β-Carotene with vitamins E and C offers synergistic cell protection against NO_x

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Abstract The peroxynitrite anion and the nitrogen dioxide (radical) are important toxic species which can arise in vivo from nitric oxide. Both in vivo and in vitro cell protection is demonstrated for β-carotene in the presence of vitamin E and vitamin C. A synergistic protection is observed compared to the individual anti-oxidants and this is explained in terms of an electron transfer reaction in which the β-carotene radical is repaired by vitamin C.

Key words: β-Carotene; Lymphocyte; Peroxynitrite; Nitrogen dioxide; Vitamin E; Vitamin C

1. Introduction

It is now accepted that $NO^{\bullet}$, a radical, has a wide range of important in vivo roles, such as the control of systemic blood pressure, respiration and as a messenger molecule. Both nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are radicals which are implicated in cellular damage, and the toxic processes involving $NO^{\bullet}$ arise both via peroxynitrite production [1]

$$O_2^{\bullet-} + NO^{\bullet} \rightarrow OONO^- \rightleftharpoons^{H^+}_{pK_a=6.8} HOONO \quad (1)$$

and even via $NO_2^{\bullet}$ formation in the region of a fully activated macrophage [2]. Of course, $NO_2^{\bullet}$ is also a major environmental air pollutant and it can also be formed from $NO^{\bullet}$ reaction with oxygen in cigarette smoke. Once formed, $NO_2^{\bullet}$ radicals can initiate many damaging reactions including lipid peroxidation [3]. Peroxynitrite itself can also initiate lipid peroxidation [4] and it has been shown to inactivate sodium channels from epithelial vesicles [5].

We now report cellular studies in which we observe *synergistic* protection of human cells both in vivo and in vitro by β-carotene plus vitamins E and C against lethal damage by both $NO_2^{\bullet}$ radicals and $OONO^-$/HOONO (formed from $NO^{\bullet}$ and the superoxide radical $O_2^{\bullet-}$), so that there is a substantially increased protection against $NO_2^{\bullet}$ [6] and $OONO^-$ compared to β-carotene alone.

2. Materials and methods

We have used both pulse radiolysis [7,8] and 355 nm pulsed laser excitation to generate the $NO_2^{\bullet}$ radical and laser flash photolysis to produce the peroxynitrite anion (laser energies were 20 mJ per pulse throughout). Using pulse radiolysis $NO_2^{\bullet}$ was formed in aqueous nitrate solutions via the reaction with the solvated electron. *t*-Butanol was used to scavenge the hydroxyl radicals, producing water and the unreactive *t*-butanol radical [9].

$$e^-_{aq} + NO_3^- \rightarrow NO_3^{\bullet 2-} \xrightarrow{H_2O} NO_2^{\bullet} + 2OH^- \quad (2)$$

It has previously [6] been shown that pulsed laser excitation of nitronaphthalene (NN) in water leads to its triplet (^{3}NN) which reacts with sodium nitrite to produce the corresponding radical anion of nitronaphthalene ($NN^{\bullet-}$). This process also generates $NO_2^{\bullet}$ [6].

$$^3NN + NO_2 \rightarrow NN^{\bullet-} + NO_2^{\bullet} \quad (3)$$

The peroxynitrite anion was generated via laser excitation of an air saturated solution of sodium nitrite (NO_2^-) and sodium formate ($HCOO^-$) in Dulbecco buffer at physiological pH [10]. Under these conditions the laser converts the NO_2^- to $NO^{\bullet}$ and hydroxyl radicals ($OH^{\bullet}$):

$$NO_2^- \xrightarrow{UV\ light} {}^{H^+} NO^{\bullet} + OH^{\bullet} \quad (4)$$

and the hydroxyl radicals react with $HCOO^-$ to produce the carbon dioxide radical anion ($CO_2^{\bullet-}$), which, in turn, reacts with oxygen to yield the superoxide radical anion, $O_2^{\bullet-}$.

$$OH^{\bullet} + HCO_2^- \rightarrow CO_2^{\bullet-} + H_2O \quad (5)$$

$$CO_2^{\bullet-} + O_2 \rightarrow O_2^{\bullet-} + CO_2 \quad (6)$$

Finally, the $O_2^{\bullet-}$ and $NO^{\bullet}$ combine to give the peroxynitrite:

$$O_2^{\bullet-} + NO^{\bullet} \xrightarrow{(H^+)} OONO^- \ (HOONO) \quad (7)$$

The transient absorption spectrum of peroxynitrite, so obtained, is shown in Fig. 1.

As can be seen, our pulsed laser 'synthetic' route to $OONO^-$/HOONO involves several other reactive intermediates (radicals). The first is the very reactive hydroxyl radical ($OH^{\bullet}$) which is certainly potentially damaging to cells. However, we used 0.1 M formate so that all of the $OH^{\bullet}$ radicals are scavenged by the formate in less than 1 ns, i.e. at the ratio of formate to cell number (see later) all the $OH^{\bullet}$ radicals are scavenged by the formate. Similarly, at the oxygen concentrations used all the $CO_2^{\bullet-}$ radicals react with oxygen to produce superoxide. For the superoxide radical we calculated the maximum amount which may be produced (related to the oxygen concentration) and, in a control experiment, using potassium superoxide, we showed that this level of $O_2^{\bullet-}$ led to no significant increase in cell killing (3.2%) compared to the control after preparation (2.8%). Calculation of the amount of $NO^{\bullet}$ radical generated (based on solution volume, number of laser pulses, volume irradiated by each pulse and light scattering) and corresponding control experiments suggested no significant increase in cell killing (3.0%) by this level of $NO^{\bullet}$. Finally, with respect to control experiments, there exists the possibility of $NO^{\bullet}$ reacting with O_2 to give $NO_2^{\bullet}$. However, as noted above, at the formate concentration used all $OH^{\bullet}$ is converted to $CO_2^{\bullet-}$ so that there is an approximately equal concentration of $NO^{\bullet}$ and $CO_2^{\bullet-}$ and $CO_2^{\bullet-}$ reacts very quickly with O_2 (2.4×10^9 M^{-1} s^{-1} [9]) while the $NO^{\bullet}/O_2$ reaction is a third order process.

Two cell types were used in the experiments. For the in vivo studies we used human lymphoid cells (3×10^7 cells/ml) [11] taken from the blood after treatment with 2 weeks of 150 mg/day of β-carotene (Carotaben, Hermal), *RRR*-D-α-tocopherol 800 mg/day (Vitamin E, Natur, Jenapharm) and 1000 mg/day ascorbic acid (Vitamin C, Boots). Control experiments were performed on cell samples of the same

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person without carotenoid/antioxidant supplements. For the in vitro studies we used lymphocytes from a cell line (Jurkat, 3×10^7 cells/ml) incubated with 20 μM β-carotene (water soluble Roche preparation), 25 μM DL-α-tocopherol (Aldrich, purified by chromatography to >99% before use, 0.1% DMSO) and 100 μM ascorbic acid (Sigma) for 1 h at room temperature followed by three washes. Cell staining with eosin (see table legend) was used to show which cells have membrane destruction leading to cell death (according to [12]). All results (mean of at least 24 measurements for each experiment) were corrected for the small percentage of stained cells due to the special preparation technique. The standard deviation was always less than 3.0.

3. Results and discussion

Our major finding is that cells which are treated with the β-carotene *plus* vitamins E and C (the anti-oxidants) in vivo and exposed to the $NO_2^{\bullet}$ radical show cell staining of 6.0% whereas, without the anti-oxidants the cell staining was 61.4%. That is, the presence of all three of the anti-oxidants leads to a protection factor of 10.2. Protection by β-carotene alone gave a protection factor of only 2.0, for α-tocopherol alone it was 1.8 and for ascorbic acid it was 1.2. These results are given in Table 1.

For the in vitro experiments the corresponding cell staining was 5.3% and 52.9%. That is, the anti-oxidant combination leads to a protection factor of 10.0. With β-carotene alone as the anti-oxidant the protection factor was only 3.5, while for α-tocopherol alone it was 3.6 and for ascorbic acid alone there was no significant protection.

A second major finding is that cell protection was also observed against the peroxynitrite anion. Thus, in vivo, the staining increased from 5.2% with the anti-oxidants to 43.3% without the anti-oxidants (giving a protection factor of 8.3). For the in vitro experiments the corresponding cell staining was 7.3% and 59.5%, i.e. a protection factor against $OONO^-$ of 8.2, as shown in Table 1. We found significant differences with $P<0.01$ or less (Mann-Whitney *U*-test) between the percentage of stained cells with and without antioxidant incubation (except for vitamin C and $NO_2^{\bullet}$) as well as between the cases of single application of antioxidants and the use of all three together. There were no significant differences between the cell killing rates of cells without antioxidant incubation.

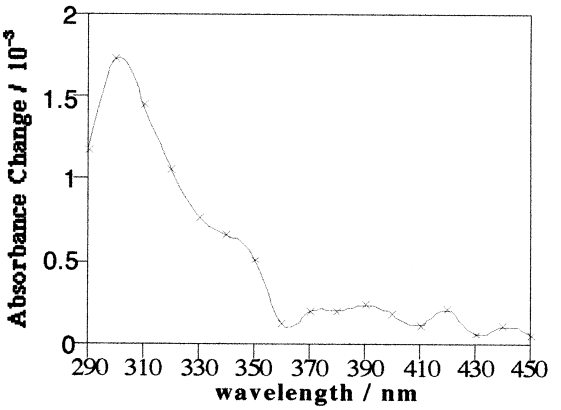

Fig. 1. Spectrum of $OONO^-$, obtained 0.4 ms after laser excitation of a solution of sodium nitrite and sodium formate in Dulbecco buffer.

For the toxic oxidising $NO_2^{\bullet}$ radical, we observed a marked synergism in cell protection by the anti-oxidant combination of β-carotene with vitamins E plus C in both in vivo and in vitro experiments. In a related series of experiments we used pulse radiolysis (1×10^{-4} M β-carotene in 2% Triton X-100 detergent micelles with $NO_2^{\bullet}$ generated as in Section 2 above) to show a reaction between $NO_2^{\bullet}$ and β-carotene leading to the β-carotene radical cation ($CAR^{\bullet+}$)

$$NO_2^{\bullet} + CAR \rightarrow NO_2^- + CAR^{\bullet+} \quad (8)$$

the characteristic absorption in the near infra-red (~930 nm) of $CAR^{\bullet+}$. Of course, α-tocopherol (TOH) is well established as an effective anti-oxidant against oxy-radicals in general ($RO_2^{\bullet}$)

$$RO_2^{\bullet} + TOH^- \rightarrow RO_2^- + TO^{\bullet} + H^+ \quad (9)$$

so that a combination of carotene and vitamin E might well offer an additive protection against cell damage. However, in the presence of ascorbic acid we observe a synergistic rather than an additive effect in our cellular studies (see Table 1) and this implies an interaction between these individual anti-oxi-

Table 1
Cell membrane protection by antioxidants against NO_x

Cells incubated with:	Reactive species	Percentage of stained cells	Protection factor
β-Carotene+vitamins E+C in vivo	$NO_2^{\bullet}$	**6.0 (without 61.4)**	**10.2**
β-Carotene in vivo	$NO_2^{\bullet}$	26.9 (without 53.2)	2.0
Vitamin E in vivo	$NO_2^{\bullet}$	28.4 (without 50.1)	1.8
Vitamin C in vivo	$NO_2^{\bullet}$	41.0 (without 51.0)	1.2
β-Carotene+vitamins E+C in vitro	$NO_2^{\bullet}$	**5.3 (without 52.9)**	**10.0**
β-Carotene in vitro	$NO_2^{\bullet}$	14.6 (without 51.6)	3.5 [5]
Vitamin E in vitro	$NO_2^{\bullet}$	14.8 (without 53.0)	3.6
Vitamin C in vitro	$NO_2^{\bullet}$	48.0 (without 48.9)	1.0
β-Carotene+vitamins E+C in vivo	**$OONO^-$**	**5.2 (without 43.3)**	**8.3**
β-Carotene in vivo	$OONO^-$	32.4 (without 55.1)	1.7
Vitamin E in vivo	$OONO^-$	27.1 (without 53.8)	2.0
Vitamin C in vivo	$OONO^-$	36.1 (without 50.9)	1.4
β-Carotene+vitamins E+C in vitro	**$OONO^-$**	**7.3 (without 59.5)**	**8.2**
β-Carotene in vitro	$OONO^-$	38.1 (without 49.1)	1.3
Vitamin E in vitro	$OONO^-$	14.0 (without 48.0)	3.4
Vitamin C in vitro	$OONO^-$	34.9 (without 47.9)	1.4

Cell membrane destruction is shown by cell staining with eosin (2 μl 1% eosin to 50 μl cell suspension, 5 min). The results ($s<3.0$) are based on the mean of at least 24 measurements. They were corrected for the small percentage (<3) of stained cells due to the preparation technique and after incubation with the reactants prior to irradiation.

dant components. This cellular result is in agreement with our previous pulse radiolysis results [13] which show that β-carotene radical cation is repaired by ascorbate in Triton X-100 solutions and by ascorbic acid in methanol according to the processes below.

$$CAR^{\bullet+} + AscH^{-} \rightarrow CAR + Asc^{\bullet-} + H^{+} \quad (10)$$

$$CAR^{\bullet+} + AscH_2 \rightarrow CAR + AscH^{\bullet} + H^{+} \quad (11)$$

While we have direct evidence for regeneration of the parent carotenoid from its radical cation via ascorbic acid, an interaction between vitamin E and carotene is more speculative. In polar environments $TOH^{\bullet+}$ would be rapidly deprotonated [14] so that no interaction between $TOH^{\bullet+}$ and CAR would be possible. Indeed, for the water soluble vitamin E analogue, Trolox C, we see the quenching of $CAR^{\bullet+}$ by Trolox C. However, in non-polar environments (e.g. hexane) we observe $TOH^{\bullet+}$ with a lifetime of about 6 μs [15] and the reaction of this species with β-carotene to give β-$car^{\bullet+}$ which can only occur in a specific non-polar region of the cell membrane. For the protection to operate in a synergistic way, i.e. for the carotenoid to enhance the cell protection by vitamin E and C (the direct interaction of $TO^{\bullet}$ with $AscH_2$ is already established [16],) an interaction between the lipophilic carotene and the water soluble ascorbic acid is required. We propose that the positively charged nature of the carotene radical cation ($CAR^{\bullet+}$) makes it much more likely that the carotene radical cation will orientate itself in the membrane so that the positive charge is much nearer to the polar interface than the parent, uncharged β-carotene molecule itself. A key feature of our hypothesis for synergistic behaviour is that the carotene radical is a charged species and this facilitates its reorientation and its reaction with ascorbic acid. From these results we would expect a particularly efficient synergistic effect in the eye due to the presence of hydroxy-substituted carotenoids such as zeaxanthin and lutein and this is possibly essential to protect the eye against a highly oxidising environment.

Niki and co-workers [17], using AMVN (2,2′-azobis(2,4-dimethylvaleronitrile)) as a radical initiator and conventional spectroscopic techniques, suggest that any protective effect of β-carotene which they observe is *not* due to $CAR^{\bullet+}$ production, but rather due to formation of *uncharged* species via addition reactions of the type:

$$LO_2^{\bullet} + CAR \rightarrow LOO{-}CAR^{\bullet} \rightleftharpoons^{O_2} LOO{-}CAROO^{\bullet}$$

where $LO_2^{\bullet}$ is a peroxyl radical deep inside the non-polar region of the membrane.

However, our pulse radiolysis and laser flash photolysis results unambiguously show the efficient formation of $CAR^{\bullet+}$ from $TOH^{\bullet+}$. In addition, we have also seen $CAR^{\bullet+}$ formed from $NO_2^{\bullet}$ (as noted above) and from $^{\bullet}O_2CCl_3$ [18]. Also Willson and co-workers [19] observe $CAR^{\bullet+}$ formation from oxy-sulphur radicals, $RSO_2^{\bullet}$, as do Mortenson and Skibsted [20] from phenoxyl radicals. Thus for a wide range of oxidative radicals the charged $CAR^{\bullet+}$ is formed and, in all such cases, we suggest a synergistic protective role involving 'repair' of CAR from $CAR^{\bullet+}$ by ascorbic acid. Of course, for other radicals which form uncharged products with carotenoids any anti-oxidative effect will only be additive rather than synergistic.

Our results on the cell protection against the peroxynitrite anion can be interpreted in terms of β-carotene quenching of $OONO^{-}$/HOONO as reported by Kikugawa et al. [21]. Again, we see a marked synergism with our antioxidant combination. As noted above the peroxynitrite anion exists as the acid (pK_a=6.8) so that in physiological conditions we can expect similar amounts of the $OONO^{-}$ and OONOH. It may be that these react differently with carotene. However, the nature of any reaction of peroxynitrite with carotenoids is not yet known [22].

Overall, our major findings are that β-carotene with vitamins C and E offer a synergistic cell protection against both $NO_2^{\bullet}$ and $OONO^{-}$ but that this is somewhat greater for the $NO_2^{\bullet}$ system.

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