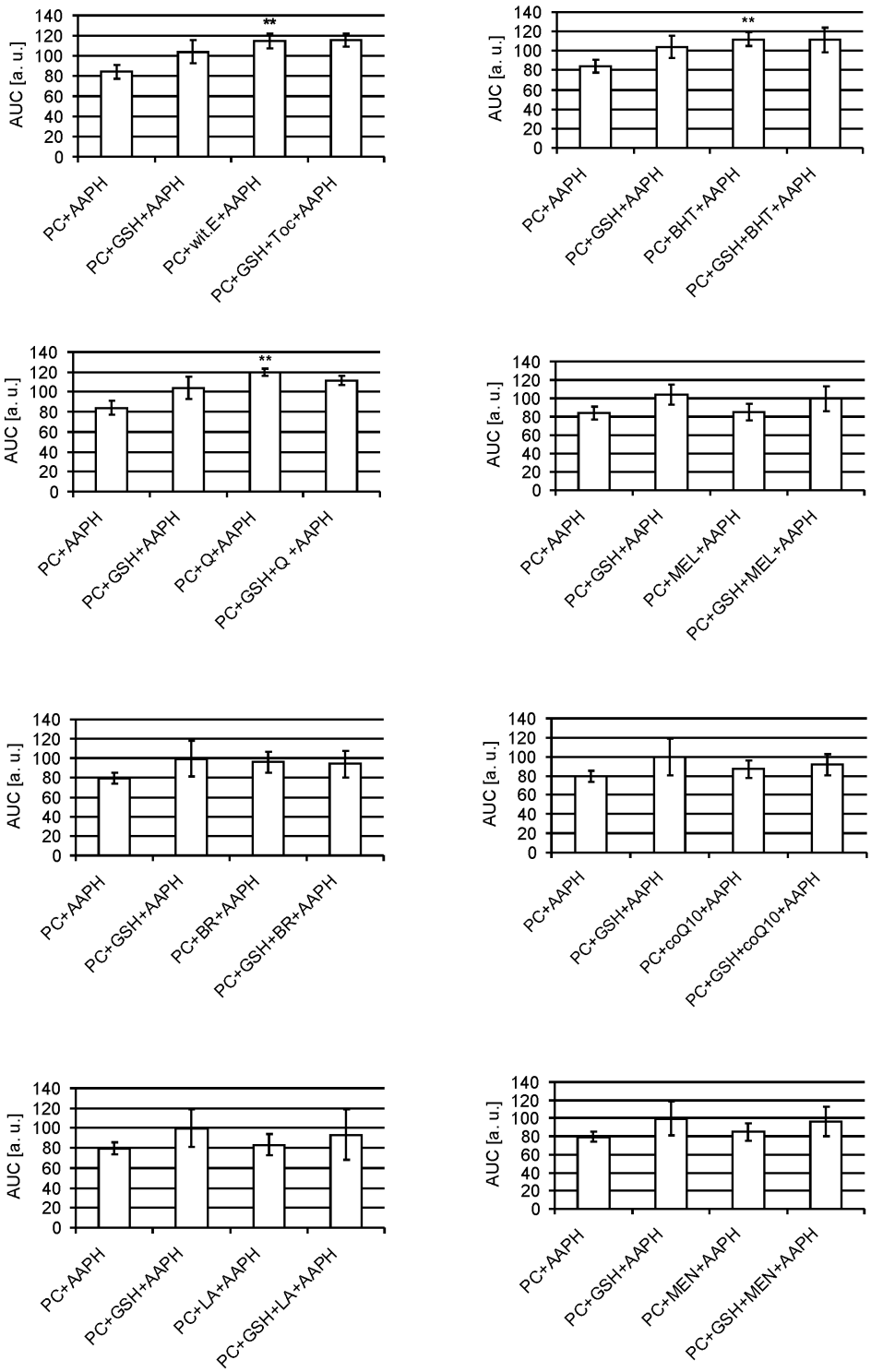

Fig. 2. Effect of antioxidants on the area-under-curve of C_{11}-BODIPY$^{581/591}$ fluorescence. BR, bilirubin; GSH, glutathione; LA, lipoic acid; MEL, melatonin; MEN, menadione; PC, phosphatidylcholine; coQ10, coenzyme Q_{10}; Q, quercetin; BHT, butylated hydroxytoluene; Toc, α-tocopherol. * $P < 0.05$, ** $P < 0.02$ (with respect to PC + AAPH).

to the aqueous phase). Hydrophobic antioxidants, on the other hand, are expected to participate mainly in reactions (9)–(11). They may also participate in reaction (8) but the importance of this reaction is diminished by their low abundance in the liposomal membranes, in comparison with the lipids.

The results obtained confirm that the reduced forms of antioxidants are necessary to protect against AAPH-induced oxidation (lack of effect of menadione and lipoic acid). They demonstrate also not only the lack of synergistic effects between the hydrophilic and hydrophobic antioxidants but even subadditive effects of such combinations of antioxidants in many cases in the applied system. Several explanations can be put forward to explain this situation but the main reason may be the high sensitivity of the BODIPY probe to free radical oxidation (MacDonald *et al.*, 2007). BODIPY is oxidized in reactions (3) and (6); the rate constant of reaction (3) can be expected to be high with respect to that of reaction (4) for the bulk of membrane lipids. If reaction (12) is important for at least some antioxidants, one can expect significant oxidation of BODIPY even in the presence of antioxidants. These results may confirm the conclusion that the drawback of the BODIPY probe is its high sensitivity to oxidation. However, the behaviour of this probe may reflect the situation of membrane components which are more easily oxidizable than most of the membrane lipids.

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