β-Carotene enhances hydrogen peroxide-induced DNA damage in human hepatocellular HepG2 Cells

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Abstract In this study, the alkaline version of the comet assay has been used to determine the effect of β-carotene supplementation (10 μM) on peroxide-initiated free radical-mediated DNA damage in human HepG2 hepatoma cells. In supplemented cells, β-carotene failed to afford any protection against hydrogen peroxide-induced DNA strand breaks. Indeed, levels of strand breaks in supplemented cells were significantly higher than in cells exposed to hydrogen peroxide alone, especially after a long incubation period. In contrast, β-carotene afforded significant levels of protection against DNA strand breaks when cells were treated with *tert*-butyl hydroperoxide. In this case, the level of protection increased as supplementation continued.

Key words: β-Carotene; HepG2; Peroxide; Comet assay

1. Introduction

It has now been well-established that a diet rich in fruit and vegetables is inversely associated with the risk of development of certain cancers [1–5]. Factors in the diet thought to contribute to this protective effect include minerals such as selenium, vitamins C, D and E, polyphenols and dietary carotenoids [6]. Carotenoids are fat soluble pigments synthesised by plants and micro-organisms. Of the 600 or so identified to date, only a small number (≈19) have been detected in human tissues and the role played by these molecules in human disease prevention has only begun to be elucidated for a select few in recent years.

Several large scale human intervention trials using isolated components from fruits and vegetables, notably β-carotene, have produced conflicting results, showing either positive, no effects or even negative effects of β-carotene supplementation on chronic human disease, such as cardiovascular disease and incidence of tumours [7–9]. In addition, experiments using cells in culture have also generated conflicting data regarding the efficacy of β-carotene. The most recent experiments have demonstrated the protective effects of dietary fruit and vegetables on oxidatively-induced DNA damage [10]. Oxidative stress and free radical attack are believed to be major factors in diseases such as cardiovascular disease and cancers.

The aim of this study was to investigate the effect of β-carotene on the modulation of oxidatively-induced DNA strand breaks in HepG2 cells using the single cell gel electrophoresis (SCGE) assay [11]. Hydrogen peroxide (H_2O_2) and the synthetic *tert*-butyl hydroperoxide (tBOOH) were chosen to induce DNA breaks. H_2O_2 is produced in tissues during normal cellular respiration and metabolism, usually as a result of the dismutation of superoxide. Organic peroxides are usually formed in cell membranes [12]. Both H_2O_2 and tBOOH have been widely used as model compounds to investigate the effects of oxidative stress in a cell culture. The most significant DNA lesion produced by both peroxides appears to be the single strand break, either by production of the hydroxyl (OH^b) radical via Fenton type reactions or, possibly in the case of tBOOH, by lipid peroxidation and reactions involving calcium [13–15].

Human HepG2 hepatoma cells retain many of the functions of a normal liver cell including the manner in which they respond to hormones and cytokines. They express an inducible cytochrome P450 system and secrete plasma proteins and lipoproteins. In addition, they have been used in previous studies of β-carotene protection of oxidatively-induced effects [14,16]. In the present study, we have used the alkaline version of the comet assay to determine whether long-term supplementation of these cells with β-carotene can protect against DNA strand breaks induced by either H_2O_2 or tBOOH.

2. Materials and methods

2.1. Chemicals

All chemicals and cell culture media were obtained from the Sigma Chemical Company (Dorset, UK), unless otherwise stated. Cell culture plastics were supplied by Costar. All-*trans* β-carotene was a kind gift from F. Hoffman-La Roche (Basel, Switzerland). Both tBOOH and H_2O_2 were prepared in ice cold sterile PBS immediately before use. Tetrahydrofuran (THF) was stored under argon and contained 0.025% (w/v) butylated hydroxytoluene (BHT). BHT is a relatively inert antioxidant which prevents peroxides forming in THF. Experiments using untreated cells were always run in parallel with the β-carotene-treated and THF only-treated cells in order to monitor the effects of the solvent.

2.2. Cell culture

Human hepatoma HepG2 cells were maintained in William's medium E (Sigma Chemical, Poole, UK) supplemented with 10% foetal calf serum (FCS) and 2 mM L-glutamine. Cells were subcultured every 7–10 days and were grown in a humidified incubator at 37°C in an atmosphere of 95% air, 5% CO_2. Cells were maintained in the absence of antibiotics and screened for mycoplasma by a hoechst staining method. Cells were plated at a density of approximately 2×10^4 cells/cm^2 for experiments. Carotenoid treatment was in tissue culture medium containing 2.5% FCS, peroxide treatment was in serum-free medium.

2.3. Purification of β-carotene

Immediately prior to use, carotenoids were purified and epoxides removed by eluting in hexane from a small neutral alumina (Brockman grade III) column. The concentration of carotenoid was determined by spectrophotometric means using the published extinction coefficient (A1%/1 cm 2592, hexane) [17] before being divided into samples, dried under a stream of oxygen-free N_2 and stored under N_2 at −80°C. The carotenoid samples were stored in this way for no

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more than 10 days and a fresh sample was used for each experiment. The β-carotene uptake into HepG2 cells was analysed by HPLC as previously described [18].

2.4. Toxicity studies and analysis of DNA strand breaks

The cell viability was determined using both the fluorescein diacetate (FDA/EtBr) assay [19] and the MTT assay, which was performed as previously described [20]. The IC_{50} value was the concentration of peroxide required to inhibit the cell proliferation by 50%. The FDA/EtBr technique was used mainly to measure the viability of cells immediately prior to processing in the comet assay in order to rule out DNA fragmentation as a result of cell death. DNA strand breaks were analysed using a modified version of the SCGE assay [21–23].

2.5. Data Analysis

Cells were visualised using a Nikon labophot fluorescence microscope fitted with a CCD camera. The comet images were captured and analysed using the Fencomet v.2.2 image analysis package (Kinetic Imaging, Liverpool, UK). 100 nuclei were analysed per sample as previously described [24]. Where appropriate, data were analysed using ANOVA followed by the Dunnett's test to compare differences between the negative controls and treatments. All results reported are the mean ± S.E.M. of at least three independent experiments unless otherwise stated.

3. Results

3.1. Effect of peroxides on HepG2 cell DNA strand breaks and viability

A 30 min treatment with tBOOH (1–100 μM) resulted in a dose-dependent increase in DNA strand breakage. The level of strand breaks reached a plateau at tBOOH concentrations higher than 25 μM. In contrast, treatment with H_2O_2 resulted in a positive linear increase in the comet tail moment over the entire 1–100 μM concentration range. HepG2 cells appeared to be more resistant to the effects of H_2O_2 than tBOOH at the lower end of the dose range (0–25 μM), as only H_2O_2 concentrations of 25 μM and above gave tail moments significantly different from those of the controls (Fig. 1). The presence of THF (0.2% v/v) in the culture media had no effect on peroxide-induced DNA strand breaks.

The effect of H_2O_2 and tBOOH on the cytotoxicity under the same experimental conditions was assessed using the MTT assay. There was a significant decrease in the surviving fraction ($P < 0.01$) relative to the negative controls at the highest concentration (500 μM) of H_2O_2 (surviving fraction: 0.38 ± 0.06) but not tBOOH (surviving fraction: 0.7 ± 0.09). When THF (0.2% v/v) was added to the culture media, there was no effect on the peroxide-induced cytotoxicity (surviving fraction for H_2O_2 and tBOOH, respectively, in the presence of THF: 0.27 ± 0.09 and 0.67 ± 0.07)

From these data, a concentration range of 0–50 μM H_2O_2 and 0–25 μM tBOOH was chosen for the experiments with β-carotene. A final concentration in tissue culture media of 10 μM β-carotene was chosen from the literature [25] to reflect the levels of β-carotene in plasma following supplementation.

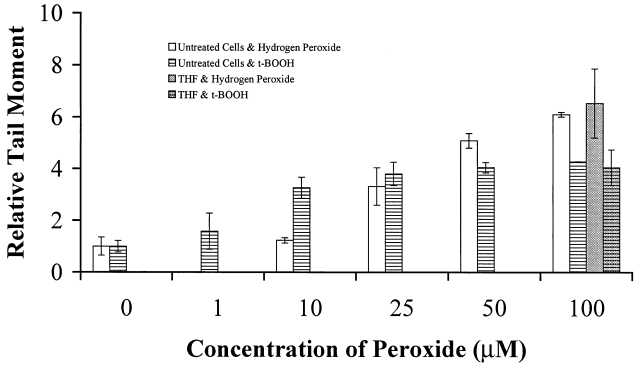

Fig. 1. The effect of a 30 min treatment with either H_2O_2 or tBOOH on HepG2 cells as measured by the ASCGE assay. Human hepatoma HepG2 cells were seeded at a density of approximately 2×10^4 cells/cm^2 in 35 mm culture dishes and allowed to grow overnight (37°C/5% CO_2). The growth media were removed and the cells washed twice in serum-free medium before being treated with peroxides for 30 min (37°C) in serum-free medium. After this time, 20% FCS was added to the dishes, the cells were washed twice in ice cold PBS prior to processing in the ASCGE assay. Data represent the mean ± S.E.M. of 3•4 independent experiments.

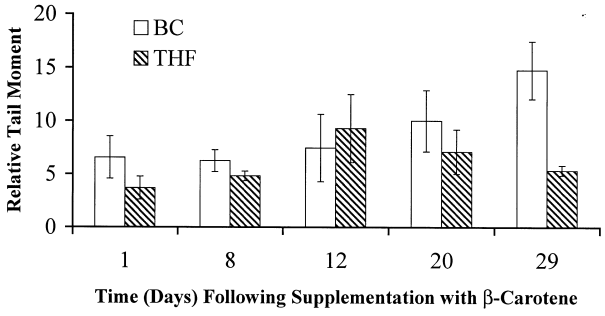

Fig. 2. The effect of continuous supplementation with 10 μM β-carotene on DNA single strand breaks induced by a 30 min tratment with H_2O_2. Cellls were seeded into T25 flasks and grown in the presence or absence of 10 μM β-carotene. The growth media were changed every second day. At the indicated time points, samples were removed and seeded into 35 mm culture dishes (2×10^4 cells/cm^2) for treatment with 50 μM H_2O_2. DNA strand breaks were measured using the ASCGE assay. Data represent the mean ± S.E.M. of three independent experiments.

3.2. Uptake of β-carotene into HepG2 cells

β-Carotene was added to the culture media (5 ml) in a small volume of THF (10 μl) to give a final concentration of 10 μM (5.36 μg/ml). This resulted in an actual concentration of 164.4 ng of β-carotene/ml cell culture media and 41.2 ng of β-carotene/2×10^6 cells.

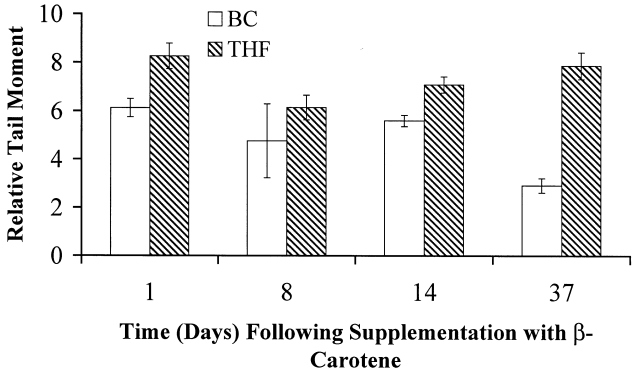

Fig. 3. The effect of continuous supplementation with 10 μM β-carotene on DNA single strand breaks induced by a 30 min treatment with tBOOH. Cells were seeded into T25 flasks and grown in the presence or absence of 10 μM β-carotene. The growth media were changed every second day. At the indicated time points, samples were removed and seeded into 35 mm culture dishes (2×10^4 cells/cm^2) for treatment with 10 μM tBOOH. DNA strand breaks were measured using the ASCGE assay. Data represent the mean ± S.E.M. of three independent experiments.

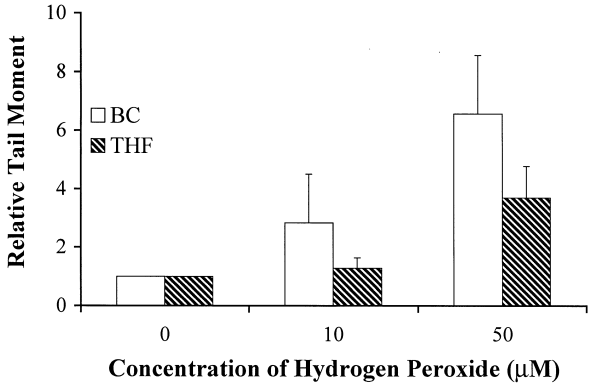

Fig. 4. The effect of a 24 h treatment with 10 μM β-carotene on DNA single-strand breaks induced by a 30 min treatment with either 10 μM or 50 μM H_2O_2. Human hepatoma HepG2 cells were seeded at a density of approximately 2×10^4 cells/cm^2 in 35 mm culture dishes and allowed to grow for 24 h in the presence or absence of 10μM β-carotene (37°C/5% CO_2). The growth media were removed, and the cells washed twice in serum-free medium before being treated with H_2O_2 for 30 min (37°C) in serum-free medium. After this time, 20% FCS was added to the dishes, the cells were washed twice in ice cold PBS prior to processing in the ASCGE assay. Data represent the mean ± S.E.M. of three. Human hepatoma HepG2 cells were seeded at a density of approximately 2×10^4 cells/cm^2 in 35 mm culture dishes and allowed to grow overnight (37°C/5% CO_2). The growth media were removed and the cells washed twice in serum-free medium before being treated with peroxides for 30 min (37°C) in serum-free medium. After this time, 20% FCS was added to the dishes, the cells were washed twice in ice cold PBS prior to processing in the ASCGE assay. Data represent the mean ± S.E.M. of 3–4 independent experiments.

3.3. *The effect of pre-treatment with β-carotene on the survival of HepG2 cells treated with peroxides*

Although the viability of HepG2 cells did not decrease following a 30 min challenge with tBOOH, when the cells were subsequently allowed to recover for 48 h in peroxide-free media, their survival was dramatically reduced (IC_{50} value: 46.5 ± 1.1 μM).

Moreover, under these conditions, tBOOH proved to be more toxic than H_2O_2, which did not generate an IC_{50} value over the concentration range of H_2O_2 used. When the HepG2 cells were incubated with 10 μM β-carotene for 24 h prior to peroxide challenge, the cytotoxic effects of tBOOH were decreased by 2.5-fold over the 48 h recovery period (IC_{50} value: 117.3 ± 25.8 μM). In contrast, the toxicity of H_2O_2 was increased (IC_{50} value: 227.5 ± 2.5 μM).

3.4. *The effect of continuous supplementation with β-carotene on peroxide-induced DNA strand breaks in HepG2 cells*

Cells were incubated with either 10 μM β-carotene or THF alone and samples were removed at different time intervals for treatment with 50 μM H_2O_2 and processing in the ASCGE assay. Addition of THF to the culture medium over the 29 day supplementation period did not appear to have any detrimental effects as the relative tail moment for THF-treated HepG2 cells following a 30 min treatment with 50 μM H_2O_2 averaged 6.05 (± 0.98), which was in agreement with the mean value obtained from the dose response curves (5.08 ± 0.09). In the presence of β-carotene, the extent of H_2O_2-induced DNA strand breaks was significantly increased compared to the solvent-treated control after 20 days supplementation ($P < 0.05$, Fig. 2). The cell viability was measured by FDA/EtBr and did not fall below 85%. However, when the experiment was repeated using 10 μM tBOOH instead of H_2O_2 as the genotoxin, supplementation with β-carotene resulted in a significant decrease in DNA strand breaks (Fig. 3).

Differences between the levels of damage seen in HepG2 cells challenged with peroxide between control and supplemented cells were evident after only 24 h treatment (Fig. 2 and Fig. 3). Optimal protection against tBOOH-induced DNA damage was seen after a prolonged exposure to β-carotene (37 days, Fig. 3). However, the deleterious effect of β-carotene supplementation in cells challenged with H_2O_2 was the most pronounced after 29 days (Fig. 2). The effect was the most pronounced when higher (50 μM) concentrations of H_2O_2 were used, but increased DNA damage was also seen in the β-carotene-treated cells following treatment with lower (10 μM) doses of H_2O_2 (Fig. 4).

4. Discussion

The present study has demonstrated that pre-treatment of HepG2 cells with 10 μM β-carotene protected against tBOOH-induced strand breaks by up to 60% after 37 days treatment (Fig. 3). Previous studies by other workers have also shown that carotenoids such as β-carotene are effective in protecting cells from tBOOH-induced oxidative damage [15,26]. In contrast, under the same experimental conditions, the presence of β-carotene increased the susceptibility of HepG2 cells to the DNA damaging effects of H_2O_2 when compared to either untreated or solvent control cells. Moreover, pre-treatment with β-carotene enhanced the H_2O_2-induced cytotoxicity as measured by the MTT assay, whereas the tBOOH-induced cytotoxicity was decreased 2.5-fold under the same experimental conditions.

It has been reported that human lymphocytes did not acquire resistance to oxidative damage induced by H_2O_2 following consumption of vegetables [10]. Moreover, it has also been shown that whereas β-carotene treatment decreased the number of sister chromatid exchanges induced by H_2O_2 in Chinese hamster ovary cells, the number of H_2O_2-induced chromosome aberrations was significantly increased [27].

The mechanisms by which β-carotene could enhance the H_2O_2-induced cellular damage but not tBOOH-induced damage in vitro remain unclear, however possibilities have been put forward. A role for β-carotene as a pro-oxidant has already been proposed [28]. Another group showed that treatment of cells in culture with high concentrations (10 μM) of β-carotene results in an enhancement of the pro-oxidant effect of paraquat toxicity due to induction of catalase and superoxide dismutase and depletion of glutathione peroxidase [29].

The failure of β-carotene and lycopene to protect human cells against the DNA damaging effects of H_2O_2 have recently been demonstrated for HT29 cells [30]. Lowe and colleagues [30] supplemented cells with 1–10 μM carotenoid for only 30 min prior to challenging the cells with xanthine/xanthine oxidase and observed that β-carotene and lycopene afforded protection only at low concentrations (2–3 μM being optimal). At higher concentrations (4–10 μM), the ability of carotenoids to protect against the damaging effects of H_2O_2 was rapidly lost.

β-Carotene could be expected to quench peroxyl radicals produced by t-BOOH [31]. However, the most likely mechanism for an observed increase in DNA damage in vitro in the

presence of H_2O_2 is the production of a carotene secondary radical following the interaction of β-carotene and OH^b (produced by a Fenton type reaction) [28]. The data presented here suggest that such a combination may be genotoxic to cells in culture. It is clear that dietary carotenoids such as β-carotene (and indeed lycopene [30]) may at best fail to protect against DNA damage and may in fact aggravate the damaging effects of H_2O_2 in human cells.

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