REVIEW

Interactions of dietary carotenoids with activated (singlet) oxygen and free radicals: Potential effects for human health

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Molecular mechanisms associated with the anti-/pro-oxidative properties of carotenoids (CARs) are described in organic solvents, micro-heterogeneous environments and model lipid membranes and in cellular suspensions. Singlet oxygen is important in the skin and eye and CARs are efficient singlet oxygen (SO) quenchers with corresponding rate constants near diffusion controlled (typically app. $10^{10}\,M^{-1}\,s^{-1}$) with lycopene (LYC) exhibiting the most efficient quenching in organic solvents. However, in membrane environments there is little or no difference in the quenching efficiency between the dietary CARs. Furthermore, aggregation of CARs, particularly those in the macula (lutein and zeaxanthin), markedly reduces SO quenching efficiency. Free radical interactions with CARs leads to at least three processes, electron and hydrogen atom transfer and adduct formation. The most studied is electron transfer where the CAR loses an electron to become a radical cation. The reactivity/lifetime of such CAR radicals may lead to a switch from anti- to pro-oxidant behaviour of CARs. These reactions are related to CAR redox potentials with LYC being the lowest (most easily oxidised) allowing LYC to reduce/repair all other CAR radical cations and LYC 'sacrificed' where mixtures of CARs are present in oxidative environments. Such redox-controlled reactions may lead to deleterious as well as beneficial health effects.

Keywords:
Anti-oxidants / Free radicals / Pro-oxidants / Reactive oxygen species / Singlet oxygen

1 Introduction

Since carotenoids (CARs) such as β-carotene (β-CAR), lycopene (LYC), lutein (LUT) and astaxanthin (ASTA) (see Fig. 1 for structures) are widely used as food colourants and, increasingly, as dietary supplements it is important to establish the benefit/risk ratio of these pigments. While much evidence to date supports a beneficial role for CARs many such studies involve whole or processed food [1–5]. For example, studies of LYC in human health have often concerned processed tomato products, which contain other nutritional components. Research is accumulating that some of these components are behaving synergistically to enhance the chemopreventative effects. Substantial human intervention studies are needed to strengthen the existing evidence. Indeed, this need is emphasised by the well-publicised trial of β-CAR as a protective nutrient against lung cancer [6, 7]. This trial, using only β-CAR, showed the reverse of the expected protective benefit in a small subgroup, those who were very heavy smokers. Of course, heavy smokers are almost certainly deficient in vitamin C [8], which may affect the behaviour of the β-CAR, as discussed below.

We review the fundamental chemical behaviour of CARs as singlet oxygen (SO) and free radical ($R^{\bullet}$) (reactive oxygen

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Abbreviations: AMD, age-related macular degeneration; **ASTA,** astaxanthin; **CAN,** canthaxanthin; **β-CAR,** β-carotene; **CAR,** carotenoid; **^{3}CAR,** carotenoid lowest excited triplet state; **$CCl_3O_2^{\bullet}$,** trichloromethyl peroxyl radical; **DHIR,** 3,3′-dihydroxyisorenieratene; **EPP,** erythropoietic protoporphyria; **LUT,** lutein; **LYC,** lycopene; **MED,** minimal erythema dose; **MMP,** metalloproteinase; **$NO_2^{\bullet}$,** nitrogen dioxide; **PP,** protoporphyrin IX; **$R^{\bullet}$,** free radical; **$RO_2^{\bullet}$,** peroxyl radical; **ROS,** reactive oxygen species; **$RSO_2^{\bullet}$,** thiyl sulphonyl radical; **SO,** singlet oxygen; **UVR,** ultraviolet radiation; **XANs,** xanthophylls; **ZEA,** zeaxanthin

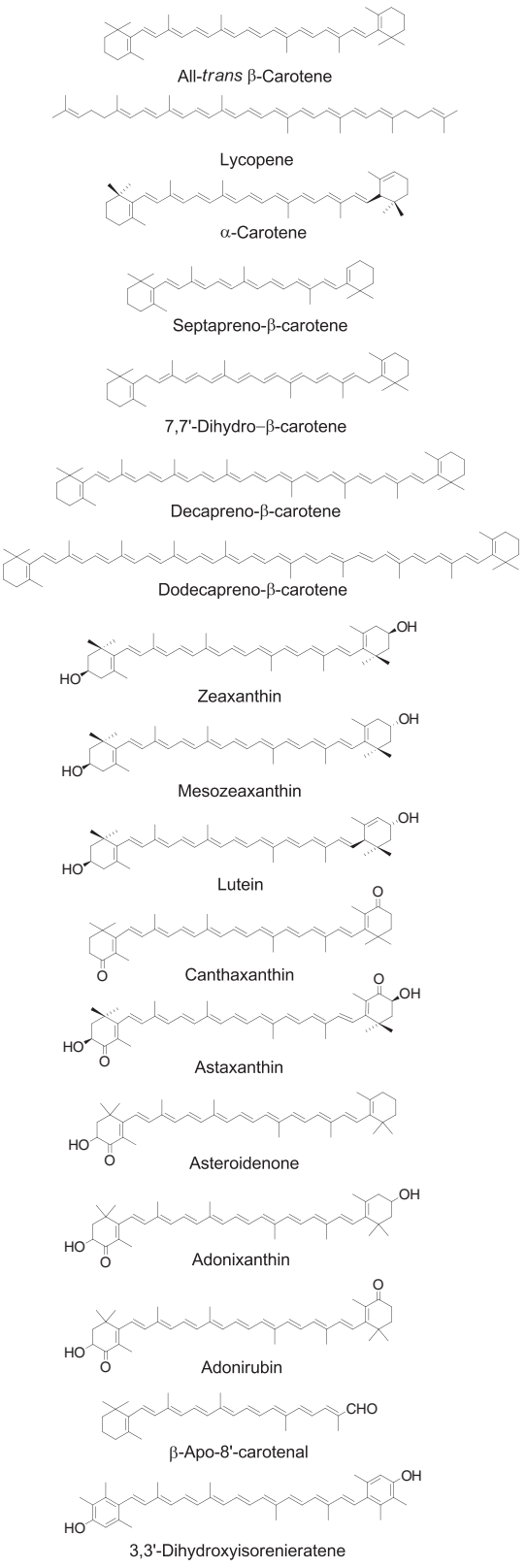


Figure 1. Structures of the carotenoids discussed in this article.

species, ROS) scavengers in simple solutions and, for $R^{\bullet}$ quenching, the reactivity of the subsequently formed CAR radicals with other biosubstrates (including vitamin C). Corresponding studies in micro-heterogenous environments such as liposomes as well as cellular protection studies of CARs with vitamins E and C are also discussed. Finally, the recent results on CARs, especially LYC, with respect to the oxidative stress, together with the rather varied data from human trials will also be reviewed. Of course, there are many other possible effects of CARs (a recent review of CARs as modulators of molecular pathways involved in cell proliferation and apoptosis has been given by Palozza et al. [9]).

Molecules that quench both SO and free radicals are frequently called anti-oxidants. However, the quenching of an oxidising $R^{\bullet}$ always produces another $R^{\bullet}$ and so may produce a pro-oxidant and therefore, lead to confusion. In this respect, it is useful to distinguish between the interactions of dietary CARs with SO and the more complex processes, which arise between free radicals and dietary CARs.

2 Carotenoids as SO scavengers

The C_{40} CARs and their oxygenated derivatives (xanthophylls – XANs) are one of nature's major anti-oxidant pigments. They efficiently quench SO, a non-radical ROS. The SO mainly arises from sunlight absorption by chromophores (e.g. porphyrins, chlorophylls and riboflavin) and CARs protect chlorophylls, proteins, lipids and DNA from SO damage. The overall quenching process simply converts the excess energy of SO into heat via the CAR lowest excited triplet state (^{3}CAR)

$$CAR + SO \rightarrow {}^3CAR + O_2 \quad (1)$$

$$^3CAR \rightarrow CAR + heat \quad (2)$$

The detailed mechanism for (1), the energy transfer, is quite complex [10] and involves singlet and triplet encounter complexes leading to the formation of ^{3}CAR.

Such processes are essential for virtually all life on Earth and, for these specific quenching processes, the CARs are behaving more-or-less as 'true' anti-oxidants with no pro-oxidative processes involved.

In organic solvents all dietary CARs with 10 or 11 conjugated double bonds quench SO at near the diffusion limit with LYC being the most efficient [11–15]. The rate constants for the quenching of SO by many dietary, and for comparison, some non-dietary CARs are given in Table 1.

Recently [16], such SO quenching data has been extended to a naturally occurring CAR with a rather unusual bifunctional structure – 3,3′-dihydroxyisorenieratene (DHIR). This has been isolated from the bacterium *Streptomyces mediolani*. The effect of the bifunctional character (conjugated and phenolic systems) is of interest for both SO and $R^{\bullet}$ quenching. In dichloromethane the SO quenching

Table 1. Second-order quenching rate constants for the quenching of singlet oxygen by various carotenoids

Carotenoid	n_c	$k_q/10^9\,M^{-1}\,s^{-1}$
7,7′-Dihydro-β-carotene	8	0.3 (Benzene) [11]
Septapreno-β-carotene	9	1.38 (Benzene) [11]
α-Carotene	10	12.0 (Benzene) [11]
		19.0 (Chloroform:ethanol:water, 50:50:1) [12]
β-Apo-8′-carotenal	10	5.27 (Benzene) [11]
Lutein	10	6.64 (Benzene) [11]
		8.0 (Chloroform:ethanol:water, 50:50:1) [12]
β-Carotene	11	13.0 (Benzene) [13]
		14.0 (Chloroform:ethanol:water, 50:50:1) [12]
		5.0 (Chloroform) [14]
Lycopene	11	17.0 (Benzene) [13]
		31.0 (Chloroform:ethanol:water, 50:50:1) [12]
		9.0 (Chloroform) [14]
Zeaxanthin	11	12.6 (Benzene) [13]
		8.0 (Chloroform:ethanol:water, 50:50:1) [12]
Canthaxanthin	11 (+2 C=O)	13.2 (Toluene) [13]
Astaxanthin	11 (+2 C=O)	14.0 (Benzene) [13]
		24.0 (Chloroform:ethanol:water, 50:50:1) [12]
Asteroidenone	11 (+1 C=O)	14.8 (Benzene) [15]
Adonixanthin	11 (+1 C=O)	12.3 (Benzene) [15]
Adonirubin	11 (+2 C=O)	10.4 (Benzene) [15]
Decapreno-β-carotene	15	20.0 (Benzene) [13]
Dodecapreno-β-carotene	19	23.0 (Benzene) [13]

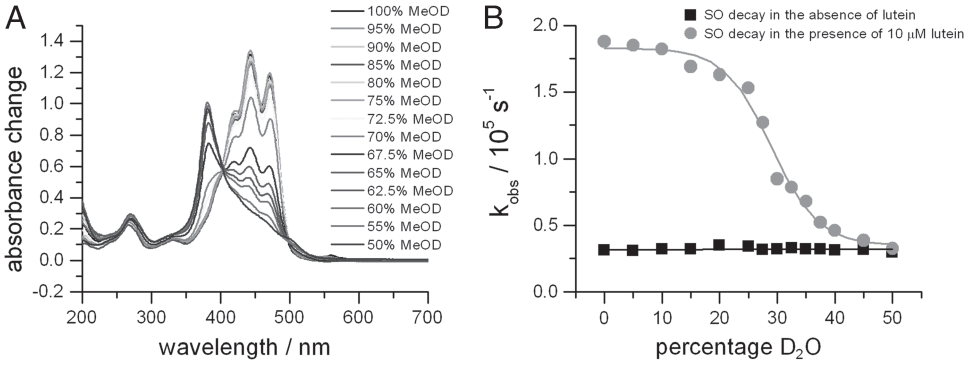


Figure 2. (A) Ground state absorption spectra of 1×10^{-5} M LUT in various MeOD/D_2O mixtures. (B) The effect of increasing D_2O (inducing LUT aggregation) on the SO deactivation efficiency by LUT.

rate constant is very near $1 \times 10^{10}\,M^{-1}\,s^{-1}$. As can be seen from Table 1, this is typical of many (conjugated) CARs.

Extrapolation of SO quenching data to in vivo systems may suggest that such quenching is an important 'valve' mechanism for the dissipation of potentially damaging excess energy and this is certainly so for photosynthetic systems. However, it must be remembered that CARs can aggregate and can span cell membranes and this can lead to inefficient SO quenching.

Aggregation of CARs is well known in simple solvent–water mixtures and the change in absorption spectrum and corresponding reduction in the efficiency of quenching of SO is shown in Fig. 2A and B. Where aggregation is precluded, efficient quenching can be observed in mainly aqueous systems as shown by Kanofsky and Sima [17] using canthaxanthin (CAN) complexed to a cyclo-dextrin. Such solubilised CAR systems may have clinical benefits in due course [18].

As well as aggregation, CARs and especially XANs can span cell membranes and hence change membrane structure and properties [19]. It is established that such processes reduce the quenching of SO as shown for zeaxanthin (ZEA) [20] in Fig. 3.

Thus, several reports of extremely efficient quenching of SO by CARs in 'simple' organic or detergent environments do not necessarily mean CARs are effective SO quenchers in vivo.

3 Carotenoids as R• scavengers in simple solutions

While the interaction of dietary CARs with potentially damaging ROS is believed to be of major importance for the protective role of CARs other mechanisms [9] have been proposed such as enhanced gap junction intercellular communication [21].

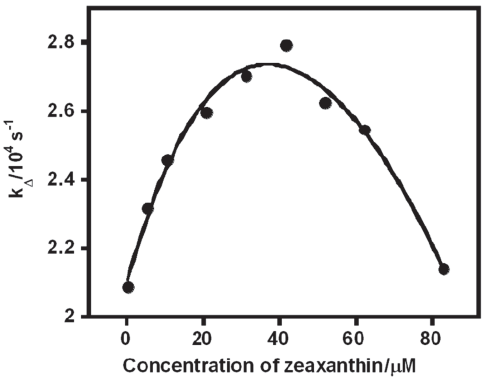


Figure 3. Rate of decay of SO against ZEA concentration in air-saturated solutions of unilamellar liposomes using rose bengal as SO sensitiser, modified from Fig. 5 in [20].

The reactions of CARs with free radicals are much more complex than with SO and depend mostly on the nature of the $R^\bullet$ rather than the CAR. Free radicals are, of course, characterised by an unpaired electron and, in all such reactions, the unpaired electron of the $R^\bullet$ is transferred to the CAR so that a new CAR radical (or CAR adduct radical) is produced and the original $R^\bullet$ is 'quenched'. This CAR radical may have properties quite different from the original $R^\bullet$. Whether or not the overall effect of the CAR is anti-oxidant (beneficial) or pro-oxidant (damaging) depends on a combination of the properties of the various radicals involved in the whole process in the specific environment of the CAR. Clearly, this is not easy to predict and hence, to refer to CARs as 'anti-oxidant due to quenching of free radicals' is possibly incorrect and confusing.

At least three different reactions have been proposed between (oxidising) free radicals and CARs:
Electron transfer

$$CAR + R^\bullet \rightarrow CAR^{\bullet+} + R^- \quad (3)$$

(e.g. $CCl_3O_2^\bullet$, $NO_2^\bullet$, $RSO_2^\bullet$)
Adduct formation

$$R^\bullet + CAR \rightarrow [CAR - R]^\bullet \quad (4)$$

(e.g. $CCl_3O_2^\bullet$, $RS^\bullet$).
Hydrogen abstraction

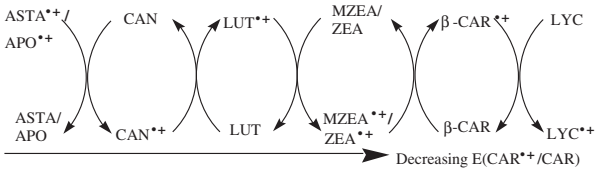
$$R^\bullet + CAR \rightarrow CAR^\bullet + RH \quad (5)$$

(e.g. $OH^\bullet$).

Electron transfer occurs when the reduction potential of the $R^\bullet$ is more oxidising than that of the CAR radical cation itself and has been the most studied of reactions 3–5. There have also been several studies of CAR–radical adducts, especially for sulphur-containing radicals. However, the first observation of the neutral radical, CAR, has only been reported very recently [22].

Fortunately, the radical cations of CARs are rather easy to study via direct time-resolved techniques such as pulse radiolysis and laser flash photolysis because of their distinct and strong absorption bands in the near infra-red. Most of the results have come from pulse radiolysis studies of peroxyl radicals ($RO_2^\bullet$) such as trichloromethyl peroxyl radical ($CCl_3O_2^\bullet$) and aryl peroxyl radicals ($ArO_2^\bullet$), as well as thiyl sulphonyl ($RSO_2^\bullet$) and nitrogen dioxide ($NO_2^\bullet$) [15, 23, 24].

As well as allowing spectral and reactivity parameters of CAR radical cations to be established, pulse radiolysis has also allowed direct measurement of the reduction potentials of several dietary CAR radical cations [25, 26]. These are all rather similar and lie in the range 1025 ± 50 mV, that is, all dietary CAR radical cations are themselves strongly oxidising species. Pulse radiolysis kinetic studies, together with comparison of electron transfer direction between pairs of dietary CARs show clear evidence that the LYC radical cation has the lowest value, i.e. it is the most strongly reducing and it is formed by electron transfer from all other CAR radical cations [27]. In particular, such studies show LYC efficiently quenches the radical cations of all the XANs studied (including those containing only hydroxyl groups and no carbonyl groups, i.e. LUT, mesozeaxanthin (MZEA) and ZEA), whereas β-CAR reduces only the radical cations of XANs containing carbonyl moieties (i.e. ASTA, β-apo-8′-carotenal (APO) and CAN). Overall, this leads to the following scheme showing the relative ordering of these one-electron reduction potentials



So that in a mixture of dietary CARs and free radicals, it is the LYC that is oxidised, protecting/repairing all the CARs present. A typical earlier study [28], also suggests LYC is the most readily oxidised CAR. More recently [29], a theoretical study, based on density functional theory, gave good agreement for the relative values obtained via the experimental (pulse radiolysis) work as shown in the above scheme. However, the values for these reduction potentials are so close, that in the 'real life ' situations the relative orders may be disturbed by other factors such as spatial distribution of the CARs in a membrane [30]. The high reduction potentials of dietary CAR radical cations, including LYC, are sufficiently oxidising to damage some biosubstrates. Furthermore, CAR radical cations are rather long-lived, particularly in aqueous micellar solutions, with a lifetime of up to half a second in some detergents [26]. The combination of long lifetime and high oxidising potential make them particularly reactive as biosubstrate oxidants. Indeed a good example is the oxidation of tyrosine (TyrOH) and cysteine [26] by CAR radical cations at pH 7:

$$CAR^{\bullet+} + TryOH \rightarrow CAR + TryO^\bullet + H^+ \quad (6)$$

Of course, the radical $R^{\bullet}$ is also sufficiently reactive to oxidise TyrOH but the lifetime of the $R^{\bullet}$ may be too short in a particular environment so, as noted above, it is the combination of the properties of CAR radicals (in this case, reduction potential and lifetime) that determine whether a given CAR in a given environment is an anti- or pro-oxidant.

Vitamin C has been shown to react with CAR radical cations, reducing their lifetime. This minimises the possibility of CARs showing pro-oxidant behaviour [31]. Indeed, there are claims that vitamin C and the CARs show synergistic benefits for removal of free radicals in cellular studies discussed below. Another example of synergistic behaviour comes from the work of Stahl et al. who suggested that specific positioning of the different CARs in multi-lamellar liposomes (models of cell membranes) leads to synergistic benefits, especially for the combination of LUT (with hydroxyl terminal groups) and the hydrocarbon, LYC [32]. A more recent example shows the repair of CAR radical cations by isoflavonoids [33]. In addition, there have been observations of synergistic anti-oxidative behaviour of LYC with a range of other bioactive compounds especially with respect to the oxidation of low-density lipoproteins [34].

For the nucleic acid bases it is established that guanosine has the lowest redox potential but this is still too high to be oxidised (damaged) by CAR radical cations, However, now the CAR is able to repair the oxidised guanosine and this gives an additional route to nucleic acid protection [35]. This result is in agreement with earlier findings by Astley and Elliot [36] showing CARs, including LYC, are capable of exerting two overlapping, but distinct effects: anti-oxidant protection by scavenging DNA-damaging free radicals and modulation of DNA repair mechanisms.

Both electron transfer and adduct formation have been seen for some $R^{\bullet}$ reactions with CARs. These include both $CCl_3O_2^{\bullet}$ and $RSO_2^{\bullet}$ radicals [23, 24]. Sulphonyl radicals form both an adduct absorbing around 400–500 nm and an unidentified species absorbing around 700–800 nm, which can decay to the radical cation [24]. The addition reaction between $RO_2^{\bullet}$ and β-CAR was first studied by Burton and Ingold [37] who suggested a resonance stabilised carbon-centered adduct radical ($ROOCAR^{\bullet}$) was formed and such processes were responsible for the chain breaking anti-oxidation exhibited by such CARs.

Some radicals, such as alkyl- and phenylthiyl species, including the species derived from glutathione, react only to give adducts ($R\text{-}CAR^{\bullet}$) [24, 38]. These adducts absorb in a similar spectral region to the CAR itself and were analysed via bleaching of the parent CAR absorption. The rate of such reactions was found to be more or less independent of the CAR structure.

Several radicals such as benzyl and benzylperoxyl were found to be unreactive to CARs but acetyl peroxyl radicals formed by the addition of oxygen to acyl radicals, such as phenyl acetyl peroxyl radicals, do form addition complexes with CARs [39]. Because of spectral overlap factors, these workers mainly studied 7,7′-dihydro-β-carotene and discussed the site of the addition of the radical to the CAR polyene chain with electron density and steric hindrance being considered although no definite structural assignments could be made.

Until recently, there was no direct evidence for the formation of neutral CAR radicals via hydrogen atom transfer to oxidising radicals despite being previously suggested, without spectral evidence [39, 40]. However, Skibsted and co-workers [22], studying the reaction between β-CAR and the hydroxyl radical, observed such a neutral radical. This short-lived species (a lifetime of about 150 ns) has an absorption maximum about 750 nm. Corresponding neutral radicals formed by reaction of hydroxyl radical with CAN and ASTA were not observed, suggesting a role for the 4,4′ hydrogen atoms in the formation of such radicals. No information was obtained concerning the fate of the neutral radical but its lifetime clearly precludes dimerisation processes, indeed the short lifetime may suggest a reaction with the solvent.

As mentioned above, a novel bifunctional CAR (DHIR) has been studied for its anti-oxidative and pro-oxidative properties [16]. Several assays were used, e.g. via measurement of cumene hydroperoxide inhibition time – this was 3–5 times longer for DHIR than for other CARs. However, when the phenolic group of DHIR was methylated the anti-oxidant capacity was reduced by about three times, suggesting the oxidation of the phenolic moieties (to give a conjugated quinoid) is an additional anti-oxidant mechanism not available to non-phenolic CARs. Other assays including inhibition of ABTS oxidation and the formation of thymidine dimers, as well as the rate of oxygen consumption via partial pressure measurements all show the same excellent anti-oxidant properties of this CAR. Overall, these results suggest DHIR may well be a useful natural food colourant, cosmetic and be invaluable in the treatment of degenerative diseases, such as macular degeneration.

This review is concerned with CARs as reactive oxy-species scavengers, however, there are other mechanisms which could be of importance such as modulation of redox thiols and modulation of cell signalling and gene expression [9, 41].

4 Carotenoids in cellular environments

While studies of anti- and pro-oxidant characteristics of CARs in ‘simple’ organic solvents and cell membrane models are useful in assessing the possible role of these processes in disease (and in mechanisms in photosynthesis) clearly a model environment nearer the in vivo situation may help further in unravelling the roles of CARs. There is little or no doubt that CARs and their oxidation products exhibit important bioactivities in cell lines and the use of cell cultures to interrogate the mechanisms of action may well help in designing sensible clinical trials. The major interest

in pro- and anti-oxidant mechanisms in cellular studies focus on (i) the reduction in oxidative stress (on lipoproteins, DNA, etc.) as measured by a range of biomarkers such as 8-oxo-2′-deoxyguanosine, the lack of such a beneficial effect and the switch from anti-oxidative to pro-oxidative effects, (ii) synergistic situations in which the beneficial properties of CARs are augmented by other species and the range of mechanisms leading to such synergism. Most studies of CARs in cellular environments concern one or more of these features.

Cell protection concerns both the quenching of SO and reduction of oxidising free radicals. Of course, the main interest in SO is because of the effect of sunlight and artificial light sources (phototherapy, photochemotherapy and cosmetic procedures in solariums). However SO can also arise in the dark [42] and this has been used as a useful experimental tool [43].

4.1 Reduction in oxidative stress and the switch from anti- to pro-oxidant

Numerous reactive species (which include free radicals and SO) produced both in the body and by external environmental factors can cause alterations to the structure of DNA. Furthermore, DNA damage is generally regarded as an indicator of disease risk. While CARs can modulate redox-controlled signalling pathways, although this is unlikely to involve the anti- and pro-oxidative properties relevant to this review [44]. Two of the main methods for the assessment of DNA damage are the comet assay [45] and the measurement of oxidised bases such as 7-hydroxy-8-oxo-2′-deoxyguanosine derivative. There have been many studies of the roles of CARs, and, especially recently, of LYC, and the protection against DNA damage with a lack of total agreement on the effect of the CARs, possibly due to the differing biomarkers used. Indeed whether LYC (and CARs in general) act as anti- or pro-oxidants for cell cultures is an on-going debate. This has been recently reviewed by Bowen [44] and emphasises the importance of CAR concentration with a switch from anti- to pro-oxidation observed in several systems as the CAR concentration increases. Of course, even the earliest work in non-cellular environments by Burton and Ingold showed the importance of oxygen concentration in a switch from anti- to pro-oxidative behaviour of CARs [37].

There have also been studies [46] of the effects of CARs on γ-radiation-induced oxidative stress [47]. The study by Saada et al. [47] concluded that LYC may protect the intestine against radiation-induced damage and this is in agreement with the work of Ito et al. and Srinivasan et al. [48, 49].

4.2 Synergistic Effects

It is important to carefully distinguish synergistic properties from simple additive effects. As noted above, there are several possible mechanisms by which CARs can exert beneficial effects. If more than one of these arise, there is a possible synergistic benefit. So, as well as direct quenching of SO or a (primary) $R^{\bullet}$, other protective mechanisms may include (i) repair of CAR free radicals by, for example, vitamin C [31], (ii) specific (and distinct) positioning of the different CARs [32], (iii) the repair of a biomolecule radical such as oxidised guanosine by a CAR and (iv) beneficial effects, by totally different (non-radical) mechanisms [9], such as increased cell–cell communication, reduced cholesterol synthesis and lowering the circulating levels of growth factors such as insulin-like growth factor-1 (IGF-1).

Böhm et al. [50] irradiated protoporphyrin IX (PP) to generate SO and uroporphyrin I (UP) to generate free radicals. Combinations of β-CAR, vitamin E and vitamin C gave a synergistic protective effect for UP-mediated damage (i.e. via free radicals) but only additive protective effects for PP-mediated damage via SO. Electron transfer to the carotene radical cation by vitamin C is a possible mechanism of such a synergistic effect.

Pulsed lasers have been used to generate specific free radicals, and to allow investigation of synergistic effects of CARs with vitamins C and E for protection of lymphocyte cells. Briefly [51], using standard cell staining methods with rose bengal to measure the cell kill, a 'protection factor' (PF) was obtained by comparing cell damage with and without anti-oxidants. Typical results for $NO_2^{\bullet}$ and β-CAR, vitamin E and C gave a PF of 2.0, 1.8 and 1.2, respectively, for the individuals anti-oxidants but 10.2 for the combination of all 3. A similar result was obtained for the non-radical peroxynitrite ($ONOO^-$) oxidising species. In this work, Böhm et al. used anti-oxidants via both incubation of the cells and via dietary supplementation. So, the synergistic effect reported for the presence of all the three anti-oxidants implies that there are interactions between these anti-oxidants leading to a beneficial effect. Interaction between α-tocopherol radical and ascorbic acid is well established [52] and the interaction between CAR radical cations (produced when a CAR is oxidised by $NO_2^{\bullet}$) and vitamin C is described above [31]. No interactive mechanisms have been proposed to date to explain the synergistic behaviour with $ONOO^-$ and vitamins C and E.

Krinsky and co-workers [53] studied synergistic interactions of anti-oxidant nutrients, including β-CAR, ascorbic acid and α-tocopherol in a biological model system based on reconstituted human serum. Several anti-oxidant combinations were shown to synergistically protect the reconstituted serum including β-CAR and ascorbic acid and β-CAR and α-tocopherol at physiological concentrations. Their results suggest a wide anti-oxidant network between water- and fat-soluble anti-oxidant nutrients in a biological system. Of course, they also point out that more work is needed to understand these processes in vivo. The possible role of CARs as trans-membrane radical channels has also been reviewed [54]. Palozza et al. [55] have recently reviewed the mechanisms of atherosclerosis prevention by LYC based on

cell culture studies. While there is an increasing evidence that LYC may protect, the authors show that the exact mechanism is far from understood. The review summarises the experimental evidence for a role of LYC in the different phases of the atherosclerotic process (prevention of endothelial injury, modulation of lipid and cholesterol metabolism, inhibition of low-density lipoprotein oxidation, prevention of oxysterol-induced cell damage and inhibition of foam cell formation, inhibition of smooth muscle proliferation) as well as the effect on ROS and remind us of the problems associated with such work due to the lack of solubility of LYC in aqueous systems and that it is essential to assess the LYC chemical stability when added to cell cultures – otherwise effects may, of course, be due to LYC metabolites. Indeed, several groups suggest a pivotal role for CAR metabolites and recently apo-lycopenals, in particular, as chemoprotective agents [56–58]. This possibility, as well as the wide range of possible synergistic effects should be taken into account in attempting to unravel the mechanisms of LYC protection in such a chronic disease.

In summary, several cell-based studies show similar overall behaviour to those seen in simple solvent and cell membrane model studies. For example, synergism is often reported although the mechanisms may be much more complex than just electron transfer as often suggested in solvent work. A switch from anti- to pro-oxidant behaviour first reported in solution environments over 27 years ago [31] is also seen in cellular environments and this switch may well be related to CAR concentration.

4.3 Skin model studies

There are many studies of model biological systems as well as animal and human trials associated with skin exposure to sunlight. Excess sunlight exposure, UVA (315–400 nm) and UVB (280–315 nm), causes DNA strand breaks, chromosomal aberrations and tumorigenic transformation in HaCaT skin keratinocytes [59] and leads to erythema. It is more or less accepted that this increases the risk of skin cancer later in life. Daily exposure to normal sunlight leads, via different mechanisms involving ROS and, especially SO, to loss of collagen fibres and loss of skin elasticity, that is, to skin ageing. A complex series of reactions take place following sunlight absorption [60] but in this review we are concerned only with the role of ROS (SO and various oxy-radicals) such as $RO_2^{\bullet}$ [61] and with the use of CARs to quench such ROS. Much of the early work on nutritional protection against skin damage has been reviewed by Sies and Stahl [62] starting with the clinical use of β-CAR to ameliorate erythropoietic protoporphyria (EPP) [63] about 35 years ago. Amongst the topics reviewed was a skin fibroblast study, which showed a switch from a beneficial role for β-CAR and LYC to adverse effects depending on CAR concentration [64]. This work complements the studies of the switch from anti- to pro-oxidant processes noted above for simple organic solvent systems. An interaction between LYC, β-CAR, vitamin E and vitamin C was also reported from skin fibroblast studies by Offord et al. [65]. In this work, LYC and β-CAR failed to protect the skin fibroblasts when used alone but did protect (e.g. via reduced induction of metalloproteinase-1 (MMP-1)) in the presence of vitamin E and with a very strong protection shown by the combination of LYC with vitamin E and vitamin C. Another mechanism associated with skin ageing has been suggested by Berneburg et al. Here, SO was shown to mediate the UV-induced generation of the photoageing-associated mitochondrial common deletion [43]. An interesting experimental aspect of this study was the generation of SO in the dark via thermodecomposition of an endoperoxide providing clear evidence of a role for SO in this overall process. Clearly, SO in human skin is of pivotal importance with respect to the photoageing and its quenching is likely to be an effective strategy for protection of human skin from photoageing. Commercial preparations involving several anti-oxidants such as Seresis containing both lipid and water-soluble compounds (β-CAR, LYC, vitamins C and E, selenium and proanthocyanidins at physiological concentrations) slow down UVB-induced erythema and MMP-1 decreased (it increased in the placebo group and there was no effect on MMP-9) [66].

5 Clinical trials

Oxidative stress has been implicated in a wide range of diseases such as various cancers, cardiovascular diseases, neurodegenerative diseases, age-related macular degeneration (AMD) and cataract formation. The possible therapeutic efficacy of anti-oxidants, including the dietary CARs, is being investigated for most of these diseases.

Damage to the skin and eyes involves photochemically generated free radicals and certainly involves SO. As described above, all dietary CARs are extremely efficient quenchers of SO in organic solvents and micro-heterogeneous environments such as detergent micelles and lipid membrane models. Furthermore, there have been direct observations of SO quenching by CARs on cell surfaces [67].

One of the earliest and most important trials developed by the Fitzpatrick group concerned β-CAR therapy for EPP. This hereditary disease is linked to a defect in haem synthesis and leads to the accumulation of PP in the skin. The PP absorbs light and is an efficient generator of SO which causes rapid skin damage – indeed, such damage was first demonstrated about 100 years ago by Meyer-Betz with a similar porphyrin–haematoporphyrin [68]. Mathews-Roth and co-workers treated patients with a high concentration (up to 180 mg/day) of β-CAR for many weeks and observed significant amelioration of the disease (84% of the patients with EPP increased their ability to tolerate sunlight by a factor of 3). Similar results have been reported by other groups [69, 70]. The trial by Gollnick et al. [69] concerned only 20 healthy young females exposed to 13 days of

sunlight exposure in Eilath (Red Sea), Israel to evaluate the efficiency of 10 weeks of 30 mg/day β-CAR dosage. Clearly, while the results from such a small trial may well not be statistically significant, the authors claim they show that β-CAR plus a topical organic and inorganic sunscreen had a multifactorial potency in preventing acute UV-induced skin damage and, the authors claim, also probably preventing long-term skin aging. Of course, while this early result appears to add weight to the value of CARs in skin protection it is not easy to speculate on molecular mechanisms. However, it is noteworthy that in a few trials, using much shorter treatment times, little or no protection was observed [71, 72].

There have been several subsequent trials showing beneficial effects for CARs, especially LYC and CARs in combination with other anti-oxidants such as vitamins C and E, proanthocyanins and selenium [66]. Clearly, such mixtures may allow synergistic mechanisms, such as those discussed above to operate but little or no definite evidence is available to date. The recent results of Rhodes and co-workers [73] reported a study of 20 healthy females who ingested 55 g tomato paste (16 mg LYC) daily for 12 weeks. Ultraviolet radiation (UVR)-induced erythemal sensitivity was assessed visually as the minimal erythema dose (MED). Biopsies were taken from unexposed and UVR-exposed skin pre- and post-supplementation, and analysed immunohistochemically for procollagen, fibrillin-1 and matrix metalloproteinase (MMP)-1, and for mitochondrial DNA damage. The outcome was MED was significantly higher following tomato paste versus control. The dietary supplementation led to a reduction in UVR-induced MMP-1, the UVR-induced reduction in fibrillin-1 was similarly abrogated, and an increase in pCI deposition was also observed. Furthermore, DNA damage following 3xMED UVR was significantly reduced by the tomato paste.

The stated conclusion from this trial was tomato LYC provides protection against acute and potentially longer term aspects of photodamage and probably shows a combination of protective effects. Probably, ROS in general are reduced by LYC leading to less erythema and, due to less SO, reduced MMP-1. Clearly, a larger trial is needed to confirm these observations of multiple effects of LYC with respect to ROS and it may well be worthwhile to investigate further protective improvements via some of the synergistic combinations discussed above.

Overall, there is significant evidence for CAR protection against sunlight-induced skin damage both in the short and long term. However, here, as with other clinical trials discussed below, the efficacy depends on several factors, length of treatment being particularly important in this case.

AMD is the leading cause of blindness in elderly people in the Western World – its prevalence increases with age and women are at greater risk than men due to reduced macular pigment. The macular region contains ZEA and LUT (usually referred to as the *macular pigment*), which are believed to mitigate light damage by quenching ROS. And there are reports that diets rich in LUT and ZEA are inversely associated with the prevalence of AMD [74, 75]. However, the macular contains no LYC or β-CAR so it is surprising that trials suggest benefits in the fight against AMD by diets supplemented by these hydrocarbon CARs [76].

The studies of Cardinault [77] concerned 37 people with AMD and 24 controls without AMD. Their results agree with the work of Mares-Perlman et al. [78] and showed that LYC was the only CAR altered (decreased) both in serum and lipoparticles of the AMD patients – in particular, there was no change in the levels of ZEA and LUT. This work suggests that even if LYC is not directly involved in the prevention of AMD, it can help by preserving the minimal available level of LUT and ZEA. This is very much in agreement with the relative redox potentials and electron transfer between CAR radicals discussed above (see electron transfer scheme above) with LYC being the most easily oxidised and therefore, possibly acting as a 'sacrificial' CAR which consequently protects other CARs from oxidation. Indeed, in the skin, resonance Raman spectroscopy has shown β-CAR is not degraded in the presence of LYC [79].

Model studies in which LYC is excluded from the diet and β-CAR is present should show the same effect and would be useful in attempting to confirm such a molecular mechanism based on electron transfer. Of course, the spatial arrangements of the different CARs could also be important – indeed, these could facilitate the electron transfer possibilities.

As well as reducing erythema and possibly long-term skin ageing CARs have also been shown to improve 'skin health' such as a reduction in skin roughness [80, 81]. These studies involved different methods to measure the CARs in the skin, one involving the use of resonance Raman spectroscopy [80] and the other via HPLC [81]. Also, they suggest anti-oxidants other than CARs, such as vitamins C and E may contribute to this beneficial effect. Finally, it is likely that damaging processes arise from UVA other than only via ROS – such as UVA-induced DNA strand breaks [59].

The well-known β-CAR intervention trial leading to increased lung cancer in sub-populations such as heavy smokers [6, 7] has lead to much debate. The details of such trials are discussed by Albanes and Wright [82]. A ROS-based electron transfer mechanism was proposed based on the extremely low vitamin C levels in smokers [83]. In this proposal, a typical radical in cigarette smoke ($NO_2^{\bullet}$) was shown to react with β-CAR to give its radical cation [51] and, in turn, this was shown to oxidise amino acids [26]. Of course, there are alternative mechanisms to explain these effects – thus the radical cation of β-CAR, which is the first intermediate in the oxidation of β-CAR, may convert to other oxidative products that modulate the redox status and intracellular ROS which can have several roles in intracellular signalling and regulation. Furthermore, non-redox mechanisms can be important via direct modulation of the expression of proteins and transcription factors involved in cell proliferation, differentiation and apoptosis, as reviewed

by Palozza et al. [9] for both redox and non-redox mechanisms. As Albanes and Wright [82] point out, given the huge amount of epidemiological information suggesting beneficial roles for CARs, within the normal dietary range, the opposite conclusion from the large β-CAR supplementation trials (called ATBC Study and CARET) were both unanticipated and disappointing, However, they have helped in understanding the risk factors associated with β-CAR.

Partly because of these β-CAR trials many subsequent clinical trials have concerned the use of LYC instead of β-CAR. The possibility that LYC could cause similar damaging effects is extremely unlikely because far less of this CAR is taken up by the human body than β-CAR.

The epidemiological work of Giovannucci et al. [84, 85] showed evidence that tomato-rich diets led to lower incidence of prostate cancer. However, some recent results do not agree [86, 87], while others do! [88].

Small human intervention trials of LYC on patients with established prostate cancer [89, 90] have used quite different assessment methods. Kucuk studied patients who were to have a radical prostatectomy and who were given 15 mg per day of tomato LYC. Comparing with placebo results the prostates were examined after removal. Significant benefits were observed for the patients taking the LYC supplement compared to the placebo. Barber and co-workers undertook a longer (one year) trial and used prostate-specific antigen (PSA) measurements to study the effects of 10 mg per day tomato LYC plus 200 mg per day vitamin C supplementation. A significant benefit was claimed for about two-thirds of the patients on this trial in terms of PSA velocity. Both these studies used a tomato extract so that several other components as well as LYC were present. Both were very small trials, with less than 80 men, but the authors claim they are statistically significant, with the work of Barber including a careful analysis based on several statistical tests. Nevertheless, much larger trials are needed to be convincing.

Another role for LYC in the prostate has been reported by Schwarz et al. [91] who found LYC inhibited progression in patients with benign prostate hyperplasia. This small pilot trial (40 men) appears not to be related to IGF changes but no anti-oxidative method was discussed. Again, while this trial is too small to be certain of the value of the results, the authors do give a statistical analysis based on *p*-values.

Because LYC is an efficient anti-oxidant it has been suggested that one role may be to contribute to the prevention of atherosclerosis by increasing resistance to LDL oxidation [92]. A recent survey by Palozza et al. [55] has summarised the experimental evidence for the other possible benefits of LYC in the different phases of the atherosclerotic process. This review calls for an in vivo well-controlled clinical and dietary intervention study, which takes into account both the possible synergistic interactions and LYC derivatives (such as apo-carotenals).

As noted above, there are several other chronic diseases for which CARs and LYC in particular are claimed to be beneficial. These are reviewed in four text books [1–4] and the recent review article by Burton-Freeman and Reimers [5].

There is much confusion in the literature on the benefits, or otherwise, of CARs, particularly LYC, in clinical trials. Many different CAR concentrations are studied, with different end points, length of trial, type of patient, etc., so it is not surprising that a wide range of conclusions have been presented. In addition, the role of oxidative metabolites [93] and the degree of isomerisation of the CARs are further complications [94].

6 Summary

In this review, we have attempted to correlate anti- and pro-oxidative properties of dietary CARs in model environments with the results of clinical trials. Some interesting possibilities arise such as the efficiency of LYC compared with other CARs as both a SO quencher and $R^{\bullet}$ scavenger (where it is the sacrificial CAR), the role of LYC in AMD and the role of vitamin C (and other antioxidants) to enhance the value of CARs and avoid possible deleterious effects due to the formation of oxidising CAR radical cations.

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7 References

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