Although the role of free radicals, lipid peroxidation and antioxidants in the desaturation process of EFA formation still requires elucidation, there is strong evidence that lipid peroxidation has a deleterious effect and will consequently reduce the availability of EFA synthesized *de novo*.

The enhanced free radical load of smokers, especially if combined with their low antioxidant intake, will therefore result in increased peroxidation of their low EFA reserves, with increased production of cytotoxic hydroperoxides and aldehydes. This illustrates a potential inter-relationship of three of the major CHD risk factors.

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Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers

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Abstract

Singlet molecular oxygen ($^1O_2$) has been shown to be generated in biological systems and is capable of damaging proteins, lipids and DNA. The ability of some biological antioxidants to quench $^1O_2$ was studied by using singlet oxygen generated by the thermodissociation of the endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate ($NDPO_2$). The carotenoid lycopene was the most efficient $^1O_2$ quencher ($k_q + k_r = 31 \times 10^9$ M^{-1} s^{-1}). Tocopherols and thiols were less effective. The singlet oxygen quenching ability decreased in the following order: lycopene, γ-carotene, astaxanthin, canthaxanthin, α-carotene, β-carotene, bixin, zeaxanthin, lutein, bilirubin, biliverdin, tocopherols and thiols. However, the compounds with low quenching rate constants occur at higher levels in biological tissues. Thus, carotenoids and tocopherols may contribute almost equally to the protection of tissues against the deleterious effects of $^1O_2$. The quenching abilities of carotenoids and tocopherols were mainly due to physical quenching. In case of some thiols chemical quenching also plays a significant role. Carotenoids and tocopherols have been reported to exert a protective action against some types of cancer.

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Abbreviations used: $^1O_2$, singlet molecular oxygen; NDP, 3,3'-(1,4-naphthylidene) dipropionate; $NDPO_2$, endoperoxide of NDP.

Introduction

Singlet molecular oxygen is generated in biological systems by photochemical reactions through transfer of excitation energy from a suitable triplet state sensitizer (photoexcitation) or by dark reactions (chemiexcitation) which include enzymatic reactions or radical interactions [1]. This $^1O_2$ species is capable of diffusing an appreciable distance in membranes and is capable of damaging biological molecules including proteins, enzymes and DNA. It has been implicated in several pathological processes like lung oxidant injury, skin photosensitivity and erythropoietic porphyria [2–5].

There is increasing interest in the role of diet and nutrition in the pathogenesis and possible prevention of cancer [6]. An inverse relationship between β-carotene intake and the incidence of certain types of cancer, such as lung and intestinal tract cancer, has been observed. Animal experiments also have revealed the anticarcinogenic properties of carotenoids [7, 8]. The biological activity of the prominent carotenoid, β-carotene, has been attributed to its ability to

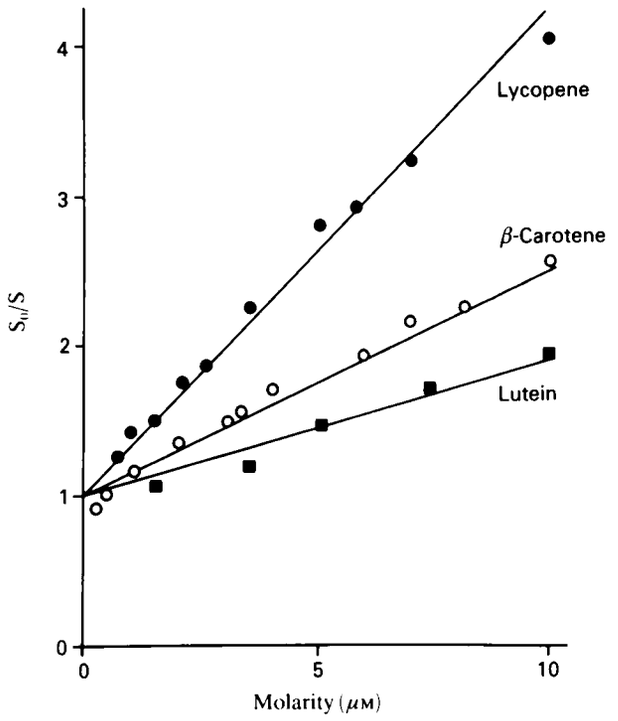

Fig. 1. *Stern–Volmer plot for the quenching of singlet oxygen by three carotenoids*

At 37°C, 3 ml of ethanol/chloroform (1:1, v/v) was placed in a 6 ml thermostated glass cuvette. 20 μl of a 0.4 M-$NDPO_2$ solution, kept at 2°C, was added to the solution. At the height of monomol emission, carotenoid was injected. For other details see Materials and methods.

quench $^1O_2$. The lipophilic tocopherols and hydrophilic thiols also have significant antioxidant properties and also react with $^1O_2$ [9–11].

However, unravelling the $^1O_2$ quenching abilities of these antioxidants has been hampered by the difficulties to obtain $^1O_2$ free from further reactive species. As a reproducible and clean source of $^1O_2$ for the investigation of the role of this oxygen species in biological systems, we used the thermodissociation of the endoperoxide of $NDPO_2$, a method through which $^1O_2$ can be obtained in an easy and simple way without reactive intermediates or byproducts. Using this we have studied the possible protection afforded by the biomolecules as measured by their quenching abilities.

Materials and methods

Generation and quenching of singlet molecular oxygen. The generation of $^1O_2$ was performed by the thermodissociation of the water soluble endoperoxide $NDPO_2$. The sodium salt of 3,3'-(1,4-naphthylidene) dipropionic acid (NDP) was prepared and its endoperoxide synthesised by the H_2O_2/Na_2MoO_4 method. The product was identified by ^1H n.m.r. and i.r. spectroscopy. $NDPO_2$ dissociates, yielding NDP and molecular oxygen [12].

The $^1O_2$-quenching ability of the biomolecules were determined by using a liquid nitrogen-cooled germanium photodiode detector equipped with an optical chopper. Monomol emission at 1270 nm is directly proportional to $^1O_2$ production.

$$O_2(^1\Delta_g) \rightarrow O_2(^3\Sigma_g^-) + h\nu(1270\ nm)$$

At the maximum of the monomol signal, achieved within 5–6 min, the quencher was injected and the overall quenching constant $(k_q + k_r)$ calculated using the Stern–Volmer plots. The chemical reaction rate constants of the tocopherols and thiols were calculated by following the depletion of these compounds [9, 11].

Table 1. *Singlet oxygen quenching constants, chemical reaction rate constants and content in mammalian tissues of carotenoids, bile pigments, tocopherols, thiols and related compounds*

The $(k_q + k_r)$ values were obtained from Stern–Volmer plots, as exemplified by Fig. 1 (see Materials and methods). ND — not determined. From [9, 11, 13]; singlet oxygen lifetime (10 μs) used for calculations.

Compound	$(k_q + k_r)$ $10^6\ M^{-1}\ s^{-1}$	k_r $10^6\ M^{-1}\ s^{-1}$	Content in tissues
Lycopene	31000	ND	0.5–1.0 μM, plasma
γ-Carotene	25000	ND	—
Astaxanthin	24000	ND	—
Canthaxanthin	21000	ND	—
α-Carotene	19000	ND	0.5–1.0 μM, plasma
β-Carotene	14000	ND	0.3–0.6 μM, plasma
Bixin	14000	ND	—
Zeaxanthin	10000	ND	43 ng/retina
Lutein	8000	ND	80 ng/retina
Cryptoxanthin	6000	ND	
Bilirubin	3200	ND	5–20 μM, plasma
Biliverdin	2300	ND	—
Bilirubin ditaurate	1200	ND	
Crocin	1100	ND	
Dithiothreitol	977	2.8	—
Dithioerythritol	744	2.9	—
Trolox	470	ND	
α-Tocopherol	280	3.6	15–31 μM, plasma
β-Tocopherol	270	0.2	—
Cysteine	236	70	30–100 μM, tissues
γ-Tocopherol	230	2.8	
δ-Tocopherol	160	1.7	—
Lipoate	130	ND	—
Coenzyme A	89	1.2	0–250 μM, tissues
Glutathione	59	1.7	0.5–10 mM, tissues
WR-1065	53	7.0	—
Cysteamine	41	11	3–13 μM, tissues
Mesna	24	2.8	—
Dihydrolipoate	5	5	—
Dimesna	6	ND	—
Captopril	4	ND	—

Results and discussion

Ranking biomolecules as singlet molecular oxygen quenchers. We have investigated the relative quenching ability of various naturally occurring antioxidants. The quenching abilities of the tested compounds differed considerably (Table 1). Lycopene, the open chain isomer of β-carotenes showed the greatest quenching ability. The $(k_q + k_r)$ value for lycopene was more than double that of β-carotene. The highest quenching ability of lycopene was followed by γ-carotene, astaxanthin, canthaxanthin, α-carotene, β-carotene, bixin, zeaxanthin, lutein, the bile pigments, bilirubin, biliverdin, dithiols, tocopherols and other thiols [9, 10, 11, 13]. The dithiol dihydrolipoate was an exception with low quenching ability. The carotenoids phytoene and phytofluene, derived from plants, had much lower singlet oxygen quenching abilities, $(k_q + k_r)$ less than $10^7\ M^{-1}\ s^{-1}$.

Our results indicate that the $^1O_2$ quenching properties of carotenoids reside not only on the triplet energy state, i.e., the length of the conjugated double bond system, but also on the functional groups and thus perhaps on the oxidation potential. While the role of carotenoids in protecting plants against photosensitization by their own chlorophyll as well as in the treatment of patients with photosensitivity diseases is

established, the mechanism by which β-carotene exerts a protective function against cancer remains unknown. However, there are several lines of evidence which suggest that the generation of reactive oxygen species may play an important role in the development of cancer [14]. The present work emphasizes that attention should be extended from β-carotene to lycopene and other carotenoids. Lycopene has a plasma concentration slightly higher than β-carotene and both these carotenoids were found in low-density lipoproteins [15].

The relative physical quenching abilities of the tocopherol homologues decreased in the following order: α, β, γ, and δ-tocopherol. With the tocopherols, the ability of $^1O_2$ quenching depends on a free hydroxyl group in position 6 of the chromane ring. Chemical reactivity of the tocopherol homologues were low, accounting for 0.1 to 1.5% of the physical quenching.

Among the biological thiols, cysteine was the most effective quencher of $^1O_2$, followed by lipoate (disulphide form of the dithiol lipoate), coenzyme A, glutathione, cysteamine and dihydrolipoate. Pharmacologically active thiols like *N*-acetylcysteine, mesna, WR-1065 and captopril significantly differed in their quenching abilities. The pD dependence of the chemical quenching indicated that $^1O_2$ reacts with the thiolate anion. As compared to their overall quenching abilities, cysteine, WR-1065, cysteamine, mesna and dihydrolipoate had significant chemical quenching abilities. other thiols tested had a chemical quenching rate less than 5%, most of them less than 1%, of their overall quenching ability.

Compared to carotenoids, other classes of compounds, e.g. bilirubin, tocopherols and thiols were less active in singlet oxygen quenching. But these may also be biologically important in $^1O_2$ quenching because of their higher concentration and/or different subcellular location in biological targets, besides solubility characteristics.

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Free radicals, myocytes and reperfusion injury

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There are several clinical settings in which the myocardium is exposed to transient ischaemia including evolving myocardial infarction, myocardial stunning and coronary thrombosis. On reperfusion, the sudden re-introduction of normotensive molecular oxygen may be detrimental to the previously ischaemic myocardium leading to suboptimal myocardial salvage. The myocardial response to ischaemia is highly dependent on the extent and duration of the ischaemia and the severity of coronary flow reduction.

Evidence for free radical involvement in reperfusion injury

There is much direct and indirect evidence for the contribution of radicals species to myocardial damage. The direct evidence comes from the application of techniques such as e.p.r. spectroscopy [1–3] which has confirmed the involvement of free radicals in *in vivo* animal models of coronary occlusion as well as in many isolated heart studies. Indirect evidence arises from protection afforded by specific scavengers of oxygen radicals and inhibitors of putative radical-generating systems in reducing infarct size and post-ischaemic contractile dysfunction. Recently the studies of Bolli *et al.* [3] have investigated the time window during which free radicals are generated in an open-chest dog model *in vivo*. The thiol-containing antioxidant compound, *N*-mercaptopropionyl glycine, was administered as an intracoronary infusion to dogs undergoing 15 min coronary occlusion and the drug infusion started at various specific time-points before and after reperfusion. Assessment of recovery of contractile function in terms of wall thickening and of inhibition of free radical production by e.p.r. after intracoronary infusion of a spin trap indicates that most of the damage responsible for myocardial stunning develops in the initial seconds after reperfusion and can be prevented by antioxidant therapy started just below reflow.

In addition, earlier studies of others [4–8] had shown attenuation of the incidence of arrhythmias and other markers of reperfusion damage by anti-radical interventions in a range of animal models. Such compounds included superoxide dismutase and catalase, several hydroxyl radical scavengers and the iron chelator desferrioxamine. All of these studies suggest that attenuation of these events by incorporation of appropriate anti-radical interventions in combination with thrombolytic therapy, for example, may help overcome the cellular damage that occurs secondarily to the initial pathology in the clinical condition.

Thus, a detailed understanding of the processes leading to the radical-dependent pathology in reperfusion injury, as well as the nature and sources of the toxic species, are crucial for the design of effective intervention strategies.