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DEVELOPMENT OF A CELLULAR MODEL TO EVALUATE THE HYPOTHESIS THAT DIETARY CAROTENOIDS ARE ANTIOXIDANTS

by

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Numerous epidemiologic studies support an inverse association between consumption of carotenoid-rich fruits and vegetables and the incidence of some degenerative diseases in humans. One proposed mechanism centers on the role of carotenoids as antioxidants independent of their role as retinoid precursors. Antioxidants prevent the formation and quench the propagation of free radicals, the species that are generally considered as the etiologic basis for many diseases. The HepG2 liver and Caco-2 intestinal human cell lines were selected as potential models for investigating the role of carotenoids as antioxidants. Caco-2 cells spontaneously differentiate into polarized, enterocyte-like cells. HepG2 cells exhibit many of the activities of normal human liver parenchymal cells and secrete lipoproteins and numerous plasma proteins. Initially, the susceptibility of HepG2 and Caco-2 cells to several commonly used free radical generating pro-oxidants was examined. Both structural and functional parameters, e.g., plasma membrane permeability and amino acid and glucose transport, were adversely altered in HepG2 cells exposed to the pro-oxidants. In contrast, Caco-2 cells were relatively resistant to the damaging effects of the pro-oxidants. These data supported the usefulness of the HepG2 human cell line as an appropriate model for investigation of potential cytoprotection conferred by carotenoids against free radical-mediated damage.

Next, I examined the ability of HepG2 cells to accumulate β-carotene and lutein, carotenoids with and without pro-vitamin A activity, respectively, from micellar preparations after overnight incubation and the potential role of the carotenoids as cellular antioxidants. Cells were also incubated with α-tocopherol as a positve control, since the antioxidant properties of this compound are well established. HepG2 readily accumulated both carotenoids and α-tocopherol. Control and treated cells were exposed to a pro-oxidant stressor and the impact on several parameters of cellular function and plasma membrane integrity were evaluated. β-Carotene and

lutein, like α-tocopherol, markedly attenuated the adverse impact of pro-oxidant exposure on nutrient transport and cellular integrity. Protein synthesis, glucose oxidation and mitochondrial activity were not impaired by the pro-oxidant, suggesting that the plasma membrane was the site of damage.

Research using cell culture systems has been problematic due to the considerable hydrophobicity of carotenoids in aqueous systems and the difficulty of presenting carotenoids in a bioavailable form. These compounds are usually introduced into tissue culture medium either in organic solvents or as micelles, whereas carotenoids are localized in lipoproteins in plasma. Thus, we speculated that carotenoid-enriched human lipoproteins would be an effective vehicle for delivery of β-carotene and α-tocopherol to HepG2 cells. Volunteers ingested either β-carotene supplements (60 mg/day) or placebo for 4 weeks to enrich plasma lipoproteins with β-carotene. The concentration of β-carotene in plasma of supplemented subjects was significantly increased ($p < 0.05$) by greater than 11-fold after 4 weeks. After isolating total lipoproteins, the stability of β-carotene and α-tocopherol in micelles and human lipoproteins was compared. Micellar β-carotene, but not lutein and α-tocopherol, was markedly oxidized under cell-free culture conditions. In contrast, β-carotene was relatively stable in cell-free medium containing total lipoproteins isolated from β-carotene supplemented individuals. Cellular acccumulation of β-carotene and α-tocopherol was proportional to the concentrations of these lipophilic compounds in total lipoproteins, low density lipoproteins, or high density lipoproteins. These and other data show that lipoproteins represent a stable, physiologically relevant vehicle for delivery of β-carotene and α-tocopherol to HepG2 human liver cells.

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DEDICATION

I would like to dedicate this work to my beloved family. I express my deepest gratitude and sincere appreciation to my family for their unending love and support. Their patience, tolerance and understanding of the importance of education in my life have strengthened my educational experience and career pursuits.

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CHAPTER I

INTRODUCTION

OVERVIEW

Since their initial discovery in the mid-19th century, hundreds of carotenoids have been identified and characterized. All carotenoids are variants of the parent $C_{40}H_{56}$ compound. Carotenoids are synthesized by all plants and photosynthetic microorganisms, but not by mammals (Pfander, 1992). Humans ingest several milligrams of carotenoids daily by eating fruits and vegetables (Lachance, 1988). Because approximately 10% of the known carotenoids, and particularly β-carotene, possess provitamin A activity, consumption of carotenoid-enriched foods is of particular interest in developing countries as a means of alleviating vitamin A deficiency (Solomons and Bulux, 1993).

One focus of recent inquiry concerns the potential antioxidant activity of carotenoids. These pigments are involved in light harvesting reactions and quench singlet oxygen-induced damage to sensitive plant organelles. Because free radical-mediated damage to mammalian tissues is believed to be the etiologic basis for the aging process and for degenerative diseases such as arthritis, cataracts, some forms of cancer and coronary heart disease (Canfield et al., 1992; Thumham, 1994), it has been proposed that dietary carotenoids also may function as antioxidants in mammalian tissues. This possibility is supported by numerous epidemiologic studies that have demonstrated an inverse correlation between the intake of fruits and vegetables and the incidence of diseases such as cancer (van Poppel and Goldbohn, 1995).

Possible mechanisms by which carotenoids may protect tissues against oxidative damage include their inherent antioxidant activity, interactions with other antioxidants, conversion to retinoids or apocarotenoids, and modulation of enzyme activities that are part of the host antioxidant system (Canfield et al., 1992). Additionally, various studies have supported the roles for carotenoids as: a) blockers of neoplastic transformation (Bertram and Bortkiewicz, 1995), b) inducers of remission of oral leukoplakia (Garewal, 1995), c) quenchers of free radicals such as singlet oxygen (Sies and Stahl, 1995), and d) modulators of immune activity (Meydani et al., 1995).

In order for dietary carotenoids to protect cells and tissues against oxidative stress, foods containing these compounds must first be digested to release the carotenoids for their incorporation into lipid micelles in the small intestine. Intestinal mucosal cells then transfer the intact carotenoid or its metabolic products to lymphatic and/or portal circulation for delivery, utilization and storage in peripheral tisues (Erdman, 1988). Carotenoids are either absorbed intact or cleaved to retinoids or apocarotenals and packaged into chylomicrons for transport via lymph. Peripheral tissues such as adipose and the adrenal glands accumulate relatively high levels of these hydrophobic compounds. The liver also accumulates carotenoids via the uptake of chylomicron remnants. Specific characteristics and regulation of many of these processes of carotenoid transport and metabolism remain largely unknown, primarily because of the lack of appropriate animal models until recently. Although there is a plethora of literature using laboratory rodents, these animals do not absorb carotenoids as efficiently as humans (Erdman, 1988). The ferret, preruminant calf and Mongolian gerbil are now recognized as excellent animal models for the study of carotenoid absorption and metabolism. Unfortunately, the availability and/or special maintenance requirements for these animals limits their use.

The development of alternative models for investigating carotenoid function and metabolism in humans is necessary. Human cell lines would appear to represent potential models. The availability of highly differentiated human cell lines with phenotypic properties similar to enterocytes and hepatocytes appear to be particularly relevant. The intestine is constantly exposed to ingested xenobiotics, transition metals and oxidants such as lipid hydroperoxides that are present in the typical diet and may cause free radical-mediated tissue damage. Similarly, the liver is an active site of xenobiotic metabolism that generates high levels of free radical compounds. Finally, both the intestine and the liver also have important roles in the transport and metabolism of dietary carotenoids.

STRUCTURE OF CAROTENOIDS

Over 700 distinct carotenoids have been identified and characterized as either hydrocarbon carotenes or oxygen-containing xanthophylls (Khachik, 1995). Approximately 10% of the carotenoids are carotenes and the remaining 90% are xanthophylls. The diversity of the carotenoids centers on the potential for numerous structural modifications and stereochemical variations of the parent compound lycopene. Lycopene, a red pigment found in tomato products, possesses eight isoprenoid units and a series of 13 conjugated double bonds. Cyclization, hydrogenation, dehydrogenation, bond migration and spatial rearrangement are responsible for the diverse family of carotenoids (Fig. 1.1). Olson (1995) has estimated that there may be more than 220,000 different carotenoids in nature. Common structural features within this family of compounds include a 40 carbon backbone with eight isoprenoid units forming an extensive conjugated diene system, molecular symmetry, and, typically, a cyclic β-ionone ring system at either end of the molecule. Derivatives in which the carbon skeleton has been shortened (<8 isoprenoid units) or elongated (>8 isoprenoid units) are named apocarotenals and

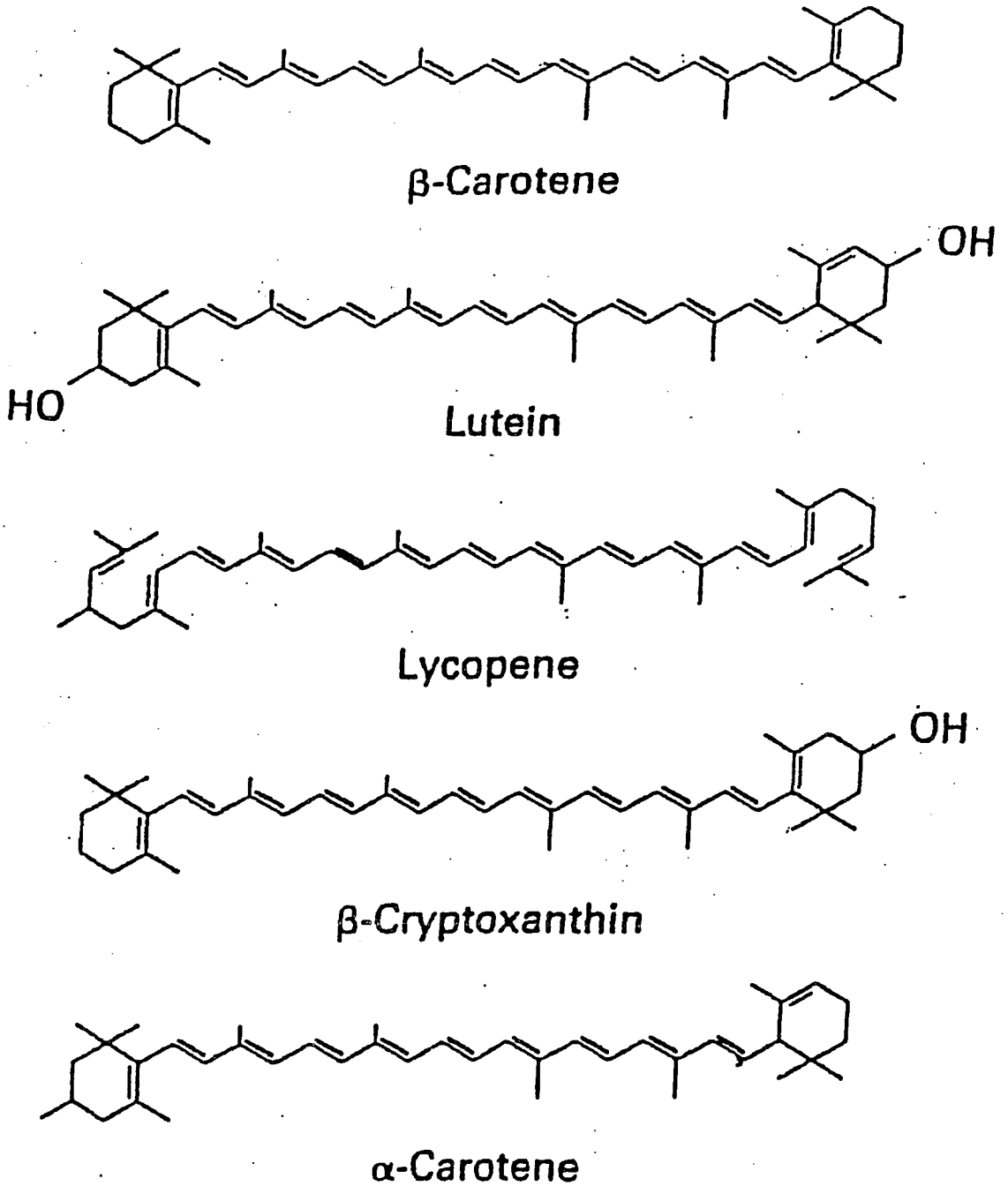

Fig. 1.1. *Structures of five carotenoids found in human plasma*

homocarotenals, respectively. Cleavage of the central 15,15' bond produces two molecules of retinal, or vitamin A, in compounds with unsubstituted rings. Cleavage of oxycarotenoids containing substituted ring systems does not generate vitamin A in mammals.

AVAILABILITY, ABSORPTION AND TISSUE DISTRIBUTION OF CAROTENOIDS

Carotenoids are synthesized by plants and photosynthetic microorganisms from acetyl-coenzyme A by a series of well defined condensation reactions (Olson, 1989). Annual production of carotenoids is estimated at 100 million tons arising predominantly from algae in the oceans and plants on land (Daun, 1988). Carotenoids are present in vegetables, vegetable oils and animal products where they impart the readily observable yellow, orange and red colors. While plants typically contain a variety of carotenoids, specific carotenoids may predominate in a food item, e.g. β-carotene in carrots, lycopene in tomatoes, and lutein in kale. Green leafy vegetables contain 80-90% xanthophylls and 10-20% β-carotene, whereas yellow and orange fruits and vegetables are poor in oxycarotenoids but rich in the hydrocarbon carotenes. In green plants, the pigment chlorophyll typically obscures the presence of the colorful carotenoids. Although carotenoids naturally occur in the food supply, only three carotenoids have been approved as food additives by the Food and Drug Administration, viz., β-carotene, canthaxanthin and β-apo-8'-carotenal.

The types and amounts of carotenoids in foods are affected by many factors, including variety of crop, cultivation methods, and season and stage of maturity at harvest (Gerster, 1993). The potential for variation of carotenoid composition in foods has been one of many considerations in attempts to accurately estimate the intake of carotenoids by individuals in the U.S. Technological advances in instrumentation and development of improved analytical

procedures have resulted in the availability of more accurate and complete food composition tables (Chug et al., 1993).

Approximately 100 carotenoids are present in the typical U.S. diet (Chug et al., 1993). Current estimates indicate a daily consumption of carotenoids in the U.S. of 1.5-2.0 mg. The Food and Nutrition Board of the National Academy of Sciences has concluded that approximately one-third of ingested β-carotene is absorbed and about one-half of this is converted to retinoids, resulting in 15-20% efficiency of utilization. Although β-carotene is a source of vitamin A, there is no RDA for β-carotene or other carotenoids at this time. Review of suggested dietary practices by the National Cancer Institute and the United States Department of Agriculture, as well as epidemiological and food disappearance surveys, have defined a range of optimal intake that is associated with reductions of many disease processes. The disparity between actual consumption patterns and the suggested intake of 5.2-6.0 mg/day have revealed a "carotene gap." Unfortunately, only 9% of the U.S. population consumes the recommended 3-5 servings of fruits and vegetables suggested in the food guide pyramid (Frei, 1994). Moreover, the most commonly consumed fruit or vegetable is the potato, a poor source of carotenoids (Lachance, 1988).

While fruits and vegetables offer a plethora of potentially beneficial phytochemicals, β-carotene supplementation has become common due to its increased availability and apparent lack of toxicity (Diplock, 1995). This practice has also been promoted by recent data indicating that consumption of increased amounts of plants by vitamin A deficient individuals is less effective at improving retinoid status than oral supplementation with β-carotene (de Pee et al., 1995). Supplementation of $\leq$180 mg/day daily for years does not appear to produce detrimental side effects in humans, although nontoxic yellowing of the skin may occur at intakes >30

mg/day. Treatment of patients with erythropoietic protoporphyria (EPP) who have ingested up to 300 mg β-carotene/day for long periods of time has resulted in few side effects, e.g. carotenodermia and mild occassional gastrointestinal distress. Other carotenoids have not been investigated in detail. However, it is assumed that they will be as safe as β-carotene because of their structural similarities (Krinsky, 1988).

Humans appear to absorb both carotenes and xanthophylls in a relatively non-specific fashion. Ingested carotenoids are emulsified into amphipathic micelles in the lumen of the small intestine by their interaction with pancreatic and biliary secretions (Erdman, 1988). Approximately 10-50% of ingested carotenoid may be taken up by mucosal cells. However, a number of factors can influence absorption and utilization of carotenoids. These include the amounts of dietary carotenoids and fat, the types of dietary fatty acids, digestability of food, presence of other food components including antioxidants and fiber, vitamin A, protein and zinc status, disease states, use of therapeutic drugs, hormone status and weight changes (Erdman, 1988). After uptake by the enterocyte, carotenoids may be cleaved centrally or excentrically by the putative 15, 15'-dioxygenase (EC 1.13.11.21) to form chain shortened apocarotenals or polar retinoids, or remain intact and combine with phospholipids, triglycerides, cholesterol and its esters to form chylomicrons. After movement across the basolateral surface of the enterocyte, chylomicrons are transported via the lymphatic system to the peripheral circulation. The endothelium-anchored enzyme lipoprotein lipase cleaves triglycerides and the released fatty acids and possibly carotenoids are transferred from the lipid particle to the peripheral tissues. The resultant chylomicron remnant is taken up by the liver by receptor-mediated endocytosis. In this tissue, the newly acquired carotenoid may be cleaved to retinoids or other metabolites, stored intact, or incorporated into other lipoproteins, viz., very low density (VLDL), low density (LDL) or high density lipoproteins (HDL), for distribution to extrahepatic tissues.

Plasma carotenoids are distributed among the various classes of lipoproteins based on relative polarity of the individual carotenoid. Hydrophobic carotenes (e.g. β-carotene) are located primarily in LDL (~70%) with lesser amounts in HDL (~20%) and VLDL (~10%). In contrast, polar xanthophylls are distributed more evenly among lipoprotein classes with ~45% present in LDL and HDL with the balance in VLDL. Carotenoids concentrate in those tissues expressing large numbers of LDL receptors (Gerster, 1993). Recent studies have shown that carotenoids, viz., β-carotene, may be transferred or exchanged between groups of lipoproteins in circulation (Wang, 1994).

Human plasma contains about 1% of the total body pool of carotenoids (100-150 mg) and contains a complex mixture of structurally diverse carotenoids that generally reflect dietary intake over recent weeks or months (Packer, 1989). Approximately 21 carotenoids have been identified in human plasma with six comprising >90% of the total amount present. These include β-carotene (~22%), lutein (~20%), lycopene (22-35%) , β-cryptoxanthin (~10%) and smaller amounts of zeaxanthin and α-carotene. Typical plasma levels are 200-400 μg/dL and are highly dependent on diet. Factors such as destruction in the gastrointestinal tract, efficiency of absorption and metabolism, and rate of tissue uptake may affect plasma levels of carotenoids (Gerster, 1993).

Humans can absorb small, but significant, amounts of non-modified carotenoids and accumulate very high concentrations of the carotenoids in peripheral tissues without toxic effects (Wang et al., 1992). Levels and profiles of carotenoids in tissues likely reflect the pattern of consumption of fruits and vegetables over many years (Packer, 1989). The whole body content of carotenoids has been estimated at 140 mg with about 84% present in adipose tissue and 10%

in liver. In fact, some 30 different carotenoids have been identified in adipose tissue in humans and the levels can vary by as much as a 40-fold between individuals. Although the accumulation of carotenoids in tissues is generally nonspecific, several examples of tissue selectivity are known. The macular pigment and the retina primarily contain zeaxanthin and lutein, respectively (Thurnham, 1994). In contrast, lycopene represents 60-80% of the carotenoids present in the testes and adrenal glands (Gerster, 1993). The biochemical basis for the observed specificity of carotenoid accumulation by some tissues is unknown.

The metabolic fate of ingested carotenoids in human organisms remains virtually unknown, except for the conversion of provitamin A carotenoids to vitamin A metabolites (i.e., retinol and retinyl ester). Xanthophylls such as lutein have no detectable provitamin A activity (Gerster, 1993). Biotransformation of β-carotene to apocarotenals and retinoids has been recently demonstrated in human intestinal homogenates (Wang, 1991). Animals such as fish may convert carotenoids that are generally assumed to lack provitamin A actvity in humans to retinoids.

PROPOSED FUNCTIONS OF CAROTENOIDS

a. Provitamin A activity of carotenoids

The classic pathologic consequence of vitamin A deficiency, viz., blindness, has been recognized for millenia. Evidence collected over the past 20 years has shown that subclinical deficiency of vitamin A adversely affects normal growth, resistance to infection, and maintenance of ocular integrity, particularly in children in developing countries (Sommer, 1989). Provitamin A carotenoids such as β-carotene are oxidatively cleaved to form vitamin A and

shorter apocarotenals (12', 10', 8') by the partially characterized enzyme 15,15'-dioxygenase in intestinal cells and the liver (Gerster, 1993). There is no other apparent metabolic route to vitamin A in animals.

In vivo studies have shown that conversion of β-carotene to vitamin A via excentric cleavage is only 40-60% as efficient as central cleavage. Wang (1994) has reported cleavage of β-carotene and β-apocarotenals to vitamin A in intestinal homogenates from several species including humans. Additionally, the dioxygenase activity appeared to be inversely related to the levels of carotenoids in the diet in several animal studies. Animal studies have also shown that β-carotene is capable of rapidly compensating localized vitamin A deficiency induced by carcinogens by promoting the expression of genes controlling cell differentiation (Van Poppel, 1993).

b. Immunomodulatory activity of carotenoids

Characteristics of the immune system include adaptation, specificity, and long-term memory to protect the host against viruses, pathogenic microorganisms and cancer cells. The immune response often centers on the cell-mediated and humoral branches that are composed of two populations of morphologically indistinguishable lymphoid cells, viz., the B and T lymphocytes, respectively. In addition to lymphocytes, the immune system contains accessory cells such as plasma cells, monocytes, basophils, polymorphonuclear leukocytes, dendritic reticular cells, and large granular lymphocytes (natural killer cells).

The cellular redox status can affect the integrity and function of membrane lipids, cellular proteins, and nucleic acids, signal transduction processes and gene expression in

immune cells (Meydani et al., 1995). The role of carotenoids in the enhancement of the immune system has been investigated using several models. For example, supplementation studies with humans have revealed that retinol and β-carotene increase the number and activities of natural killer cells and the percentages of cells expressing surface activation markers such as transferrin and interleukin-2 receptors. Supplementation with β-carotene has also been shown to increase the percentage of T helper cells ($CD4^+$) without adversely affecting the population of T cytotoxic cells ($CD8^+$).

In several animal studies, the carotenoids β-carotene and canthaxanthin have been shown to enhance mitogenic reactivity of T and B lymphocytes. β-Carotene supplementation of hamsters with chemically-induced tumors markedly increased the numbers of cytotoxic T cells. Krinsky et al. (1991) reported that supplementation with β-carotene slowed the growth and induced the regression of virus-induced tumors implanted in mice. Supplementation with β-carotene also increased immunoresponsiveness upon subsequent rechallenge with tumors. β-Carotene has been shown to protect murine thymus, lymphocytes and macrophages against stress-, radiation- and oxidant-induced damage, respectively (Thurnham, 1994). Numerous in vitro and in vivo studies have shown that β-carotene protects phagocytic cells from autooxidative damage, stimulates effector T cell function, enhances the bactericidal capacity of macrophages, cytotoxic T cells and natural killer cells, and increases the production of interleukins such as tumor necrosis factor. The ability of β-carotene and carotenoids without provitamin A activity to modulate immune responses provides evidence that this activity is not necessarily dependent on the production of retinoids (Bendich, 1988).

c. Antioxidant activity of carotenoids

Numerous studies have utilized both pro- and nonprovitamin A carotenoids to investigate the antioxidant function of carotenoids and the relative importance and dependence on provitamin A activity for this action. The uniqueness of the carotenoids has centered on their diversified roles in light energy absorption, oxygen transport, protection against photosensitized oxidation, provitamin A activity, use as food colorants, quenchers of singlet oxygen, regulators of plant growth, and more recently as inhibitors of chemical carcinogenesis (Daun, 1988). Recognition that the antioxidant capacity of β-carotene was linked to the length of the polyene chain led to the demonstration that non-β-carotene compounds possessing at least 7-9 conjugated double bonds were useful as quenchers of singlet oxygen and free radical traps. The literature contains numerous studies that have considered the putative antioxidant activity of the carotenoids. Selected examples from human, animal and cellular studies are discussed below.

Human studies. Overall, evidence from retrospective studies in which estimated food intake of patients with cancer prior to or at the time of diagnosis was compared with estimates of intake of matched subjects strongly suggests that consumption of dark leafy or dark yellow-orange vegetables decrease the risk of some cancers, e.g., lung cancer (van Poppel and Goldbohn, 1995). Review of prospective dietary studies have supported the contention that the increased risk of some cancers, and particularly of the lung, decreases markedly with increased intake of fruits and vegetables. Most epidemiologic studies concerned the requirement for conversion of carotenoids to retinoids for anticarcinogenic action. Despite its important role in normal cell differentiation, increased intake of preformed vitamin A has not been associated with significant decreases in the risk of some cancers.

Individuals who smoke or chew tobacco products exhibit increased formation of micronucleated cells, which is indicative of chromosomal damage due to the nitrosoamines and other carcinogens in tobacco (Garewal, 1995). The presence of micronuclei and nuclei with changed textures precedes development of oral leukoplakia. β-Carotene (180 mg/week) and vitamin A supplementation significantly reduced the frequency of micronuclei in the oral mucosa of betel quid chewers and snuff dippers. β-Carotene supplementation also induced regression of new leukoplakia, even in subjects who continued to use tobacco products. Dysplasia of the uterine cervix is another marker of preneoplastic lesions (Meyskens and Manetta, 1995). In a group of females attending a family planning clinic, those with cervical dysplasia had significantly lower plasma levels of β-carotene than those exhibiting normal cytology of the cervix. The disease-free interval was prolonged after supplementation with 24 mg β-carotene and 36 mg canthaxanthin daily, for several months in patients after surgery for cancer of the breast, lung, urinary bladder or head and neck. In another study, individuals consuming a variety of fruits and vegetables [viz., β-carotene-rich carrots, mangos, papaya, lycopene-rich tomatoes, and lutein-rich dark green leafy vegetables] exhibited inverse relationships between intake of β-carotene and the occurrence of lung cancer (van Poppel and Goldbohm, 1995). There also was a strong impact of consumption of lycopene and lutein enriched vegetables against the incidence of cancer. The greatest protective effect was observed for individuals consuming combinations of all vegetables indicated above. Several other studies have shown that increased intake of fruits and vegetables is associated with elevated blood levels of carotenoids and the decreased occurrence of some cancers (Gerster, 1993).

The role of carotenoids in protecting delicate plant organelles against potentially damaging photosensitized molecules is widely accepted. Matthews-Roth (1982) proposed that since carotenoid pigments could protect algae and bacteria from the harmful effects of light,

these pigments might also protect human tissues from similar oxidative stress. This led to the widespread and therapeutic use of high doses of β-carotene combined with canthaxanthin for patients with the genetic photosensitivity disease erthyropoietic protoporphyria. The successful outcome of therapeutic use of carotenoids has prompted further studies in animals.

Age related macular degeneration (AMD) is a degeneration of the retina and the retinal pigment epithelium in the macula region of the mammalian eye. It is the leading cause of blindness in the U.S. and no treatment is currently available (Snodderly, 1995). An extensive body of literature from experiments with primates, rodents and other vertebrates shows that the retina is damaged by visible light. One mechanism that has been proposed is the peroxidation of polyunsaturated membrane lipids in the retina. Epidemiologic data indicate that individuals with low plasma concentrations of carotenoids and antioxidant vitamins are at increased risk for AMD. Lutein and zeaxanthin are selectively accumulated by the retina and increasing evidence suggests that these carotenoids may help retard some of the destructive processes associated with AMD (Snodderly, 1995).

The requirement of the mammalian eye for oxygen and light and the potential for uncontrolled production of reactive oxygen species appears to be related to the development of cataracts. In fact, depletion of antioxidants including the carotenoids compromises the function of the lens (Taylor et al., 1995). In human studies, individuals with high plasma levels of total carotenoids had <20% the prevalence of cataracts compared with persons with relatively lower total plasma carotenoid levels. Additional studies report decreased incidences of cataracts and associated problems in individuals with relatively higher plasma levels of carotenoids.

Numerous epidemiologic studies have supported the protective role of carotenoids in delaying and preventing the development of cardiovascular disease (Manson et al., 1993). Epidemiologic research has included ecologic examination, case-control studies, cross sectional and cohort studies for elucidating the inverse relationship between increased dietary consumption and plasma levels of carotenoids and mortality due to cardio- and cerebro-vascular disease (Kohlmeier and Hastings, 1995). Participants who had previously had a cardiovascular event (stable angina or coronary revascularization) and were supplemented with β-carotene (50 mg every other day) experienced almost 50% fewer subsequent problems than those receiving placebo only (Thurham, 1994). Initiation of cardiovascular disease is highly correlated with the oxidation, uptake and accumulation of plasma lipoproteins by immune cells. Oxidized LDL is avidly scavenged by macrophages and subsequently converted to foam cells in the arterial intima where they form fatty streaks and later atherosclerotic plaques. In vitro susceptibility of LDL to oxidation has been shown to correlate with clinical severity in persons recently having a heart attack. Two studies (viz., Jialal and Grundy, 1991; Lavy et al., 1992) reported increased resistance of LDL to oxidation in the presence of β-carotene. Similarly, lutein markedly extended the lag phase of Cu-stimulated LDL oxidation. Studies with adult smokers and cystic fibrosis patients have provided conflicting evidence that β-carotene supplementation increased LDL resistance to oxidative stress (Thurnham, 1994). LDL from smokers supplementing with β-carotene was not protected against oxidative stress. In contrast, the oxidation of LDL in cystic fibrosis patients supplementing with β-carotene was significantly reduced when compared to control subjects. Five prospective case-controlled studies reported that decreased plasma β-carotene levels were associated with increased risk of lung cancer. The majority of epidemiologic evidence supports the protective effect of β-carotene, carotenoids as a group, and carotenoid-rich foods against the development of cardiovascular disease (Kohlmeier and Hastings, 1995).

Animal studies. Extrapolation of animal findings to humans is limited because the uptake and metabolism of carotenoids is species-specific and the development of cancer in animals does not closely mimic carcinogenesis in humans (Gerster, 1993). In numerous studies utilizing rodents as models, viz., hamsters, mice and rats, supplementation with β-carotene and/or canthaxanthin significantly reduced chemical- and radiation-induced tumorigenesis and carcinogenesis (Krinsky, 1991). The marked protection against carcinogenesis after supplementation with β-carotene and canthaxanthin, carotenoids with and without provitamin A activity, respectively, suggest a mechanism independent of conversion to retinoids. Carcinogenesis of epithelial origin, which accounts for >95% of cancers in man, were induced in several animal models at sites other than skin or mucosa including salivary glands, respiratory tract, colon, stomach and breast (Gerster, 1993). In studies using site-specific carcinogens, supplementation with carotenoids significantly reduced initiation and progression of chemically-induced tumors. Collectively, numerous animal studies have provided compelling evidence for the role of carotenoids as anticarcinogenic compounds.

In vitro studies. The discovery that carotenoids quench potentially damaging singlet oxygen was an important advance in the understanding of the effectiveness of carotenoid pigments in preventing damage in photobiological systems. Many in vitro studies using cell-free models including artificial membranes have been aimed at demonstrating the antioxidant function of carotenoids against free radical-mediated damage initiated by light, chemicals and radiation (Krinsky, 1989). Furthermore, investigators have studied the autoxidation and coupled oxidations of carotenoids, radical-induced oxidations, the effects of various enzymes such as lipoxidases and peroxidases on carotenoids, and the kinetics of radical-carotenoid interactions. Collectively, results from numerous in vitro studies support the function of carotenoids as quenchers of chemically-induced free radicals in cell-free systems.

Cellular studies. Cell culture and other in vitro models offer useful tools to study the molecular mechanisms involving carcinogenesis and to identify factors as potential anticancer agents even though they may represent an oversimplification of the in vivo environment (Gerster, 1993). Initial findings supporting the role of carotenoids as antioxidants focused on inhibition of carotenogenesis in pigmented yeast and subsequent exposure to oxidative stress. Inclusion of β-carotene in medium abolished the impact of oxidant-induced cytotoxicity on yeast growth (Krinsky, 1989). In several bacterial systems, incubation with carotenoids with and without provitamin A activity inhibited carcinogen- and radiation-induced mutagenesis. Extension of studies to mammalian cell culture have shown that carotenoids, viz., carotenes and xanthophylls, inhibit adverse pro-oxidant-induced biochemical and functional changes in cell models including polymorphonuclear leukocytes, fibroblasts obtained from mouse embryo and chicken, and Chinese hamster ovary cells (Krinsky, 1991; Lawlor & O'Brien, 1995). Results from studies with several human cell models have supported the putative antioxidant activity of carotenoids against singlet oxygen-induced oxidative stress and chemically-induced neoplastic transformation (Bertram and Bortkiewicz, 1995). In vitro human models have been limited to cells of neural, lymphoid and keratinocyte origin.

The similarity between retinoids and carotenoids in stabilizing carcinogen-initiated cells against subsequent transformation has lead to an interesting proposal (Betram and Bortkiewicz, 1995). Gap junctions of cell membranes serve as channels for cell to cell communication and growth regulation. Thus, upregulation of junctional communication increases formation of channels and enhances intercellular communication. The decreased proliferation of carcinogen-initiated cells inhibits progression to a neoplastic phenotype. β-Carotene and canthaxanthin were as equipotent as retinoids at elevating junctional communication, thereby inhibiting progression

of initiated cells. Upregulation of gap junctional communication parallels increased formation of connexin 43, a gene coding for structural units of the gap junction. Carotenoids have been shown to inhibit plasma membrane oxidation and induce upregulation of connexin 43 expression.

Human cell lines as models for evaluating carotenoid antioxidant activity

Caco-2 is derived from a human colonic adenocarcinoma and has proved useful in many nutrient and drug transport studies. Caco-2 spontaneously differentiates at confluency into highly polarized enterocyte-like cells possessing well developed brush borders with microvilli and tight junctions (Pinto et al., 1983). Caco-2 monolayers develop many of the morphological and functional characteristics of polarized absorptive epithelial cells, including synthesis of brush border hydrolases and basolateral secretion of lipoproteins (Hidalgo et al., 1989). HepG2 is derived from a human hepatocellular carcinoma and exhibits many of the activities of normal liver, including an inducible cytochrome P450 system and the secretion of lipoproteins and at least 17 major plasma proteins (Knowles et al., 1980; Javitt, 1993). It has been widely used for cytotoxicity studies involving xenobiotics. These two cell lines seem to represent suitable models for investigating problems related to the transport, metabolism and function of carotenoids in human intestine and liver.

Several reports on carotenoid accumulation and cytoprotection by human cells were available when this project was intiated. However, these studies focused primarily on immune- and fibroblast-derived cell lines further supporting the potential novelty of HepG2 and Caco-2 cell lines as models for assessing carotenoid metabolism and function. With this background, I defined the following Specific Aims.

Specific aims

1. To compare the relative susceptibilites of the Caco-2 and HepG2 human cell lines to various oxidants to evaluate their appropriateness as models for testing the cytoprotective potential of selected carotenoids against oxidative stress.

2. To assess the antioxidant potential of β-carotene and lutein in HepG2 cells.

3. To examine the effectiveness of organic solvents, micelles and lipoproteins as vehicles for the delivery of carotenoids to HepG2 cells.

The results from detailed studies addressing each specific aim are outlined in subsequent chapters.

CHAPTER II

SUSCEPTIBILITY OF CACO-2 AND HEPG2 HUMAN CELL LINES TO OXIDATIVE STRESS

ABSTRACT

Free radical-mediated cellular damage has been proposed to be critically linked to degenerative processes associated with various diseases. The objective of this study was to compare the impact of several pro-oxidants on two highly differentiated human cell lines, viz., HepG2 and Caco-2. Anchored, differentiated cultures were treated with various concentrations of either tert-butylhydroperoxide (TBHP), hydrogen peroxide (HPX), menadione (MD), or 2, 2'-azobis(2-amidinopropane) hydrochloride (AAPH) for 90 minutes at 37^{o}C. Pro-oxidant induced cellular damage was assessed by monitoring the following: 1) production of thiobarbituric acid reactive substances (TBARS); 2) release of lactate dehydrogenase (LDH) and ^{51}chromium (^{51}Cr) from cells; 3) ^{14}C-glucose oxidation; 4) transport of glucose and amino acid analogues; and, 5) mitochondrial activity. Exposure of HepG2 cultures to TBHP, HPX and AAPH generated higher concentrations of TBARS than in Caco-2 cultures treated with equimolar concentrations of the pro-oxidants. In contrast, MD treatment elevated TBARS to a greater extent in Caco-2 cultures than in HepG2. Cellular loss of LDH and ^{51}Cr increased significantly in HepG2 cultures, but not in Caco-2 cultures in response to 500 μmol/L TBHP, HPX and MD. Likewise, treatment with pro-oxidants generally impaired U-^{14}C-glucose oxidation and the uptake of ^{3}H-2-deoxyglucose (^{3}H-DOG) and ^{3}H-α-aminoisobutyric acid (^{3}H-AIB) to a greater degree in HepG2 cultures. The observed changes in the uptake of the glucose and alanine analogues after exposure to the pro-oxidants was not associated with a decline in mitochondrial activity. These

data demonstrate that HepG2 cultures exhibited greater susceptibility to oxidant-induced damage than cultures of Caco-2 in which pro-oxidants were added to the apical compartment.

INTRODUCTION

Free radicals have been implicated as etiologic factors for a variety of degenerative conditions, including emphysema, cataracts, arthritis and inflammatory diseases. Both enzymatic and non-enzymatic antioxidants normally inactivate free radicals, thereby preventing their deleterious effects on the structure and function of cellular components. However, excessive generation of reactive metabolites can damage DNA, proteins and polyunsaturated fatty acids in cell membranes (Jaeschke, 1995).

Isolated tissues, cells, organelles and lipoproteins have been used as models to characterize the adverse consequences and mechanisms of free radical-mediated processes. To better evaluate the potential significance of in vitro observations, it is important to study those tissues and cells that are likely targets of specific free radicals in situ. Hepatocytes are especially appropriate for investigation because they have a central role in the oxidative metabolism of nutrients, metabolites and xenobiotics. Although primary cultures of animal hepatocytes have been widely used to investigate the production, metabolism and cytotoxicity of free radicals, studies with human hepatocytes are limited (Moshage and Yap, 1992). Likewise, the intestine is exposed to relatively high levels of ingested pro-oxidants, but primary cultures of differentiated enterocytes are difficult to isolate and maintain in culture (Evans et al., 1994). Highly differentiated human cell lines such as HepG2 and Caco-2 are widely used models for in vitro studies. The HepG2 cell line is derived from a human hepatocellular carcinoma, secretes plasma proteins and lipoproteins, possesses an inducible cytochrome P450 system, and retains many other differentiated functions that are often lost by primary cultures of hepatocytes (Knowles et al. 1980; Javitt, 1990). The Caco-2 cell line is derived from a human colonic adenocarcinoma and differentiates at confluency into a polarized cell that expresses brush border hydrolases and

vectorially secretes lipoproteins (Pinto et al., 1983; Hidalgo et al., 1989). These properties have resulted in its widespread use for study of the uptake, metabolism and absorption of nutrients and drugs (Han et al., 1994).

In the present study, we have compared the consequences of exposing cultures of HepG2 and Caco-2 cells to several commonly used pro-oxidants on cellular integrity and activities. Two of the pro-oxidants selected for investigation, viz., tert-butylhydroperoxide (TBHP) and hydrogen peroxide (HPX), have been reported to generate free radicals in part via iron catalyzed processes, whereas the pro-oxidants 2, 2'-azobis(2-amidino)propane hydrochloride (AAPH) and menadione (MD) generate free radicals after thermolytic cleavage and metabolic activation with redox recycling, respectively (Nalini and Balasubramanian, 1993; Farber et al., 1990). The results indicate that HepG2 cells are generally more susceptible to oxidant-induced stress than Caco-2 cells.

MATERIALS AND METHODS

Supplies. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. AAPH was obtained from Polysciences Inc. (Warrington, PA). Uniformly labeled (U)-^{14}C-glucose (sp. act. 89 MBq/mmol), ^{14}C-mannitol (sp. act. 1 TBq/mmol), sodium ^{51}chromate (sp. act. 23.8 GBq/mg) and α-[methyl-^{3}H]-aminoisobutyric acid (sp. act. 252 GBq/mmol) were purchased from DuPont/New England Nuclear (Wilmington, DE). 2-Deoxy-D-[2,6-^{3}H] glucose (sp. act. 999 GBq/mmol) was obtained from Amersham (Arlington Heights, IL). Tissue culture flasks (75 and 25 cm^2) and microporous membrane inserts (Falcon inserts with area of 4.9 cm^2 and 3 μmeter pore) were obtained from Bectin, Dickinson & Company (Lincon Park, NJ) and

multiple well dishes (15 and 35 mm diameter) were obtained from Costar Corp. (Cambridge, MA).

Cell Culture. HepG2 and Caco-2 were purchased from American Type Culture Collection (ATCC, Rockville, MD) and stock cultures were maintained in T75 flasks. All experiments were performed using cells originating from a single ampule supplied by ATCC since some phenotypic characteristics of Caco-2 vary between batches (Herrold et al., 1993). For experiments, cells were seeded in multiwell plastic dishes (15 and 35 mm diameter) and incubated in a humidified atmosphere of air/CO_2 (95:5 v/v) at 37^{o}C. HepG2 cultures (passages 80-90) were grown in MEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mmol/L pyruvate, 2 mmol/L glutamine, 50 mg/L gentamicin, 0.5 mg/L fungizone and 15 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0. Medium was replaced every 2 days for HepG2 cultures and experiments were initiated at 1-3 days post-confluency. Caco-2 cells (passages 25-35) were grown in DMEM supplemented with 7.5% fetal bovine serum and 7.5% Fetal Clone II (Hyclone Labs, Inc., Logan, UT), 15 mmol/L HEPES, 1% (v/v) nonessential amino acids, 2 mmol/L glutamine, 50 mg/L gentamicin, and 0.5 mg/L fungizone, pH 7.0. Medium was replaced every 2 days until the monolayer was confluent. The levels of fetal bovine serum and Fetal Clone II in complete medium then were decreased to 5% each. Fresh medium continued to be provided every second day and experiments were performed at 10-12 days post-confluency when cells were highly differentiated (Hidalgo et al., 1989; Elfwood et al., 1993).

Experiments were initiated after removal of spent medium and washing cultures grown on multiwell dishes or in flasks 3 times with prewarmed Hank's Balanced Salt Solution (HBSS) without phenol red at 37^{o}C. Basal medium either alone (control) or containing indicated

concentrations of either TBHP, HPX, MD or AAPH was added and cultures were incubated at 37°C for 90 minutes.

Assessment of Oxidative Damage to Cells

a. Lipid peroxidation. To measure thiobarbituric acid reactive substances (TBARS), a modification of the method by Buege and Aust (1978) was used. Briefly, 100 µL 0.45 µmol/L butylated hydroxytoluene (BHT) in absolute ethanol was added to each well after exposure to pro-oxidant to quench further oxidative changes. After transferring one-half of the test medium to a 5 mL glass screw cap tube and discarding remaining medium, the monolayer was washed three times with HBSS. Cellular material was collected in 1 mL harvest solution (3.5 mmol/L sodium dodecylsulfate (SDS) containing 1 mmol/L disodium ethylenediaminetetraacetic acid) and sonicated once for 3-5 seconds using a Sonic 50 Dismembranator at 60% power output (Fisher Scientific, Pittsburgh, PA). One half of the sonicate (500 µL) and 2 mL TBA solution (26 mmol/L thiobarbituric acid, 920 mmol/L trichloroacetic acid (TCA), 7 mmol/L SDS in 250 mmol/L HCl) were added to the tube containing the sample of medium. Tubes were heated at 95°C for 30 minutes and cooled in ice. After mixing, aliquots were centrifuged at 16,000 x g for 10 minutes before the supernate was analyzed spectrophotometrically at 532 nm. Tetramethoxypropane was used as a standard and processed identically to test samples. Results are presented as pmol TBARS/mg cellular protein.

b. Lactate dehydrogenase (LDH) release. Aliquots of medium were centrifuged for 5 minutes at 1,000 x g at 25°C to remove any cells that may have detached. Monolayers were washed with HBSS and collected in 1 mL 1% (v/v) Triton X-100 to determine cellular LDH (EC 1.1.1.27) activity. Cellular material was sonicated once for 3-5 seconds and the samples were centrifuged

at 16,000 x g for 5 minutes. The reaction mixture contained 2.8 mL 0.1 mol/L phosphate buffer (pH 7.5) with 85 μmol/L nicotinamide adenine dinucleotide, 100 μL 23 mmol/L sodium pyruvate, and 500 μL supernate from either undiluted medium or cell sonicate that was diluted 1:50 with phosphate buffer (Glascott et al., 1992). Pilot studies showed that the activity of purified LDH in medium was not altered by exposure to either 500 μmol/L TBHP, HPX or MD, or 25 mmol/L AAPH for 90 min at 37°C. The rate of change in absorption at 340 nm was monitored for 5 min (Beckman DU-64 UV/visible spectrophotometer, Fullerton, CA). The percentage of cellular LDH released to the medium was calculated as follows:

$$\%\text{LDH release} = [\text{LDH activity in media} / (\text{LDH activity in media} + \text{LDH activity in cells})] \times (100\%)$$

c. ^{51}Chromium (Cr) release. After incubating cultures overnight (20 hr) with medium containing 37 kBq ^{51}Cr, monolayers were washed with HBSS and incubated with medium with or without test pro-oxidants for 90 min at 37°C. Aliquots of medium were collected and centrifuged at 700 x g for 5 min. Supernatent was sampled and ^{51}Cr determined by gamma ray spectrometry (Packard Cobra II auto-gamma counter, Meridan, CT). Monolayers were washed, collected and sonicated as described above (LDH release). The amount of ^{51}Cr in aliquots was measured to determine cellular ^{51}Cr. The percent of cellular ^{51}Cr released to the medium was calculated as follows:

$$\%\ ^{51}\text{Cr release} = [^{51}\text{Cr in medium} / (^{51}\text{Cr in medium} + ^{51}\text{Cr in cells}] \times (100\%)$$

d. Paracellular transport of ^{14}C-mannitol. To determine if exposure of the apical surface of Caco-2 cells to pro-oxidants adversely affected the integrity of tight junctions, cultures were grown and maintained on microporous inserts as described by Han et al. (1994). Pro-oxidants

were added to DMEM in the apical compartment and cultures were incubated for 90 min at 37°C. After washing with HBSS at 37°C, monolayers were exposed to complete medium containing ^{14}C-mannitol (9.25 Bq/well) in the apical compartment, while the basolateral compartment received complete medium without label. Mannitol is transported across the monolayer via the paracellular route (Ranaldi et al., 1992). Determination of the rate of ^{14}C-mannitol flux across the monolayer has been described by Han et al. (1994).

e. [^{14}C]-Glucose oxidation. Following exposure to the test oxidants for 90 min and washing, monolayers (T25 flasks) were incubated with 2 mL fresh MEM (5.5 mmol/L glucose) containing 10% serum and 37 kBq D-[U-^{14}C]-glucose at 37°C for 2 hr. Production of $^{14}CO_2$ was measured as described previously (Ellwood et al., 1993). Data are expressed as nmol glucose oxidized/(hour·mg cellular protein).

f. Transport of ^{3}H-2-D-deoxyglucose (^{3}H-DOG) and ^{3}H-α-aminoisobutyric acid (^{3}H-AIB). Cultures of HepG2 and Caco-2 in multiwell dishes were washed twice with HBSS at 37°C and incubated with MEM alone or containing the indicated concentrations of the pro-oxidants for 90 minutes at 37°C. After monolayers were washed twice with HBSS, cultures were incubated at 37°C with glucose-free MEM containing ^{3}H-DOG (9.25 kBq/mL) and 100 μmol/L DOG. After 10 min, cells were washed twice with ice-cold glucose-free MEM containing 100 μmol/L phloretin to inhibit ^{3}H-DOG efflux. To measure ^{3}H-AIB uptake, amino acid-free MEM containing ^{3}H-AIB (9.25 kBq/mL) with 100 μmol/L AIB at 37°C was added to washed cultures. After 3 min, radioactive medium was removed rapidly and monolayers were washed twice with ice-cold amino acid-free MEM (2 mL). Cellular material was collected in 1 mL harvest solution. Pilot studies showed that uptake of ^{3}H-AIB and ^{3}H-DOG was linear for 4 and 20 min, respectively,

using the described conditions. Cellular ^{3}H was determined by liquid scintillation counting. Uptake data are expressed as nmol/(min·mg cell protein).

g. Mitochondrial function. To examine the impact of treatment of cells with the pro-oxidants on mitochondrial activity, cells were washed and incubated with MEM alone or containing the indicated concentrations of the pro-oxidants for 90 min at 37°C. After washing cultures, MEM containing 60 μmol/L 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazaolium bromide (MTT) was added and cultures were incubated at 37°C. After 30 min, MEM was removed, monolayers were washed twice with HBSS, and 1 mL dimethylsulfoxide was added to each well to lyse cells and solubilize the reduced MTT crystals. The quantity of reduced MTT was measured spectrophotometrically at 560 nm (Scudiero et al., 1988).

Antioxidant enymes. The cellular activity of selenium-dependent glutathione peroxidase (EC 1.11.1.9) was determined by addition of aliquots of cell sonicates (5 μL) to cuvettes containing 20 μL hydrogen peroxide as substrate and 975 μL 50 mmol/L Tris buffer (pH 7.6) with 0.2 mmol/L nicotinamide adenine dinucleotide phosphate, 1 mmol/L glutathione (reduced form), 1 unit/mL glutathione reductase and 1 mmol/L disodium ethylenediaminetetraacetic acid. Absorption at 340 nm was monitored for 5 min and data are expressed as nmol H_2O_2 converted to water/(min·mg protein) (Lawrence and Burk, 1976). Catalase (EC 1.11.1.6) activity was measured by adding an aliquot of cell sonicate (50 μL) to cuvettes containing 800 μL 50 mmol/L phosphate buffer (pH 7.0) and 100 μL 20 mmol/L H_2O_2. The decomposition of H_2O_2 was monitored by the change in absorbance at 240 nm over 3 min (Aebi, 1984). Data are expressed as μmol H_2O_2 decomposed/(min·mg protein). The activity of total cellular superoxide dismutase (EC 1.15.1.1) was determined by monitoring the inhibition of pyrogallol autoxidation. Aliquots of cell sonicate (100 μL) were added to 2.9 mL 50 mmol/L Tris buffer (pH 8.2) containing 1 mmol/L

diethylenetriaminepentaacetic acid and 0.2 mmol/L pyrogallol. Absorbance at 320 nm was monitored for 5 min. Data are expressed as units/mg protein where 1 unit of SOD activity is the amount required to inhibit the oxidation of pyrogallol by 50% (Marklund and Marklund, 1974). Glutathione transferase (2.5.1.18) activity was analyzed in a reaction mixture containing 1 mmol/L 1-chloro-2,4-dinitrobenzene (25 μL), 1 mmol/L glutathione (25 μL) and 900 μL 100 mmol/L sodium phosphate buffer (pH 6.5) containing 0.1 mmol/L EDTA (Habig et al., 1974). The change in absorbance was monitored at 340 nm for 3 min. Data are expressed as μmol GSH conjugate formed/(min·mg protein).

Protein determination. Cellular protein was determined as previously described (Ellwood et al., 1993).

Statistical analysis of data. Statistically significant differences ($p<0.05$) were determined using either Student's t test or one way ANOVA with post hoc analysis using Tukey's multiple range test (SAS Institute, Cary, NC). All experiments were repeated at least twice using triplicate wells or flasks for each test variable. Data are expressed as means ± SEM.

RESULTS

Although somewhat nonspecific, the TBARS assay is useful as an indicator of the extent of lipid peroxidation resulting from oxidative stress (Halliwell and Gutteridge, 1989). Therefore, the effects of various concentrations of pro-oxidants on the generation of TBARS in HepG2 and Caco-2 cultures were examined initially. Concentrations of TBARS in control cell cultures were 34 ± 3 and 50 ± 14 pmol/mg protein for HepG2 and Caco-2, respectively. Levels of TBARS in cultures of HepG2 increased 14 and 20-fold after exposure to 125 and 250 μmol/L TBHP for 90

min, respectively (Fig. 2.1A). In contrast, exposure of Caco-2 cultures to the same concentrations of TBHP elevated levels of TBARS less than 2-fold ($p>0.05$). Exposure of HepG2 cultures to 125 and $\geq$250 μmol/L hydrogen peroxide (HPX) increased TBARS levels approximately 3 and 7-fold, respectively ($p<0.05$) (Fig. 2.1B). Levels of TBARS in Caco-2 cultures were not altered at 125 μmol/L HPX, but increased about 3-fold at $\geq$250 μmol/L HPX. TBARS levels increased linearly as the concentration of menadione (MD) increased in HepG2 cultures; TBARS were 5-fold above the basal level in cultures exposed to 750 μmol/L MD (Fig. 2.1C). Caco-2 cultures were more sensitive than HepG2 to lower concentrations of MD with the level of TBARS elevated approximately 6- and 8-fold above background after exposure to 125 and $\geq$250 μmol/L pro-oxidant, respectively. The levels of TBARS in cultures of HepG2 and Caco-2 cultures treated with AAPH increased in direct proportion to the concentration of the stressor (Fig. 2.1D). Concentrations of TBARS were increased 24- and 12-fold above control levels ($p<0.05$) in cultures of HepG2 and Caco-2, respectively, exposed to 100 mmol/L AAPH. These observations indicate that the relative susceptibility of HepG2 cultures to lipid peroxidation induced by TBHP, HPX and AAPH was greater than that in Caco-2 cultures, whereas Caco-2 cells exhibited greater sensitivity to MD than HepG2 cells.

Although increased levels of TBARS in cultures indicate that cellular components were peroxidized, it was unknown if the changes reflected a loss of cell integrity and/or function. To address these possibilities, cultures were exposed to one concentration of each pro-oxidant that had significantly increased the level of TBARS in initial studies, viz., 500 μmol/L for TBHP, HPX and MD and 25 mmol/L AAPH. Release of cytoplasmic LDH and cellular ^{51}Cr from HepG2 and Caco-2 cells into the medium were used as indicators of membrane integrity. Exposure of HepG2 cultures to TBHP, HPX and MD increased medium LDH activity 2.1, 2.3 and 1.6-fold, respectively ($p<0.05$) and ^{51}Cr release 10.6, 3.2 and 5.9-fold, respectively, above control levels

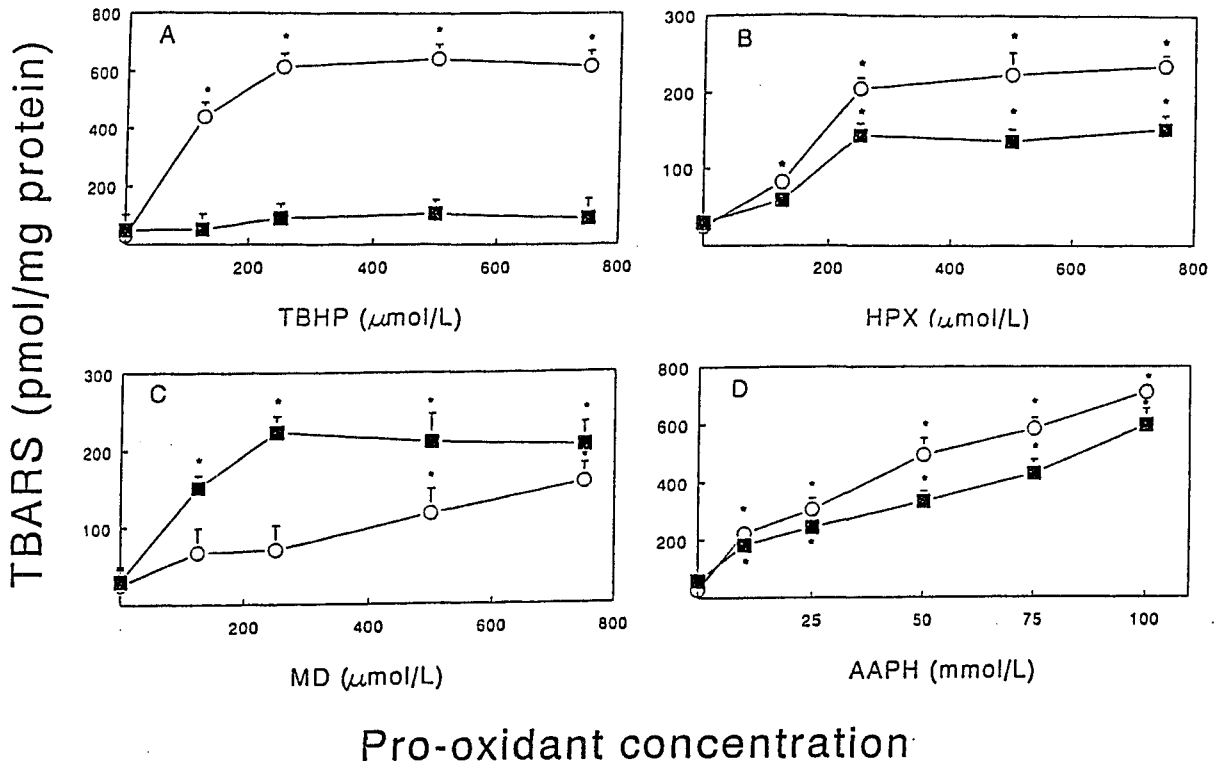

Fig. 2.1. *Effects of pro-oxidants on levels of thiobarbituric acid reactive substances (TBARS) in HepG2 (—O—) and Caco-2 (—■—) cells.*

Confluent cultures were incubated in MEM with or without tert-butylhydroperoxide (panel A), hydrogen peroxide (panel B), menadione (panel C) or AAPH (panel D) at the indicated concentrations for 90 minutes at 37°C. Cultures were washed, harvested and analyzed as described in METHODS to determine pmol TBARS/mg protein. Data represent means ± SE from at least two experiments each containing three wells per treatment. The presence of an asterisk above the error bar indicates that the level of TBARS in each of the two cell cultures differ significantly ($p<0.05$) from the means of the respective controls after exposure to the given level of pro-oxidant.

(Table 2.1). In contrast, treatment of Caco-2 cultures with TBHP and MD did not significantly ($p>0.05$) increase LDH release. Exposure to HPX increased ^{51}Cr release from Caco-2 cells by 1.5-fold. Exposure of HepG2 and Caco-2 cultures to 25 mmol/L AAPH did not increase LDH release. Likewise, apical exposure of Caco-2 monolayers grown on membrane inserts to pro-oxidants did not significantly ($p>0.05$) alter the apical to basolateral flux of ^{14}C-mannitol (data not shown).

To determine whether the pro-oxidant-induced biochemical changes were associated with altered cellular activity, U-^{14}C-glucose oxidation was examined initally. The basal rates of glucose oxidation for control cultures of HepG2 and Caco-2 were 17.2 ± 1.7 and 25.0 ± 4.0 nmol/(hr·mg protein), respectively. U-^{14}C-Glucose oxidation by HepG2 cultures treated with 500 μmol/L TBHP (Table 2.2) was only 19% that of the control cultures. Similarly, the rates of U-^{14}C-glucose oxidation after exposure to HPX, MD and AAPH were decreased to 50, 56 and 79%, respectively, that in control cultures of HepG2 ($p<0.05$). Exposure of Caco-2 cells to TBHP and HPX decreased U-^{14}C-glucose oxidation to 89 ($p<0.05$) and 65% ($p<0.05$) that by control cultures (Table 2.3); this change was less severe than that with HepG2. In contrast, U-^{14}C-glucose oxidation by Caco-2 treated with MD and AAPH was 40 and 76% that of controls ($p<0.05$), respectively (Table 2.3); these decreases are similar to those observed in cultures of HepG2 cells that had been treated similarly (Table 2.2).

The observed pro-oxidant-induced reductions in glucose oxidation may have been due to increased shunting of glucose from the glycolytic to the hexose monophosphate pathway, inhibition of cytosolic or mitochondrial enzymes participating in the glucose oxidation, or modification of plasma membrane components that are required for the transport of glucose into the cell. We assessed the latter possibility by examining the impact of pro-oxidant treatment of

TABLE 2.1

Release of lactate dehydrogenase (LDH) and ^{51}Chromium (Cr) by HepG2 and Caco-2 cells after exposure to pro-oxidants[1]

Treatment	HepG2		Caco-2	
	LDH release	^{51}Cr release	LDH release	^{51}Cr release
	(%)		(%)	
CONTROL	1.1 ± 0.1^{c}	2.2 ± 0.1^{d}	0.9 ± 0.1^{a}	2.2 ± 0.2^{b}
TBHP	2.4 ± 0.2^{a}	23.4 ± 3.8^{a}	0.9 ± 0.1^{a}	2.5 ± 0.2^{b}
HPX	2.6 ± 0.2^{a}	13.0 ± 2.0^{b}	0.9 ± 0.1^{a}	3.2 ± 0.2^{a}
MD	1.7 ± 0.1^{b}	7.0 ± 2.0^{c}	1.0 ± 0.2^{a}	2.7 ± 0.2^{b}
AAPH	1.1 ± 0.1^{c}	nd	0.8 ± 0.1^{a}	nd

[1]Cultures of HepG2 and Caco-2 were exposed to MEM alone or MEM containing either 500 μmol/L TBHP, HPX or MD, or 25 mmol/L AAPH for 90 min at 37^{o}C. LDH activity in aliquots of medium and cell sonicates were analyzed as described in Material and Methods to determine % LDH released to medium. For ^{51}chromium release studies, cultures were incubated overnight (20 hr) with MEM containing 37 kBq ^{51}Cr prior to exposure to the pro-oxidants. After collecting medium, monolayers were washed and harvested. Aliquots of cell sonicates and medium were analyzed by gamma ray spectroscopy to determine % ^{51}Cr released to medium. Data are means ± SEM for triplicate wells from a representative experiment. Different letters as superscripts within a column indicate significant differences ($p<0.05$) between means. nd=not determined.

TABLE 2.2

Oxidation of U-^{14}C-glucose and uptake of ^{3}H-2-deoxyglucose (DOG) and ^{3}H-α-aminoisobutyric acid (AIB) by HepG2 cells exposed to pro-oxidants[1]

Treatment	glucose oxidation	DOG transport	AIB transport
	nmol/(hr·mg protein)	nmol/(min·mg protein)	
CONTROL	25.0 ± 4.0^{a}	17.3 ± 1.5^{a}	5.0 ± 0.1^{a}
TBHP	4.7 ± 0.6^{c}	3.8 ± 0.5^{c}	0.5 ± 0.1^{d}
HPX	12.5 ± 1.8^{b}	10.7 ± 1.2^{b}	3.4 ± 0.3^{c}
MD	14.1 ± 2.1^{b}	4.6 ± 1.5^{c}	2.7 ± 0.3^{c}
AAPH	19.8 ± 2.3^{a}	12.0 ± 1.5^{b}	4.0 ± 0.1^{b}

[1]Cultures of HepG2 were exposed to MEM alone or MEM containing either 500 μmol/L TBHP, HPX or MD, or 25 mmol/L AAPH for 90 min at 37°C. After removing medium with oxidants, monolayers were washed and incubated with MEM containing either U-^{14}C-glucose for 120 min, ^{3}H-DOG for 10 min, or ^{3}H-AIB for 3 min to determine the effect of treatment on $^{14}CO_2$ production and the uptake of ^{3}H-DOG or ^{3}H-AIB. Data are means ± SEM from triplicate wells or flasks from three separate experiments. Different letters as superscripts indicate that significant differences ($p<0.05$) between means within a column.

TABLE 2.3

Oxidation of U-^{14}C-glucose and uptake of ^{3}H-2-deoxyglucose (DOG) and ^{3}H-α-aminoisobutyric acid (AIB) by Caco-2 cells exposure to pro-oxidants[1]

Treatment	glucose oxidation	DOG transport	AIB transport
	nmol/(hr·mg protein)	nmol/(min·mg protein)	
CONTROL	17.2 ± 1.7^{a}	17.0 ± 1.2^{a}	0.28 ± 0.02a
TBHP	15.3 ± 0.5^{ab}	15.0 ± 0.5^{b}	0.30 ± 0.01^{a}
HPX	11.2 ± 2.5^{c}	15.7 ± 1.8^{ab}	0.30 ± 0.03^{a}
MD	6.9 ± 0.2^{d}	3.6 ± 0.9^{c}	0.53 ± 0.07^{b}
AAPH	13.1 ± 2.3^{bc}	15.8 ± 0.9^{ab}	0.31 ± 0.04^{a}

[1]Cultures of Caco-2 were exposed to MEM alone or MEM containing either 500 μmol/L TBHP, HPX or MD, or 25 mmol/L AAPH for 90 min at 37°C. After removing medium with oxidants, monolayers were washed and incubated with MEM containing either U-^{14}C-glucose for 120 min, ^{3}H-DOG for 10 min, or ^{3}H-AIB for 3 min to determine the effect of treatment on $^{14}CO_2$ production and the uptake of ^{3}H-DOG or ^{3}H-AIB. Data are means ± SEM from triplicate wells or flasks from three separate experiments. Different letters as superscripts indicate that significant differences ($p<0.05$) between means within a column.

HepG2 and Caco-2 cultures on the uptake of ^{3}H-2-deoxyglucose (^{3}H-DOG), a nonmetabolizable analogue of glucose. The rate of ^{3}H-DOG uptake was only 24, 64, 25 and 65% that of control cultures after treatment of HepG2 cells with TBHP, HPX, MD and AAPH, respectively ($p<0.05$). In contrast, apical exposure of Caco-2 cultures to TBHP, HPX and AAPH had minimal affect ($p>0.05$) on the rate of ^{3}H-DOG transport (Table 2.3). However, apical exposure of Caco-2 cells to MD decreased ($p<0.05$) the rate of ^{3}H-DOG transport to 25% that of control cultures ($p<0.05$).

To determine whether oxidant-induced damage to the process of glucose uptake was specific or may have reflected a more general impairment of nutrient transport processes, cellular uptake of the amino acid analogue ^{3}H-α-aminoisobutyric acid (^{3}H-AIB) was examined. Exposure of HepG2 cells to TBHP, HPX, MD and AAPH reduced the rate of ^{3}H-AIB transport (Table 2.2) to 11, 68, 54 and 81%, respectively, that of the control ($p<0.05$). In contrast, incubation of Caco-2 cells with TBHP, HPX and AAPH did not significantly alter ($p>0.05$) the rate of ^{3}H-AIB transport (Table 2.3). Moreover, the rate of ^{3}H-AIB uptake by Caco-2 cells treated with MD was 1.8-fold higher than that by the control cells (526 ± 68 vs. 285 ± 19 pmol/mg protein, respectively).

Since many transport processes directly or indirectly require cellular energy, the impact of pro-oxidant treatment on mitochondrial activity was measured. Incubation of HepG2 cells with TBHP, HPX and AAPH increased the reduction of MTT 4-fold ($p<0.05$); exposure to MD increased MTT reduction 7-fold ($p<0.05$). Incubation of Caco-2 cells with TBHP and HPX did not not alter MTT reduction ($p<0.05$), whereas exposure to MD and AAPH increased reduction by 2.5 and 1.3-fold, respectively ($p<0.05$). Together, these data suggest that mitochondrial activity of the cell lines was not impaired by the test conditions and that the decreased rate of DOG and AIB transport was not the result of insufficient generation of cellular energy.

The ability of a cell type to resist an oxidative challenge depends in part on the activities of its antioxidant enzymes. We compared the activities of several key antioxidant enzymes in control cultures of HepG2 and Caco-2. The activities of glutathione peroxidase, catalase and glutathione transferase were similar in the two cell types (Table 2.4). Total SOD activity was 2.3-fold ($p<0.05$) higher in HepG2 than in Caco-2 cells, whereas GSH transferase activity was 1.5 times ($p>0.05$) higher in Caco-2 than in HepG2 cells.

DISCUSSION

The susceptibility of a tissue to oxidative damage is influenced by the chemical characteristics and concentration of the pro-oxidant compound and the duration of exposure, and the phenotypic properties of the target cell (e.g., levels of enzymatic and nonenzymatic antioxidants and content of unsaturated fatty acids). Because the anatomic location and the metabolic activities of the liver and intestinal mucosa result in their exposure to diverse pro-oxidant species, we compared the relative susceptibilities of commonly used differentiated human cell lines from these tissues to several pro-oxidants. More specifically, HepG2 and Caco-2 cells were selected because confluent cultures of each express many of the phenotypic characteristics of hepatocytes and enterocytes, respectively (Knowles et al., 1980, Pinto et al., 1983, Hidalgo et al., 1989). In general, our results demonstrate that differentiated monolayers of Caco-2 intestinal cells are more resistant than HepG2 liver cells to the adverse effects of pro-oxidants. Apical exposure of Caco-2 cells to 500 μmol/L TBHP and HPX and 25 mmol/L AAPH did not significantly alter membrane permeability and nutrient uptake. In contrast, incubation of HepG2 cultures with all test pro-oxidants compromised membrane integrity and decreased the uptake of glucose and alanine analogues. MD was the one pro-oxidant that increased the levels

TABLE 2.4

Antioxidant Enzyme Activities in HepG2 and Caco-2 Cells[1]

	HepG2	Caco-2
glutathione peroxidase [nmol/(min·mg protein)]	4.2 ± 0.5^a	5.8 ± 0.3^b
catalase [μmol H_2O_2/(min·mg protein)]	36.4 ± 5.1^a	41.5 ± 6.2^a
superoxide dismutase (units/mg protein)	2.8 ± 0.4^a	1.2 ± 0.1^b
glutathione transferase [μmol/(min·mg protein)]	2.7 ± 0.5^a	4.0 ± 1.5^a

[1]Activities of antioxidant enzymes were measured in control cultures of HepG2 and Caco-2 cells using standard assays as indicated in the Material and Methods section. Assays were performed using at least three monolayers from two different passages for each cell line. Data are means ± SE and significant differences ($p<0.05$) in activities between the two cell types are indicated by different superscripts.

of TBARS and decreased the uptake of glucose and alanine analogues to a greater extent in Caco-2 cultures than in HepG2 cultures. Our observations with Caco-2 cells confirm and extend the recent report by Baker et al. (1995). These investigators found that apical exposure of Caco-2 to TBHP (500 μmol/L) did not significantly alter the integrity of the cell surface, the permeability of the monolayer, or production of cellular energy. In contrast, apical exposure to 500 μmol/L MD adversely affected the plasma membrane permeability and energy production by Caco-2 cells.

Similar to our observations with HepG2 cells, others have reported that exposure of isolated rat hepatocytes to organic hydroperoxides (Rush et al., 1985) and HPX (Innes, 1988) results in peroxidation of membrane lipids and potentially lethal cellular injury. Additional studies have indicated that exposure of rat hepatocytes and HepG2 cells to free radical generating quinones, e.g., MD, (Babich and Stern, 1993), and AAPH (Nalini and Balasubramanian, 1991) also results in significant cellular damage.

Although the culture conditions we used for the growth and maintenance of the Caco-2 and HepG2 cell lines are standard, it is possible that the different composition of the media contributed to the differential responses of the cells to oxidants. For example, the formulation of the basal media, the percentage and type of sera, and the period between seeding vessels and initiating experimental analysis differed for the test cell lines (see Material and Methods). Our attempts to address this confounding factor by culturing HepG2 in DMEM with high sera and Caco-2 in MEM with 10% FBS were not successful. However, substitution of FBS for the serum substitute Fetal Clone II did not influence the relative resistance of Caco-2 cells to oxidative stress (data not shown). Another possible factor was that each cell line was used at markedly different passage numbers. However, experiments were performed at early passages after

arrival of the cell lines from the vendor (ATCC) and are similar to the ranges used by other investigators. Recognizing these potential limitations, our aim has been to compare the relative susceptibilities of two highly differentiated and widely used human cell lines, viz., HepG2 liver and Caco-2 intestinal cells, cultured under typical in vitro conditions to several pro-oxidant stressors.

One alternative possibility for the relative resistance of Caco-2 cells to iron-catalyzed, pro-oxidative stress compared to HepG2 cells, is that they may express high levels of antioxidant enzymes. Grisham et al. (1990b) compared the activities of antioxidant enzymes in Caco-2 cells to those in normal human intestinal tissue and liver. Glutathione peroxidase activity in differentiated cultures of Caco-2 and normal colonic epithelium were similar, whereas the activities of catalase and superoxide dismutase in Caco-2 cells were significantly lower than in normal human colonic epithelium. Moreover, antioxidant enzyme activities in human liver were markedly greater than in Caco-2 cells and colonic epithelium. We observed similar activities of glutathione peroxidase, catalase and glutathione transferase in HepG2 and Caco-2 cells, but higher superoxide dismutase activity in HepG2 cells than in Caco-2 cells. These data suggest that the decreased susceptibility of Caco-2 to pro-oxidants as compared to HepG2 cells is not simply due to higher activities of antioxidant enzymes in the former.

The relative resistance of Caco-2 cells to the potentially damaging influence of iron-catalyzed free radical generators, i.e., TBHP and HPX, is consistent with previous observations with homogenates of intestinal mucosa. Balasubramanian et al. (1988) reported that homogenates from rats and human small intestinal mucosa were resistant to free-radical induced peroxidation in comparison to homogenates from other tissues that were treated identically. Similarly, perfusion of intact rat intestine with HPX and xanthine-xanthine oxidase did not induce

lipid peroxidation or alter absorptive function (Nalini and Balasubramanian, 1993). In contrast, apical perfusion with MD and AAPH induced lipid peroxidation and compromised the permeability of the mucosa. This resistance of the intestinal preparations to iron-catalyzed, oxidative damage has been associated with the presence of relatively high concentrations of non-esterified, monounsaturated fatty acids in the brush border membrane (Diplock et al., 1988; Balasubramanian et al., 1989). The addition of oleate to homogenates of extra-intestinal tissues from several species, including man, conferred protection against iron-catalyzed damage by pro-oxidants. It has been suggested that the unsaturated free fatty acids such as oleate complex iron at the membrane surface, thereby inhibiting its availability for Fenton-type reactions (Simpson and Peters, 1987). Murthy et al. (1988) have reported elevated levels of oleic and palmitoleic acids in microsomes isolated from Caco-2 cells, but the levels in the brush border membrane are unknown.

The collective results with Caco-2 cells, intestinal loops and intestinal homogenates suggest that the brush border is relatively resistant to iron-catalyzed, oxidant-induced damage. Perhaps, the regular presence of dietary pro-oxidants and reactive low molecular weight complexes of iron in the intestinal lumen provided a selective pressure for the modification of brush border membrane composition, viz., the presence of high levels of monounsaturated free fatty acids, to protect against iron-catalyzed oxidative damage. Indeed, Cermak et al. (1993) have demonstrated that monolayer cultures of Caco-2 cells are relatively resistant to hydrogen peroxide-induced damage even after preincubation with iron(II)sulfate or hemin. In contrast with the reports in which pro-oxidants were added to the apical compartment, Baker and associates (1993, 1995) have shown that exposure of the basolateral surface of Caco-2 cells to pro-oxidants compromises cellular integrity and function. Similarly, excessive production of reactive oxygen species by phagocytic cells in the lamina propria can overwhelm the antioxidant capacity of

enterocytes, and adversely alter the basolateral surface (Grisham et al., 1990a). The similarity of the response of Caco-2 cells and intact intestine to pro-oxidant substances further supports the usefulness of this human intestinal cell line as an in vitro model of absorptive epithelial cells.

Oxidation of U-^{14}C-glucose to $^{14}CO_2$ was used as an initial indicator of the impact of pro-oxidants on cellular metabolism. While exposure to pro-oxidants decreased the apparent efficiency of oxidation of labeled U-^{14}C-glucose in both cell lines, the inhibition generally was greater in cultures of HepG2 than Caco-2. We found that the decline in production of $^{14}CO_2$ from U-^{14}C-glucose by pro-oxidant treated cells was correlated with a reduction in the ability of the cell to transport the glucose analogue 2-deoxyglucose. It is likely that some of the decrease in the rate of U-^{14}C-glucose oxidation by pro-oxidant treated cells also was due to shunting of glucose-6-phosphate to the hexose monophosphate pathway (Baker and Baker, 1993; Cepsinkas et al., 1994). Pro-oxidant treatment also impaired the uptake of ^{3}H-AIB by HepG2 cells. Similarly, Vroegop et al. (1995) recently reported impaired ^{3}H-DOG and ^{3}H-AIB transport in a neural hybridoma cell line exposed to several chemical oxidants. This shows that the alteration in ^{3}H-DOG transport was not specific. Together, these results suggest that the pro-oxidants adversely affected the plasma membrane. This conclusion is supported by the normal or increased mitochondrial activity in cells after exposure to pro-oxidants suggesting that cell energy status was not compromised.

The excessive generation of free radicals has been implicated in numerous pathological conditions in humans. Free radicals or their metabolites react with various cellular macromolecules and may damage cell integrity and function. Highly differentiated human cell lines provide an important tool for assessing pro-oxidant-induced damage to cells. Our studies

indicate that the brush border surface of Caco-2 cells is less susceptible to pro-oxidant-mediated damage than the surface of HepG2 cells.

CHAPTER III

β-CAROTENE AND LUTEIN PROTECT THE PLASMA MEMBRANE OF HEPG2 HUMAN LIVER CELLS AGAINST OXIDANT-INDUCED DAMAGE

ABSTRACT

Numerous epidemiological studies support a strong inverse relationship between consumption of carotenoid-rich fruits and vegetables and the incidence of some degenerative diseases. One proposed mechanism of protection by carotenoids centers on their putative antioxidant activity, although direct evidence in support of this contention is limited at the cellular level. The antioxidant potential of β-carotene (BC) and lutein (LUT), carotenoids with or without provitamin A activity, respectively, was evaluated using the human liver cell line HepG2. Pilot studies showed that 90 min exposure of confluent cultures to 500 μmol/L tert-butylhydroperoxide (TBHP) at 37°C significantly ($p<0.05$) increased lipid peroxidation, cellular leakage of lactate dehydrogenase (LDH) and impaired the uptake of ^{3}H-α-aminoisobutyric acid and ^{3}H-2-deoxyglucose. Protein synthesis, mitochondrial activity and glucose oxidation were not affected by TBHP treatment suggesting that the plasma membrane was the primary site of TBHP-induced damage. Overnight incubation of cultures with ≥1 μmol/L α-tocopherol protected cells against oxidant-induced changes. In parallel studies overnight incubation of HepG2 in medium containing micelles with either BC or LUT (final concentrations of 1.1 and 10.9 μmol/L, respectively), the cell content of the carotenoids increased from <0.02 to 0.17 and 1.93 μg/mg protein, respectively. Carotenoid loaded cells were partially or completely protected against oxidant-induced changes in lipid peroxidation, LDH release and amino acid and deoxyglucose

transport. These data demonstrate that BC and LUT or their metabolites protect HepG2 cells against oxidant-induced damage to the cell membrane.

INTRODUCTION

Carotenoids are naturally occurring pigments in plants that are involved in light harvesting reactions and protection of plant organelles against singlet oxygen-induced damage. Because free radical-induced damage to mammalian tissues is believed to contribute to the aging process and to the development of some degenerative diseases (Canfield et al., 1992), it has been proposed that dietary carotenoids serve as antioxidants in tissues (Thumham, 1994). This possibility is supported by numerous epidemiologic studies that indicate an inverse association between the increased intake of carotenoid-rich fruits and vegetables and the incidence of diseases such as cancer (Van Poppel and Goldbohm, 1995). Additionally, in vitro studies have demonstrated that carotenoids can inhibit chemically-induced neoplastic transformation (Bertram and Bortkiewicz, 1995), induce remission of oral leukoplakia (Garewal, 1995), quench free radicals such as singlet oxygen (Sies and Stahl, 1995), and modulate immune activity (Meydani et al., 1995).

Possible mechanisms of how carotenoids protect tissues against oxidative damage include their antioxidant capacity, interactions with other antioxidants and antioxidant enzymes, and conversion to retinoids and apocarotenoids (Canfield et al., 1990). Critical evaluation of the antioxidant potential of carotenoids in humans has been hampered in part by the relative lack of appropriate animal models. Human cell lines provide a relevant model for investigating carotenoid function. The availability of highly differentiated human cell lines with phenotypic properties similar to mature hepatocytes is particularly pertinent since the liver actively metabolizes drugs and other xenobiotics and generates high levels of free radicals (Moshage and Yap, 1992). We selected HepG2 human liver cells as our model because these cells secrete plasma proteins and lipoproteins, express an inducible cytochrome P450 system, and respond to

hormones and cytokines in a manner similar to normal human liver (Javitt, 1993). The objective of the present study was to determine whether pretreatment of HepG2 cells with β-carotene (BC) and lutein (LUT), i.e., carotenoids with or without pro-vitamin A activity, respectively, confers cytoprotection when cultures are subjected to oxidative stress.

MATERIALS AND METHODS

Supplies. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. D-[U-^{14}C]-glucose (sp. act. 89 MBq/mmol) and D-[6-^{14}C]-glucose (sp. act. 181 MBq/mmol) and α-[methyl-^{3}H]-aminoisobutyric acid (sp. act. 252 GBq/mmol) were purchased from DuPont/New England Nuclear (Wilmington, DE). 2-Deoxy-D-[2,6-^{3}H] glucose (sp. act. 999 GBq/mmol) and [4, 5-^{3}H]-leucine (sp. act. 6 TBq/mmol) were obtained from Amersham (Arlington Heights, IL). Tissue culture flasks (75 and 25 cm^2) and multiwell dishes were obtained from Costar Corp. (Cambridge, MA). Purified BC, LUT and echinenone were gifts from Dr. Fred Khachik, USDA, Beltsville Human Nutrition Research Center.

Cell Culture. HepG2 cells were purchased from American Type Tissue Collection (ATCC, Rockville, MD) and stock cultures were maintained in T75 flasks. For experiments, cells were seeded in multiwell plastic dishes (15 or 35 mm diameter) or T25 flasks and incubated in a humidified atmosphere of air/CO_2 (95:5 v/v) at 37^{0}C. HepG2 cultures (passages 80-90) were grown in MEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 1 mmol/L pyruvate, 2 mmol/L glutamine, 50 mg/L gentamicin, 0.5 mg/L fungizone and 15 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0 (complete medium). Medium

was replaced every two days and experiments were conducted with cultures at 1-3 days post-confluency.

Experiments were initiated by washing confluent cultures twice with Hank's Balanced Salt Solution (HBSS) without phenol red at 37°C. Serum-free complete MEM alone (control) or containing 500 μmol/L tert-butylhydroperoxide (TBHP) was added and cultures were incubated at 37°C for 90 minutes. The indicated conditions for the oxidative stress were selected as appropriate after performing pilot studies that examined the effects of varying the concentration of TBHP and length of exposure on cell viability, levels of TBARS and LDH release from cell monolayers (see below).

Micellar Preparation and Cellular Uptake of Carotenoids and α–Tocopherol. Carotenoids and dl-α-tocopherol (α-TC) were delivered to cells as micelles that had been prepared by modification of the procedure of El-Gorab and Underwood (1973). All manipulations with carotenoids were performed under yellow fluorescent lights to minimize photodecomposition. Appropriate volumes of stock solutions of the following compounds in either ethanol, hexane or chloroform/methanol were transferred to glass bottles to yield the following final concentrations after dilution with complete medium: l-α-phosphatidylcholine (16.7 μmol/L), monoolein (100 μmol/L), taurocholic acid (1.7 mmol/L), oleic acid (33.3 μmol/L) and either BC, LUT or α-TC (15 μmol/L). Pilot studies were performed to examine the cytotoxic potential of the micellar preparation at the indicated and higher concentrations in which the ratio of the individual components was maintained constant. Morphological appearance of the monolayer and release of cytoplasmic LDH (see below) were the same in cultures incubated for at least 24 hours in complete MEM alone or containing micelles with the components at the final concentrations listed above.

However, increasing the quantity of the micellar components by ≥ 50% had an adverse impact on the integrity of the monolayer.

After solvents were evaporated with a gentle stream of nitrogen, complete cell culture medium was added to the bottles. Samples were transferred to a water bath sonicator (Sonic Systems, Inc., Newtown, PA) at room temperature and sonicated for three cycles of 5 minutes each with exchange of bath water between cycles. Samples were filter sterilized (SFCA syringe filter with 0.22 μmeter pore; Nalgene, Rochester, NY) to remove microcrystalline particles.

The concentration of micellar α-TC and carotenoids in filtered medium was measured by HPLC (Bieri et al., 1979, 1985). As indicated in Table 3.1, the filtered micellar preparation contained 73% of the LUT and 94% of the α-TC originally added. In contrast, filtered samples contained only 7% of the BC added to the original sample, i.e., 1.1 μmol/L BC. Relative solubility of BC in the micellar preparation, i.e. 7%, was similar to that reported by Canfield et al. (1990), i.e. 4-13%.

To examine cellular accumulation of the lipophilic test compounds from medium with micelles, confluent monolayers of HepG2 were exposed overnight (20 hours) to MEM containing the micellar preparation alone or with either BC, LUT or α-TC. After removal of medium, monolayers were washed two times with HBSS, collected in 1 mL 0.1 mmol/L phosphate buffer containing 10% ethanol with 0.45 μmol/mL butylated hydroxytoluene (BHT) and sonicated 3-5 seconds using a Sonic Dismembranator at 60% output (Fisher Scientific, Pittsburgh, PA). Aliquots (500 μL) of the sonicate were diluted with ethanol (500 μL) containing echinenone (0.2 μg/mL) and tocol (25 μg/mL) as internal standards for analysis of carotenoids and α-tocopherol,

TABLE 3.1

Medium concentrations and accumulation of α-tocopherol, β-carotene and lutein by HepG2 cells[1]

	Post-filtered Medium	Cells
	μmol/L	nmol/mg protein
β-carotene	1.1 ± 0.31[a]	0.32 ± 0.07[a]
lutein	10.9 ± 0.17[b]	3.39 ± 0.34[b]
α-tocopherol	14.1 ± 1.49[c]	2.58 ± 0.35[c]

[1]Aliquots (n=4) of pre- and post-filtered (0.22 μmeter pore) medium were analyzed for two separate experiments and cellular content of test compounds was determined for triplicate cultures from at least two separate experiments. Cellular levels of α-tocopherol and carotenoids in medium containing micelle only were <0.03 and <0.02 μg/mg protein, respectively. Different letters as superscripts indicate significant differences ($p<0.05$) among means within a column.

respectively. Samples were extracted into hexane containing BHT (0.45 μmol/mL), dried under nitrogen, reconstituted in the appropriate mobile phase and analyzed by HPLC as described below. The concentration of LUT in cell homogenates was approximately 10 fold that of BC, whereas the concentration of α-TC was intermediate between LUT and BC (Table 3.1).

HPLC Analysis. Carotenoid and α-TC were analyzed independently using HPLC (Hewlett-Packard, Model HP-1090, Avondale, PA) with a UV/visible dual beam detector with wavelengths set at 450 or 292 nm for carotenoids and α-TC, respectively, as described by Bieri et al. (1979, 1985). The column was a Microsorb-MV 5-μm 25 cm x 0.46 cm diameter C-18 ODS (Rainin, Woburn, MA) and was protected by a C18 Adsorbosphere, 5 μm particle size, 0.75 x 0.46 cm guard column (Alltech, Deerfield, IL). The mobile phase for the analysis of carotenoids was 70:20:10 (v/v/v) acetonitrile (with 0.13% (v/v) triethylamine):methylene chloride:methanol containing 0.01% (w/v) ammonium acetate. Methanol was used as the mobile phase for the analysis of α-TC. The flow rates throughout the isocratic runs were 1.7 and 2.5 mL/min for analysis of carotenoids and α-TC, respectively. The column temperature was maintained at 20°C by using a 40 cm HPLC column water jacket (Alltech, Deerfield, IL) and a constant-temperature circulator (Fisher model 910; Fisher Scientific, McGaw Park, IL). Data were calculated based on the analysis of a series of external standards that were >94% pure as verified by HPLC.

Evaluation of cytoprotective activity of carotenoids and α-TC against TBHP-induced damage

Thiobarbituric acid reactive substances (TBARS). Peroxidation of membrane lipids was estimated by measuring the levels of TBARS in cultures (medium plus monolayer) immediately

after a 90 min exposure ± 500 μmol/L TBHP by a modification of the method of Buege and Aust (1978). Briefly, 100 μL absolute ethanol containing 0.45 μmol/mL BHT was added to wells (9.6 cm^2) containing 2 mL MEM alone or with 500 μmol/L TBHP to quench further oxidation. An aliquot (1 mL) of the test medium was transferred to a 5 mL glass screw cap tube and residual medium was discarded. The monolayer was washed three times with HBSS at room temperature. Cellular material was collected in 1 mL harvest solution containing 10% (v/v) ethanol with 0.45 μmol/mL BHT, 3.5 mmol/L sodium dodecyl sulfate, and 1 mmol/L disodium ethylenediaminetetraacetic acid. After sonication for 10-20 seconds, one-half of the sample (500 μL) was added to the aliquot of medium in the glass tube and the remaining sample was frozen at -20°C for protein analysis. Tubes were heated at 95°C for 30 minutes after addition of 2 mL TBA solution (26 mmol/L thiobarbituric acid and 920 mmol/L trichloroacetic acid (TCA) in 250 mmol/L HCl). Samples were cooled in ice, mixed and aliquots were removed and centrifuged at 16,000 x g for 10 minutes at 25°C. Supernate was analyzed spectrophotometrically at 532 nm. Tetramethoxypropane was used as a standard and various concentrations were processed identically to samples. Data are expressed as pmol TBARS/mg cell protein.

Lactate dehydrogenase (LDH) release. The release of the cytoplasmic enzyme LDH (EC 1.1.1.27) into the medium by HepG2 monolayers during exposure to TBHP was used to assess the integrity of the cell membrane. Aliquots of medium were centrifuged for 5 minutes at 700 x g at 4°C to remove debris. Monolayers were washed with HBSS (25°C) and collected after incubation for 15 minutes in 1 mL 1% (v/v) Triton X-100. Cellular material was sonicated for 3-5 seconds on ice and the samples were centrifuged at 16,000 x g for 8 minutes at 4°C. LDH activity was determined according to Glascott et al. (1992). The reaction mixture contained 2.8 mL 0.1 mol/L phosphate buffer (pH 7.5) with 85 μmol/L NADH, 100 μL 23 mmol/L pyruvic acid, and 500 μL supernate from either undiluted medium or cell homogenate that had been diluted

1:50 with phosphate buffer. The rate of change in absorption at 340 nm was monitored for 3 minutes (Beckman DU-640 UV/visible spectrophotometer, Fullerton, CA). The percentage of cellular LDH released to the medium was calculated as follows:

% LDH release = [LDH activity in medium / (LDH activity in medium + LDH activity in cells)] x 100%

Pilot studies using either various dilutions of supernate from sonicated HepG2 cells that were not exposed to TBHP or purified rabbit muscle LDH showed that enzyme activity was not altered after exposure to 500 μmol/L TBHP for 90 minutes at 37°C.

^{14}C-Glucose Oxidation. After incubating cultures with or without TBHP for 90 min, monolayers (T25 flasks) were washed twice with HBSS at 37°C. MEM (5.5 mmol/L glucose) containing 10% FBS and 37 kBq of either D-[U-^{14}C]- or D-[6-^{14}C]-glucose was added to each flask and cultures incubated at 37°C for 2 hr. Production of $^{14}CO_2$ was measured as described by Ellwood et al. (1993). Data are expressed as nmoles glucose oxidized/(hr·mg cell protein).

Uptake and Incorporation of ^{3}H-Leucine. After removal of medium ± TBHP, monolayers were washed twice with HBSS before adding MEM containing 10% FBS and 9.25 kBq [4,5-^{3}H]-leucine. Cultures were incubated for 60 min at 37°C. Medium was collected and the cell monolayer washed twice with ice cold HBSS and harvested as described previously. Cell homogenates were sonicated for 3-5 sec, and an aliquot (200 μL) added to cold TCA (306 mmol/L) to precipitate ^{3}H-protein, vortexed and stored in ice for 15 min. Precipitated material was collected by centrifugation for 10 min at 16,000 x g, washed twice with 306 mmol/L TCA and resuspended in a small volume of 1 mol/L sodium hydroxide. Aliquots were transferred to vials containing 4 mL Ultima Gold scintillation cocktail (Packard, Meriden, CT) and analyzed by liquid

scintillation spectrophotometry (Beckman model 6000SE LS counter; Fullerton, CA) to quantify ^{3}H. To determine the amount of ^{3}H-protein secreted into the medium, a 200 μL sample was added to a tube containing an equal volume of water with bovine serum albumin (1 mg/mL) as carrier and processed as described for the cell homogenates. The level of acid precipitable ^{3}H in cells and medium represents protein synthesized during the labelling period. An additional aliquot of cell sonicate also was analyzed to determine total cellular radioactivity. ^{3}H-leucine uptake was estimated by adding total cellular ^{3}H and secretory protein ^{3}H.

Transport of ^{3}H-aminoisobutyric acid (^{3}H-AIB) and ^{3}H-deoxyglucose (^{3}H-DOG). Control and TBHP-treated monolayers were washed twice with 2 mL HBSS before adding an amino acid-free formulation of MEM without FBS and containing ^{3}H-AIB (9.25 kBq/mL) and 100 μmol/L AIB. Monolayers were incubated for 3 min at 37°C, washed twice with 2 mL amino acid-free MEM at 0°C and collected in 1 mL harvest solution. To measure ^{3}H-DOG uptake, control and TBHP-treated cultures were incubated with a glucose-free formulation of MEM without FBS and containing ^{3}H-DOG (9.25 kBq/mL) and 100 μmol/L DOG for 10 minutes. Radiolabeled medium was removed and monolayers were washed twice with 2 mL ice cold, glucose-free MEM containing 200 μmol/L phloretin to inhibit DOG efflux. Pilot studies showed that uptake of ^{3}H-AIB and ^{3}H-DOG were linear for 4 and 20 minutes, respectively, using the described conditions. Cellular ^{3}H was measured as described above and data are expressed as nmol/(min·mg protein).

Mitochondrial Activity. To examine the impact of treatment of cells with TBHP on mitochondrial activity, control and treated cells were washed twice with HBSS before the addition of serum-free MEM containing 60 μmol/L 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 30, 60 or 90 minutes. At indicated times, MEM was removed from replicate wells and dimethyl

sulfoxide (1 mL) was added to lyse cells and solubilize reduced crystals of MTT. After mixing for 5 minutes, the increase in optical density of reduced MTT was determined at 560 nm by UV/visible spectrophotometry (Scudiero et al., 1988).

Protein. Cellular protein was quantified as previously described (Ellwood et al.,1993).

Statistics. Data were analyzed using either Student's t test or one way ANOVA with post hoc analysis using Tukey's multiple range test to determine statistically significant differences ($p<0.05$; SAS Institute, Cary, NC). All experiments were repeated at least twice using triplicate cultures for each test parameter. Incubation of HepG2 cultures with β-carotene, lutein and α-tocopherol independent of pro-oxidant exposure did not have a statistically significant effect on any of the parameters used (see Materials and Methods). Consequently, data are expressed as a comparison of response of TBHP exposure versus control cultures not exposed to the oxidant.

RESULTS

Prior to evaluating the cytoprotective influence of carotenoids against pro-oxidant injury, it was necessary to establish a positive control for the protection of HepG2 cultures against TBHP-induced damage. Monolayers were incubated overnight in complete medium containing micelles with increasing concentrations of α-TC to examine the relationship between cellular content of α-TC and susceptibility to TBHP-induced injury. Cellular α-TC was increased significantly when the medium was supplemented with ≥3 μmol/L α-TC (Fig. 3.1, upper panel) and was correlated positively (r=0.84) with medium α-TC between 1-30 μmol/L. The level of TBARS was measured to assess peroxidation of membrane polyunsaturated fatty acids in control and TBHP-treated samples. TBHP-induced elevation of TBARS in cultures was attenuated by pre-treatment of monolayers with medium containing 1-30 μmol/L α-TC (Fig. 3.1, lower panel). TBARS levels in TBHP-treated cultures were inversely correlated (r= -0.86) with cellular levels of α-TC. To determine if the increase in TBARS was associated with a loss of membrane integrity, release of LDH into medium was measured. Exposure to TBHP increased release of LDH from a background level of 2.4 ± 0.2% to 12.0 ± 1.2 % (Fig. 3.1, lower panel). Pretreatment of cultures with as low as 0.3 μmol/L α-TC significantly decreased TBHP-mediated release of LDH from cells, whereas overnight incubation in medium containing ≥3 μmol/L α-TC completely blocked TBHP-mediated release of LDH. Addition of micelles alone to complete MEM did not affect the increases in TBARS levels and LDH release in response to exposure to TBHP. In subsequent studies, cultures were incubated overnight in medium containing 14 μmol/L α-TC, since this concentration represents the lower range for normal human plasma, i.e., 15-40 μmol/L (Keaney and Frei, 1994).

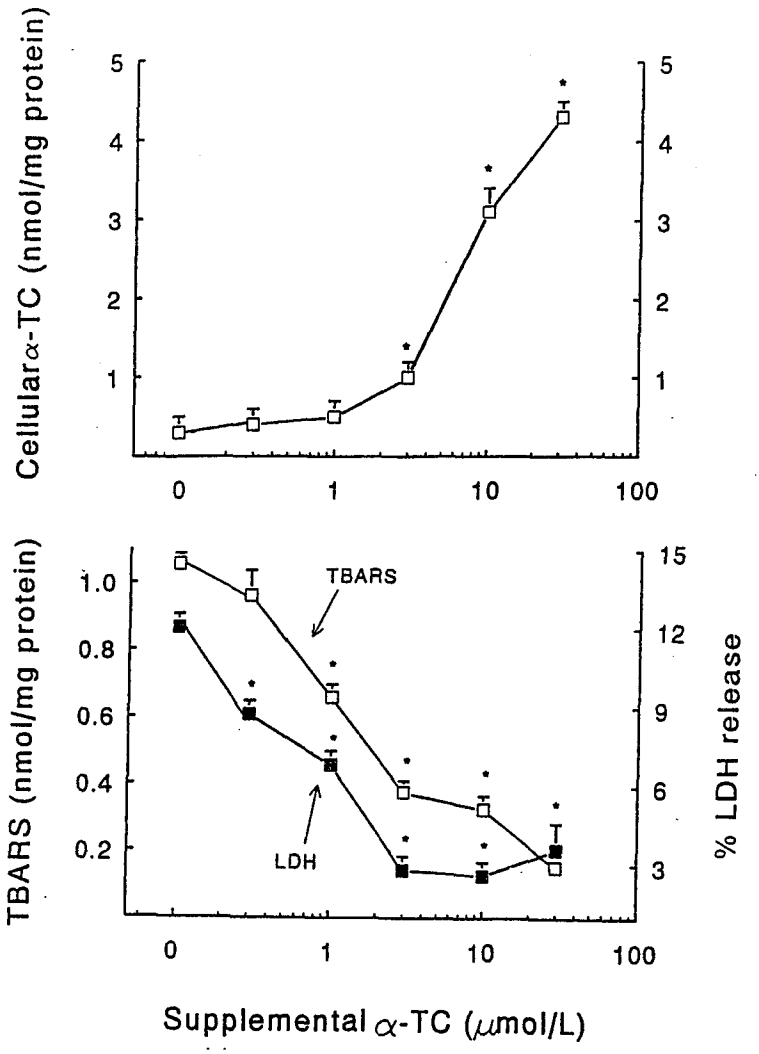

Figure 3.1. Accumulation and cytoprotective influence of α-TC in HepG2 cells incubated with supplemental α-TC.

Upper panel. Cultures were incubated for 20 hr with increasing concentrations of α-TC delivered in micelles before analyzing the cellular content of the antioxidant. Cultures incubated in complete medium with micelles but no supplemental α-TC contained 0.26 ± 0.12 nmol α-TC/mg protein. *Lower panel.* After loading cells with α-TC, replicate cultures were treated with 500 μmol/L TBHP for 90 min at 37°C before analyzing the levels of TBARS (open squares) and release of cytoplasmic LDH (closed squares). The levels of TBARS and LDH release in control cultures, i.e., not supplemented with α-TC or exposed to TBHP, were 0.15 ± 0.03 nmol/mg protein and 2.4 ± 0.2%, respectively. Data are the means ± SE from a representative experiment with at least three wells for each treatment. Significant differences ($p<0.05$) between groups treated with α-TC or micelles only are indicated by asterisks above the error bars.

Next, the impact of pre-treatment of cultures with either BC or LUT on susceptibility to pro-oxidant damage was determined. Overnight incubation in medium containing micelles with either BC (1.1 μmol/L), LUT (10.9 μmol/L) or α-TC (14.1 μmol/L) did not significantly alter basal levels of TBARS or LDH release in cultures treated with micelles only (data not shown). Prior exposure to medium containing BC and LUT attenuated TBHP-induced elevations in both TBARS (Fig. 3.2) and LDH release (Fig. 3.3), although the degree of protection conferred by the carotenoids was significantly less ($p<0.05$) than that observed for cultures that had been incubated with supplemental α-TC.

To assess whether the oxidant-induced biochemical changes outlined above were associated with alterations in cellular activities, the uptake and incorporation of ^{3}H-leucine into acid precipitable protein was compared in control and TBHP-treated cultures. Exposure of cultures to TBHP decreased cellular accumulation of ^{3}H-leucine in 60 min by 52% (15.8 ± 0.2 vs. 7.6 ± 0.4 nmol/mg protein for control and TBHP-treated cells, respectively; $p<0.05$). Overnight incubation of HepG2 with micelles containing BC, LUT and α-TC completely protected cells against TBHP-induced reduction of ^{3}H-leucine accumulation (Table 3.2). The percentage of cellular ^{3}H present in the acid insoluble fraction was similar in both control and TBHP-treated cultures suggesting that protein synthesis was not significantly altered by exposure to the pro-oxidant. These data suggested that TBHP impaired amino acid uptake. To address this possibility, the unidirectional transport of the non-metabolizable amino acid analogue α-aminoisobutyric acid (^{3}H-AIB) was measured in control and TBHP-treated cultures. As shown in Figure 4, treatment of cultures with TBHP decreased ^{3}H-AIB uptake to 16% that of control (0.71 ± 0.04 vs. 4.42 ± 0.13 nmol/(min·mg protein), respectively; $p<0.05$). Overnight incubation of cultures in medium containing BC and LUT attenuated the impact of TBHP treatment on ^{3}H-AIB transport; cellular uptake of ^{3}H-AIB by BC and LUT treated cultures exposed to TBHP was 33

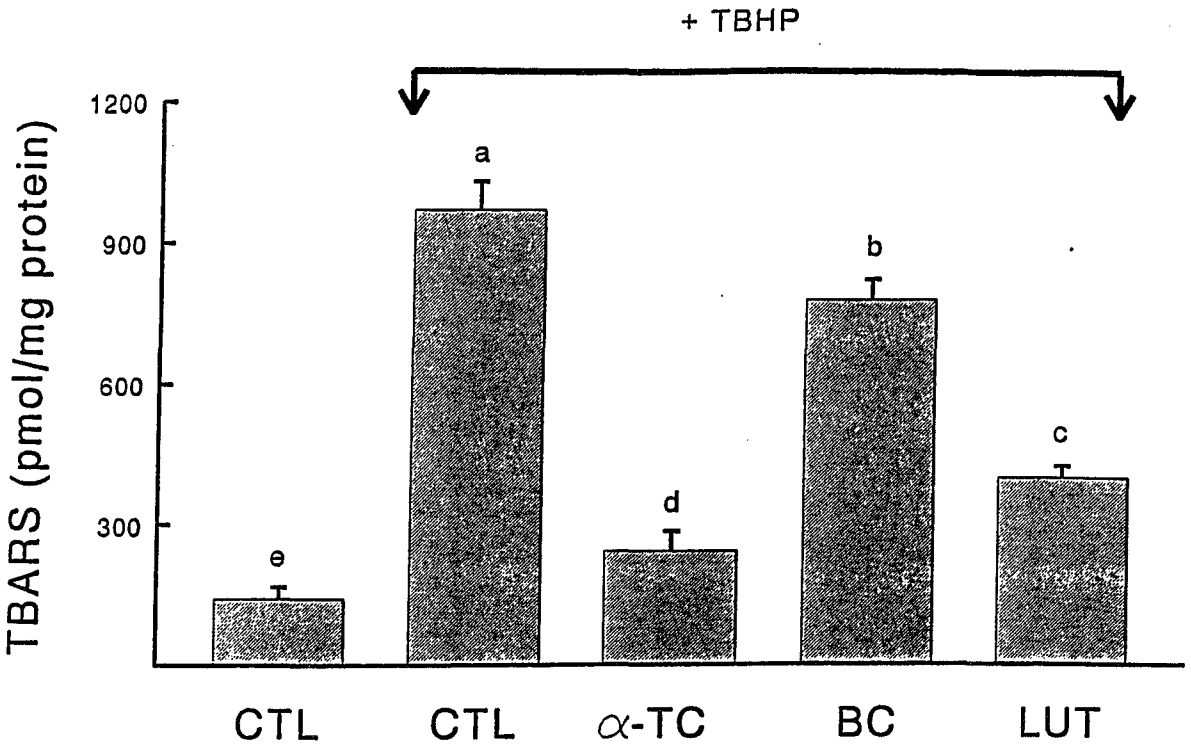

Figure 3.2. Preincubation with α-TC, BC and LUT attenuates TBHP-induced generation of TBARS in HepG2 cultures.

Cultures were incubated overnight in medium containing micelles alone or with either α-TC (14.1 μmol/L), BC (1.1 μmol/L) or LUT (10.9 μmol/L) as indicated in Table 3.1. After washing the monolayer, cultures were exposed to TBHP for 90 min and TBARS levels were measured as described in Materials and Methods. Data represent means ± SE for at least two separate experiments performed in triplicate. Different letters above the error bars indicate significant ($p<0.05$) differences between means of treatment groups.

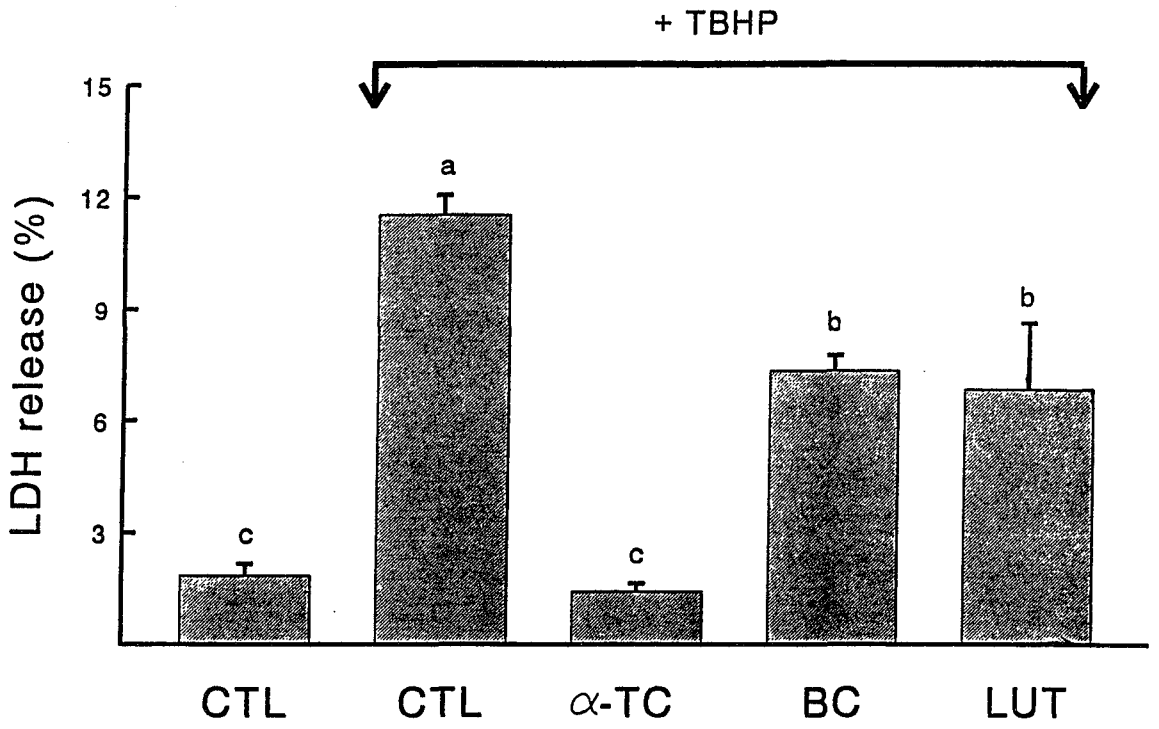

Figure 3.3. Preincubation with α-TC, BC and LUT decreases TBHP-induced release of cytoplasmic LDH in HepG2 cultures.

After overnight incubation with carotenoids or α-TC as described in the legend to Table 3.1, cultures were exposed to TBHP for 90 min and LDH release was determined. Data are means ± SE from at least two separate exeriments performed using triplicate wells. Different letters above the error bars indicate the means of the treatment groups differ significantly ($p<0.05$).

TABLE 3.2

Effect of exposure to TBHP on accumulation and incorporation of ^{3}H-Leucine in cultures of HepG2 pretreated overnight with α-tocopherol, β-carotene and lutein[1]

Treatment	-TBHP	+TBHP
Cellular accumulation of ^{3}H-Leucine	nmol/mg protein	
micelles only	202 ± 3	110 ± 5*
+β-carotene	195 ± 17	199 ± 12
+lutein	190 ± 21	172 ± 7
+α-tocopherol	217 ± 19	219 ± 12
Incorporation of ^{3}H-Leucine into protein	% acid ppt. cellular ^{3}H	
micelles only	36.3 ± 1.6	35.5 ± 1.3
+β-carotene	44.6 ± 8.3	41.2 ± 3.4
+lutein	41.3 ± 2.7	37.9 ± 2.2
+α-tocopherol	34.9 ± 1.4	31.1 ± 3.5

[1]Cultures were incubated overnight in complete medium containing micelles with or without either β-carotene (1.1 μmolL), lutein (10.9 μmol/L) or α-tocopherol (14.1 μmol/L). Medium was removed and monolayers were washed with HBSS before addition of MEM ±500 μmol/L TBHP and incubated for 90 min at 37°C. After washing the monolayers, complete MEM containing 9.25 kBq [4,5-^{3}H]-leucine was added and cultures were incubated for 1 hr at 37°C. Total cellular ^{3}H and the percentage of cellular ^{3}H incorporated into acid precipitable (ppt.) protein were determined as described in Materials and Methods. Data represent means ±SE for at least two experiments with three replicate wells per experiment. The presence of an asterisk as superscript indicates that means for control and TBHP-treated cultures differ significantly ($p<0.05$).

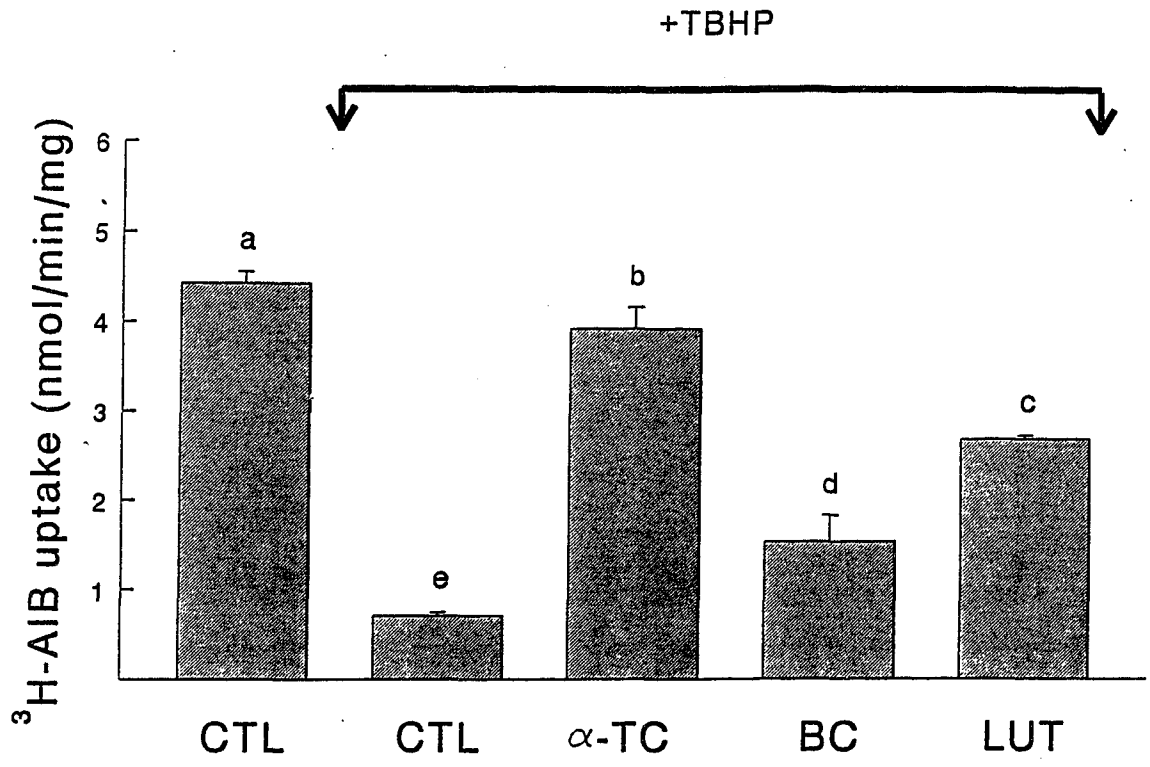

Figure 3.4. Pretreatment with α-TC, BC and LUT attenuates oxidant-induced reduction of ^{3}H-AIB transport in HepG2 cells.

Cultures were incubated overnight as described in Table 3.1. After 90 min exposure to TBHP, cultures were pulsed with MEM containing ^{3}H-AIB (18.5 kBq/well) for 3 min and uptake of the radiolabel was determined. Data represent means ± SE from at least three separate experiments each performed in triplicate. Different letters above the error bars indicate significant differences ($p<0.05$) between the means of the treatment groups.

and 60%, respectively, that of the control ($p<0.05$). Prior supplementation of cultures with α-TC was more effective than the carotenoids at blocking TBHP-induced damage to the ^{3}H-AIB transport process.

In a parallel series of studies, the impact of oxidant exposure on glucose utilization by HepG2 cells was examined. Cultures incubated with uniformly labeled (U)-^{14}C-glucose after exposure to TBHP generated only 56% as much $^{14}CO_2$ as controls ($p<0.05$). Because mammalian cells respond to many toxicants by increasing the activity of the hexose monophosphate pathway, the apparent reduction in glucose oxidation caused by TBHP may have reflected altered utilization of the substrate rather than impaired glucose oxidation. Therefore, we next compared 6-^{14}C-glucose oxidation by control and TBHP treated cultures (Figure 3.5), since the production of $^{14}CO_2$ from this compound is primarily due to glycolytic activity (Katz and Wood, 1960). Overnight incubation of cultures in medium containing BC, LUT and α-TC completely protected cells against the TBHP-mediated reduction in 6-^{14}C-glucose oxidation. Since TBHP decreased ^{3}H-AIB uptake (see above), it was necessary to consider whether the apparent decrease in ^{14}C-glucose oxidation in TBHP-treated cells might reflect impaired transport of glucose across the plasma membrane, i.e., decreased cellular acquisition of the radiolabel. Similar to results for the oxidation of 6-^{14}C-glucose, ^{3}H-DOG uptake by TBHP-treated cultures was only 55% that of the control (Figure 3.6). Overnight incubation of cultures in medium containing BC, LUT and α-TC completely protected cells against the adverse effects of TBHP on ^{3}H-DOG transport. These data suggest that glucose transport, but not its utilization, was adversely affected by treatment of cells with TBHP.

Since nutrient and electrolyte transport processes are often energy-dependent, the mitochondrial activity of control and TBHP-treated HepG2 cultures was examined. Exposure of

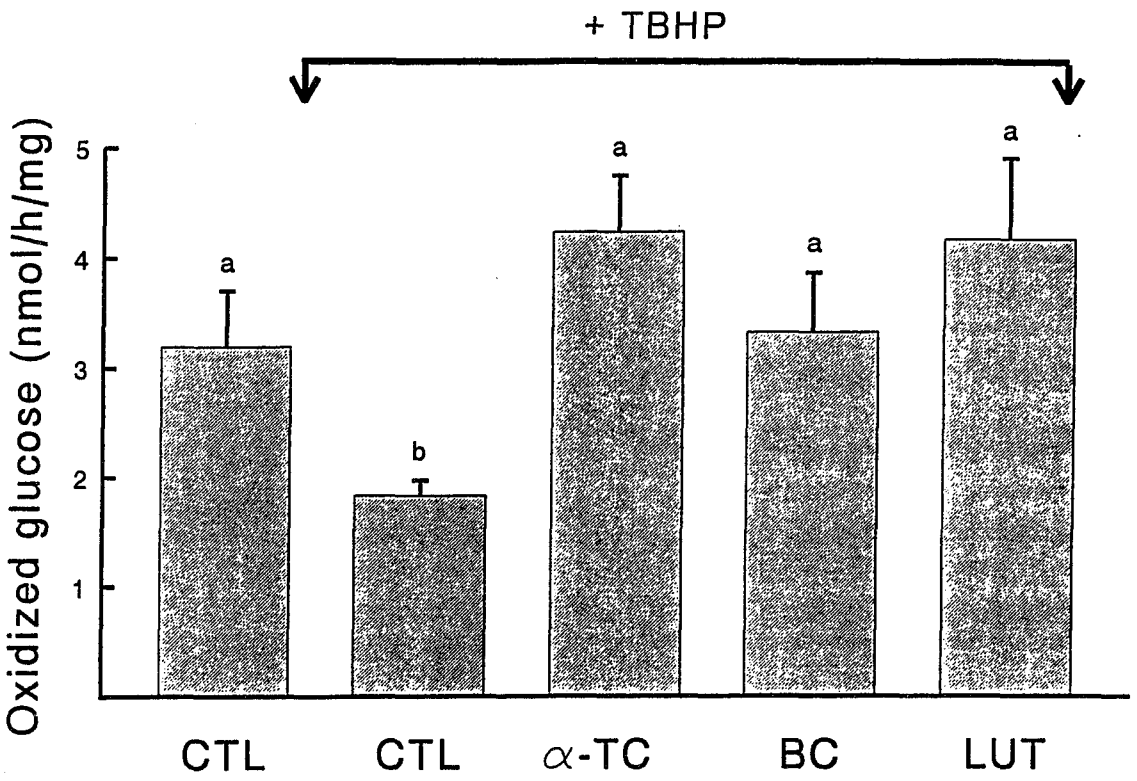

Figure 3.5. α-TC, BC and LUT protects HepG2 cells against TBHP-mediated suppression of glucose oxidation.

Cultures were incubated overnight as outlined in Table 3.1. After exposure to TBHP, production of $^{14}CO_2$ was measured in cultures incubated for 2 hr with 6-^{14}C-glucose. The data represent means ± SE from at least three experiments performed in triplicate. An asterisk above the error bar indicates that values for the treatment groups differ significantly ($p<0.05$).

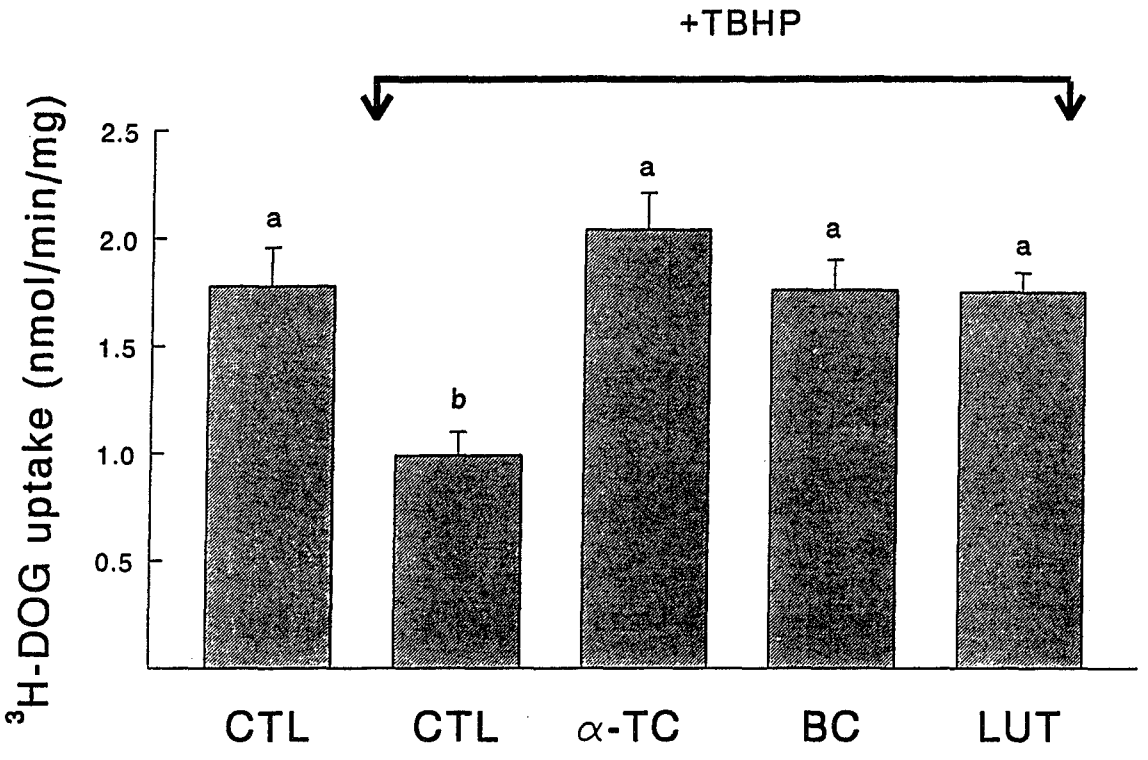

Figure 3.6. TBHP does not impair ^{3}H-DOG transport by HepG2 cells pretreated with α-TC, BC and LUT.

Cultures were incubated overnight as described in Table 3.1. Cultures were exposed to pro-oxidant and subsequently incubated for 10 min in MEM containing ^{3}H-DOG to determine cellular uptake of the glucose analogue. Data represent means ± SE from at least three experiments each performed in triplicate. Letters above the error bars indicate significant differences among the means of the treatment groups.

cells to TBHP did not decrease mitochondrial activity as assessed by metabolic reduction of the tetrazolium dye MTT (data not shown).

DISCUSSION

Our primary goal was to evaluate the cytoprotective influence of BC and LUT, carotenoids with and without provitamin A activity, respectively, against oxidative stress in a highly differentiated human liver cell model, viz., HepG2 cells. The data demonstrate that preincubation of cells in medium containing micellar preparations of BC and LUT provided cells with partial to full protection against oxidant-mediated changes in plasma membrane integrity and the uptake of analogues of glucose and alanine. This confirms and extends the observations of several other investigators who have examined the impact of carotenoid treatment on the status of animal fibroblast cell lines. Zhang et al. (1991) found that long term treatment with carotenoids (3 μmol/L), including BC and LUT introduced in acetone and tetrahydrofuran, respectively, decreased levels of TBARS in cultures of 10T1/2 mouse embryo fibroblasts. Similarly, Franke et al. (1995) reported that treatment with ≤10 μmol/L BC (ethanol vehicle) decreased lipid peroxidation in 10T1/2 cell cultures exposed to α-linolenic acid. Lawlor and O'Brien (1995a, 1995b) also have shown that ≤10 μmol/L BC and astaxanthin delivered in ethanol protected chick embryo fibroblasts against paraquat-induced changes in the activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. Our conclusion that BC and LUT protected HepG2 cells against oxidative damage is further supported by demonstration that preincubation of HepG2 cells in medium containing as low as 1 μmol/L α-TC conferred partial resistance against TBHP-mediated elevation of TBARS and LDH release. α-TC is recognized as one of the most efficient lipophilic antioxidants and protects cell membranes from peroxidative damage (Packer, 1991). These findings agree with previous reports that addition of ≤ 1 μmol/L α-TC to medium protected rat hepatocytes (Glascott et al., 1992) and HepG2 cells (Turley and Brewster, 1993) against oxidant-induced damage.

Our original objective was to compare the relative effectiveness of the two carotenoids to one another and to α-TC. We had hoped to incubate cultures in medium with similar concentrations, i.e., 15 μmol/L of carotenoids and α-TC. However, HPLC analysis of the concentration of the test compounds in filtered medium revealed that BC was poorly solubilized by micelles. As shown in Table 3.1, the final concentration of BC in filtered medium was only 1.1 μmol/L, whereas the concentrations of LUT and α-TC were 10 and 14-fold higher, respectively. The cellular concentration of BC was about 10% that of LUT, suggesting that accumulation of these carotenoids were proportional to their levels in medium. Canfield et al. (1990) reported that the efficiency of BC incorporation into micelles was maximum at 15 μmol/L, and that the relative amount of BC incorporated into micelles varied between 4 and 13% of the initial concentration. Similarly, Levin and Mokady (1995) reported that the maximum solubility of BC in micelles was 14 μmol/L and also that the isomer composition of BC mixtures affected the total amount of BC incorporated. It is likely that the low solubility of BC in our system was due in part to the need to decrease the concentrations of the components used for the preparation of micelles to prevent cytotoxicity. Also, taurocholate was the only bile acid in our preparation, whereas the formulation used by Canfield et al. (1990) contained taurocholate and 5 additional bile salts. Exclusion of the other bile acids may have affected the efficiency of BC incorporation, since the types and relative quantities of lipids will influence the structure and size of micelles (El-Gorab and Underwood, 1973). Additionally, we used a relatively short time for solubilization of these compounds into micellar preparations and filter sterilized (0.22 μmeter pore) the material before use, thereby removing microcrystalline particles.

Both BC and LUT attenuated, but did not prevent, TBHP-induced increases in TBARS and LDH release. Pre-treatment with α-TC prevented the enhanced leakage of cytoplasmic LDH and significantly reduced ($p<0.05$) the rise in TBARS after exposure to TBHP. However, various

factors preclude direct comparison of the antioxidant potency of the carotenoids and α-TC in our model. First, as discussed above, the final concentrations of BC, LUT and α-TC in medium and cells differed. Second, cellular concentration by itself does not provide insights about subcellular distribution and spatial orientation of the carotenoid within membranes (Britton, 1995). Third, we did not consider the types or concentrations of carotenoid metabolites present in the cells. Khachik et al. (1995) have discussed the potential role of carotenoid metabolites as mediators of the biological properties that are normally associated with their parent compounds. Finally, sites of toxicant-induced damage are likely to be influenced by both the chemical properties of the free radical produced and the phenotypic characteristics of the cell. Therefore, specific conditions will influence the effectiveness of antioxidants as cytoprotective agents against free radical-induced damage.

One proposed mechanism of damage by the pro-oxidant TBHP is the initiation and propagation of lipid peroxidation of polyunsaturated fatty acids in the plasma membrane. However, TBHP or its metabolites may also interact with and damage various intracellular processes (Aw and Rhoads, 1994). Olson (1994) has argued that evaluation of regulated cellular activities provides greater sensitivity for detecting oxidative insult than simply monitoring the loss of barrier integrity or lethality. Therefore, we monitored the impact of TBHP exposure on the ability of cultures to incorporate ^{3}H-leucine into protein. Exposure to the pro-oxidant decreased cellular accumulation of the amino acid, but did not alter its relative incorporation into protein. This suggested that TBHP-induced damage was localized to the plasma membrane in our model. In support of this possibility, ^{3}H-AIB uptake was suppressed by 84% after TBHP treatment. It is noteworthy that ^{3}H-leucine and ^{3}H-AIB uptake are mediated by different amino acid transporters, viz., systems L and A, respectively (Goenner et al., 1992), suggesting that TBHP adversely affected amino acid transporters. TBHP also has been reported to alter the

transport of ions in several cell types. Srivastava et al. (1989) found that TBHP treatment increased sodium entry into myocytes by altering Na^+-channels through increased lipid peroxidation. Elliott and Koliwad (1995) found that exposure of endothelial cells to TBHP markedly inhibited agonist-stimulated uptake of Ca^{2+} across the plasma membrane, and that this impairment appeared to be due to an alteration in the membrane transport protein rather than to lipid peroxidation. TBHP-mediated reduction in ^{3}H-AIB transport may result from altered membrane fluidity, conformational changes of the transporter due to peroxidation of membrane lipids in the local environment, or oxidative modification of the transporter protein itself. Since BC, LUT and α-TC all significantly ($p<0.05$) attenuated the impact of TBHP exposure on ^{3}H-AIB uptake by HepG2 cells, it appears that these compounds or their metabolites conferred partial or complete protection against oxidant-induced alterations in the plasma membrane. This protective influence was not limited to amino acid transporters, since the carotenoids also prevented TBHP-induced impairment in ^{3}H-DOG transport. These data agree with reports by Vroegop et al. (1995) who recently reported marked impairment of ^{3}H-AIB and ^{3}H-DOG transport in neuron-derived hybridoma cells exposed to the lipophilic pro-oxidant cumene hydroperoxide.

Transport activities are generally energy-dependent processes requiring ATP for function of the Na^+/K^+ pump, maintenance of electrochemical gradients and proper function of the transporters. Exposure of HepG2 cells to TBHP did not affect mitochondrial activity. Similarly, cumene hydroperoxide did not adversely affect mitochondrial function in neural hybridoma cells (Vroegop et al., 1995). Thus, impaired transport of ^{3}H-AIB and ^{3}H-DOG by TBHP-treated HepG2 cells appears to be due to direct damage of the cell surface rather than a limitation in energy required to maintain nutrient transport.

In summary, both BC and LUT partially or completely protected HepG2 human liver cells against free radical-mediated damage as assessed by a panel of biochemical and functional indicators. The protective influence of LUT indicates that the carotenoids do not need to be converted to retinoids for occurrence of this phenomenon. It is unknown if the parent compounds and/or their metabolites are the species that act as antioxidants within cells. The implications of these findings are that increased consumption of carotenoid-rich fruits and vegetables may contribute to the protection of human cells against the adverse effects of free radicals on cellular integrity.

CHAPTER IV

HUMAN LIPOPROTEINS AS A VEHICLE FOR THE DELIVERY OF β-CAROTENE AND α-TOCOPHEROL TO HEPG2 CELLS

ABSTRACT

Highly differentiated human cell lines represent a useful in vitro model for the study of carotenoid uptake, metabolism and function. However, research using cell culture systems has been hindered due to the marked hydrophobicity of these compounds in aqueous systems and the difficulty of presenting carotenoids in a bioavailable form. These compounds are usually introduced into tissue culture media either in organic solvents or as micelles, whereas carotenoids are localized in lipoproteins in plasma. Initially, the stability of β-carotene and α-tocopherol in micelles and human lipoproteins under standard tissue culture conditions was compared. After filtration (0.22 μmeter pore) and overnight incubation of β-carotene and α-tocopherol in serum-free medium without cells, recovery of β-carotene and α-tocopherol was 27 ± 2 and 73 ± 2%, respectively. In contrast, recovery of β-carotene and α-tocopherol was ≥ 88% when medium containing total lipoproteins (1 mg/mL) isolated from β-carotene supplemented individuals (60 mg/d for 4 weeks) was incubated overnight without cells. The marked loss of β-carotene from medium containing micellar preparations was the result of oxidative modification, since β-carotene recovery was increased considerably by inclusion of α-tocopherol in micelles. Overnight incubation of confluent HepG2 cultures with lipoproteins containing 1 μmol/L β-carotene or α-tocopherol increased cellular levels from < 0.02 to 0.17 and < 0.03 to 0.42 μg/mg protein, respectively. Cellular accumulation of β-carotene and α-tocopherol was proportional to the concentrations of the lipophilic compounds (r=0.94 for β-carotene and 0.74 for α-tocopherol).

Similarly, cellular levels of β-carotene and α-tocopherol were positively correlated with the concentration of the compounds in medium containing either low density lipoprotein (r=0.85 for β-carotene; r=0.74 for α-tocopherol) or high density lipoproteins (r=0.82 or β-carotene and r=0.66 for α-tocopherol). These data show that lipoproteins represent a stable vehicle for delivery of β-carotene and α-tocopherol to HepG2 human liver cells.

INTRODUCTION

Dietary carotenoids are absorbed into the blood via the lymphatic system and transported in circulation by plasma lipoproteins (Erdman et al., 1993). LDL carries 55-80% of the total carotenoids in the plasma of healthy subjects (Clevidence and Bieri, 1993). For example, Johnson and Russell (1992) reported that 79, 8 and 12% of the β-carotene (BC) in human serum was present in the low density lipoprotein (LDL), high density lipoprotein (HDL), and very low density lipoprotein (VLDL) fractions, respectively. The types and quantities of carotenoids present in many human tissues generally are reflective of those in plasma, suggesting that there is effective transfer of carotenoids from plasma lipoproteins to tissues (Clevidence and Bieri, 1993).

Detailed investigation of the transport and tissue specific accumulation of carotenoids has been hampered somewhat by the lack of appropriate animal models. Human and animal cells appear to represent useful models for studying the accumulation and metabolism of carotenoids. However, the extreme hydrophobicity and relative instability of carotenoids in air makes their delivery to cultured cells problematic. Various investigators have added carotenoids to tissue culture media using organic solvents (e.g., tetrahydrofuran, ethanol and dimethyl sulfoxide), liposomes, water miscible beadlets and micelles as vehicles (Cooney et al., 1993). Vehicle alone is usually added to replicate cultures to monitor potential cytotoxicity of the carrier. However, the long-term solubility and stability of the test carotenoids during incubation using standard tissue culture conditions has received minimal attention. Moreover, it is not clear if the carotenoids partition into lipoproteins, their physiologic carrier, in serum-containing tissue culture medium when introduced in the vehicles indicated above. In the present study, we assessed the influence of standard tissue culture conditions on the stability of BC and α-tocopherol (α-TC) added to serum-free medium as either micelles or lipoproteins. We also examined the

effectiveness of lipoproteins as vehicles for the delivery of BC and α-TC to HepG2 human liver cells. This highly differentiated cell line was selected as the in vitro model because it secretes plasma proteins, expresses an inducible cytochrome P450 system, and responds to hormones and cytokines in a manner similar to normal human liver (Javitt, 1990; Knowles et al., 1980). Moreover, HepG2 cells possess specific receptors for apolipoproteins B and E, catabolize LDL, HDL and apoprotein E-containing particles, and synthesize VLDL, LDL and HDL (Javitt, 1990).

MATERIALS AND METHODS

Supplies. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Tissue culture flasks (T75) and multiwell dishes (35 mm diameter) were obtained from Costar Corp. (Cambridge, MA). Capsules containing synthetic β-carotene (30 mg/capsule) and placebos were provided by Hoffman-LaRoche (Nutley, NJ).

Subjects. Five healthy, normolipemic adult males (25-47 yrs of age) in our laboratory volunteered to ingest either a BC supplement (60 mg/d; n=2) or placebo (n=3) at 2 meals per day for 4 wk. Blood samples were collected by a trained phlebotomist at various times during and after the supplementation period to prepare lipoprotein fractions containing different levels of β-carotene. Two independent supplementation studies were conducted with an 8 wk washout period to allow plasma BC levels of BC-supplemented subjects to return to baseline levels. In the first study, blood samples were collected at the beginning and the end of the 4 wk supplementation period from placebo and β-carotene supplemented subjects. Additional blood samples were collected at 2 and 4 weeks post-supplementation from BC-supplemented subjects to obtain total lipoproteins with different concentrations of BC. In the second study, blood samples were collected at the beginning and the end of the 4 wk supplementation period and at

2 wk post supplementation. In this study, samples of total lipoproteins were also fractionated to obtain LDL and HDL.

Isolation of lipoproteins. Blood was collected by venipuncture into vacutainer tubes containing Na_2-EDTA (1 mmol/L) as a preservative after an overnight fast. Plasma was separated from whole blood by centrifugation at 1,500 x g for 25 min at 4°C and transferred to glass vials using glass Pasteur pipets. Lipoproteins were isolated as described by Lanningham-Foster et al. (1995). Briefly, the density of plasma (d=1.006 g/mL) from each subject was elevated to 1.21 g/mL by addition of solid potassium bromide (0.3265 g/mL). Aliquots of plasma (1.8 mL) were transferred to polyallomar tubes for the Beckman 50.3 Ti rotor and overlaid with 4.3 mL of a sodium chloride solution (d=1.006 g/mL) containing 0.01% sodium EDTA. To flotate lipoprotein particles, tubes were centrifuged for 40 hr (Beckman L7-65 ultracentrifuge, Fullerton, CA) at 114,000 x g at 18°C with automatic soft-start and braking disengaged at 800 rpm. After recovery of the total lipoprotein fraction (LP) that was essentially free of albumin, the volume was readjusted to a final density of 1.21 g/mL This preparation (1.8 mL in 50.3 Ti rotor tube) was overlaid with sodium chloride solution (d=1.006 g/mL) and centrifuged for 4 hr as described above. After ultracentrifugation, the HDL and LDL lipoprotein bands were readily visualized by the presence of carotenoids in the fractions. The VLDL band was visualized as the top white layer in the centrifuge tube. The lipoprotein fractions were collected using syringe/needle assemblies, transferred to glass tubes and stored in the dark under a nitrogen blanket at 4°C for a maximum of two wk. Prior to use in experiments, KBr was removed by passing the lipoprotein sample through a gel filtration column (Econo-Pac 10DG, Bio-Rad, Hercules, CA) packed with a polyacrylamide gel matrix with a 6 kDa pore size and equilibrated with phosphate buffered saline, pH 7.5. Protein levels of total lipoprotein (LP), LDL and HDL fractions were measured by the Lowry procedure as modified by Markwell et al. (1981).

Cell culture. HepG2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and stock cultures were maintained in T75 flasks. For experiments, cells were seeded in multiwell plastic dishes (35 mm diameter) and incubated in a humidified atmosphere of air/CO_2 (95:5 v/v) at 37^{0}C. HepG2 cultures (passages 80-90) were grown in MEM supplemented with 10% fetal bovine serum (FBS), 1 mmol/L pyruvate, 2 mmol/L glutamine, 50 mg/L gentamicin, 0.5 mg/L fungizone and 15 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.0 (complete medium). Medium was replaced every two days and experiments were conducted with cultures at 1-3 days post-confluency.

Micellar preparations of BC and α-TC. Appropriate volumes of stock solutions of the following compounds in either ethanol, hexane or chloroform/methanol were transferred to glass bottles to yield the following final concentrations after dilution with complete medium: l-α-phosphatidylcholine (16.7 μmol/L), monoolein (100 μmol/L), taurocholic acid (1.7 mmol/L), oleic acid (33.3 μmol/L) and either BC or α-TC (15 μmol/L). In one study, micelles containing both BC and α-TC were prepared. After solvents were evaporated with a gentle stream of nitrogen, complete cell culture medium was added to bottles which were then transferred to a water bath sonicator (Sonic Systems, Inc., Newton, PA) at room temperature and sonicated for three cycles of 5 min each. Samples were filter sterilized (SCFA syringe filter with 0.22 μmeter pore, Nalgene, Rochester, NY) before incubating overnight in plastic multiwell dishes. In other studies (Chapter 3), it was shown that the described micellar preparation did not alter the morphological appearance of the monolayer or compromise the integrity of the cell surface.

In vitro stability of BC and α-TC. The influence of standard cell culture conditions on BC and α-TC was assessed by overnight incubation of complete medium containing either micellar

preparations or total lipoproteins (1 mg protein/well) in tissue culture dishes without cells. The lipoprotein fraction was prepared from BC-supplemented subjects (n=2 subjects) during each of the two trial periods. Aliquots of medium containing either micelle or lipoprotein were extracted and analyzed as described below immediately after filter sterilization and incubation for 20 hr at 37^{o}C in a humidified atmosphere of air/CO_2 (95:5 v/v). Each test sample was incubated in triplicate wells.

Cellular accumulation of BC and α-TC. Carotenoids and α-tocopherol were introduced into confluent cultures of HepG2 cells by using lipoproteins as vehicles. Experiments were initiated by overnight incubation of confluent cultures in serum-free medium containing 0.1% ITS (insulin, transferrin and selenium; Collaborative Research, Bedford, MA) to increase expression of lipoprotein receptors on the cell surface (Javitt, 1990). Monolayers were washed and incubated with 1 mL serum-free MEM containing 0.1% ITS (v/v) and either the indicated lipoprotein fraction or PBS vehicle (control). The concentrations of total LP, HDL and LDL were 1.0, 1.0 and 0.5 mg protein/mL, respectively. The lower concentration of LDL protein was due to the limited availability of this material. Pilot studies showed that incubation of HepG2 cultures with concentrations of total lipoproteins up to 3 mg/mL did not adversely affect morphology or cellular integrity of the monolayer. After incubation of cultures in lipoprotein-containing medium for 20 hr, monolayers were washed twice with HBSS at 0^{o}C, collected in 1 mL 0.1 mmol/L phosphate buffer (pH 7.5), and sonicated for 3-5 seconds using a Sonic Dismembranator at 60% power output (Fisher Scientific, Pittsburgh, PA). Aliquots (500 μL) of the sonicate were diluted with an equal volume of ethanol (500 μL) containing either echinenone (0.2 μg/mL) or tocol (25 μg/mL) as internal standards for analysis of carotenoids or α-tocopherol, respectively. Hydrophobic materials were extracted into hexane, dried under nitrogen, reconstituted in the appropriate mobile phase, and analyzed by HPLC as below.

HPLC Analysis. BC and α-TC were analyzed by HPLC (Hewlett-Packard, model HP-1090, Avondale, PA) with a UV/visible dual beam photometer with wavelengths set at 450 nm for carotenoids (Bieri et al., 1985) and 292 nm for α-tocopherol (Bieri et al., 1979). The column was a Microsorb-MV 5-μmeter 25 cm x 0.46 cm diameter C-18 ODS (Rainin, Wobum, MA) and was protected by a C-18 Adsorbosphere, 5 μmeter particle size, 0.75 x 0.46 cm guard column (Alltech, Deerfield, IL). The mobile phase consisted of 70:20:10 (v/v/v) acetonitrile (with 0.13% triethylamine):methylene chloride:methanol (with 0.01% ammonium acetate) at a flow rate of 1.7 mL/min for estimation of carotenoid concentrations. The mobile phase for quantification of α-TC was methanol at a flow rate of 2.5 mL/min. The column temperature was maintained at 20^{0}C using a 40 cm HPLC water jacket (Alltech, Deerfield, IL). Data were calculated based on external standards that were >94% pure as verified by HPLC. The limits of detection for β-carotene and α-tocopherol were 1 and 8 pmol, respectively. Retention times for BC and α-TC were 14.1 and 5.7 min, respectively. Retention times for other carotenoids, tocopherols and retinoids of nutritional interest were also determined using purified standards. Representative chromatographic profiles for carotenoids and tocopherols in plasma from control (placebo) and β-carotene supplemented individuals are shown in Figures 4.1-4.4 .

Chemical determinations. Triacylglycerol and cholesterol levels in plasma and lipoprotein fractions were determined by commercial kits obtained from Sigma (St. Louis, MO). The protein content of cells was determined by a modification of the method by Lowry et al. (1951) using bovine serum albumin as a standard.

Analysis of data. Student's t test was used to determine differences between means. Differences were considered significant at $p < 0.05$.

Fig. 4.1

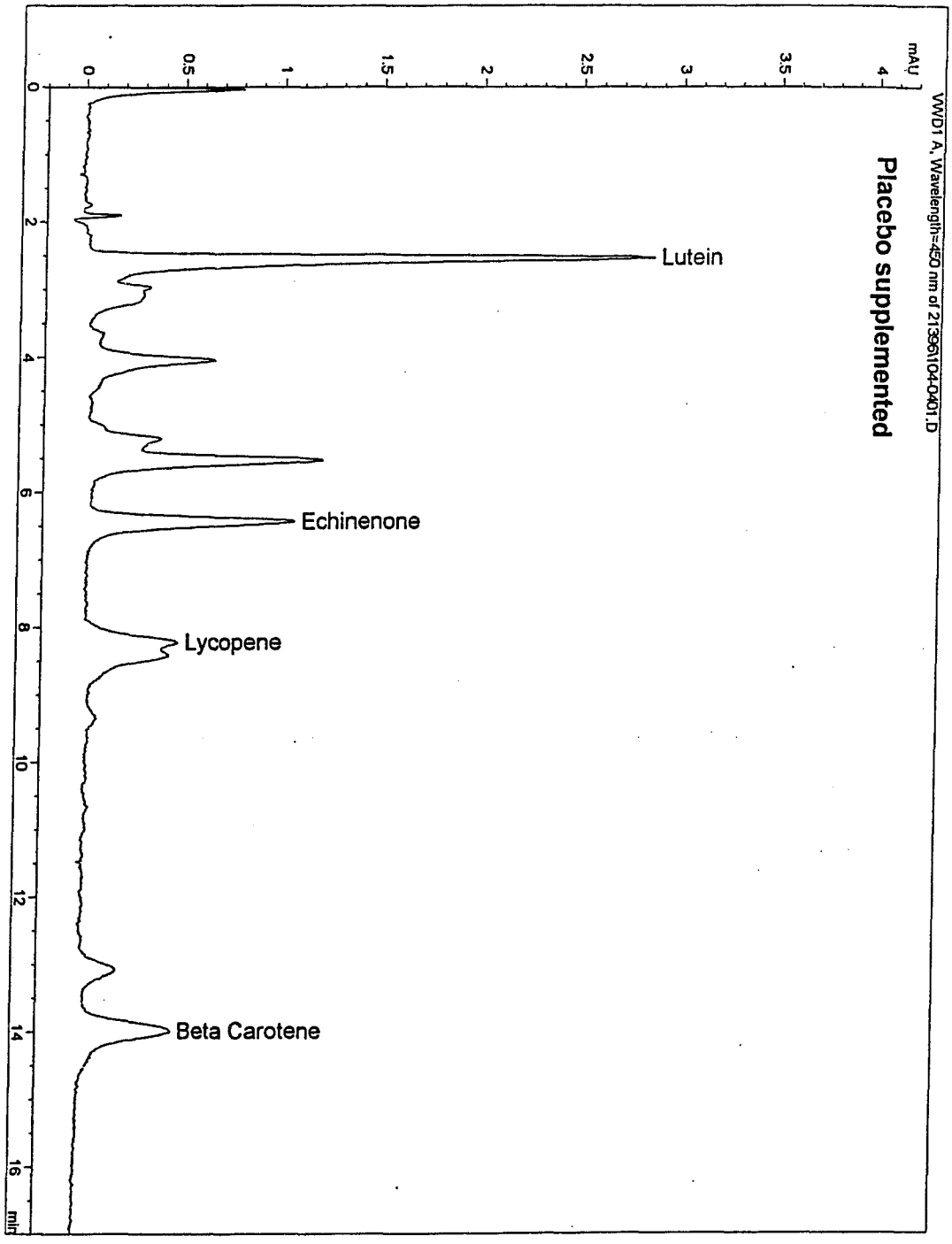

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Fig. 4.2

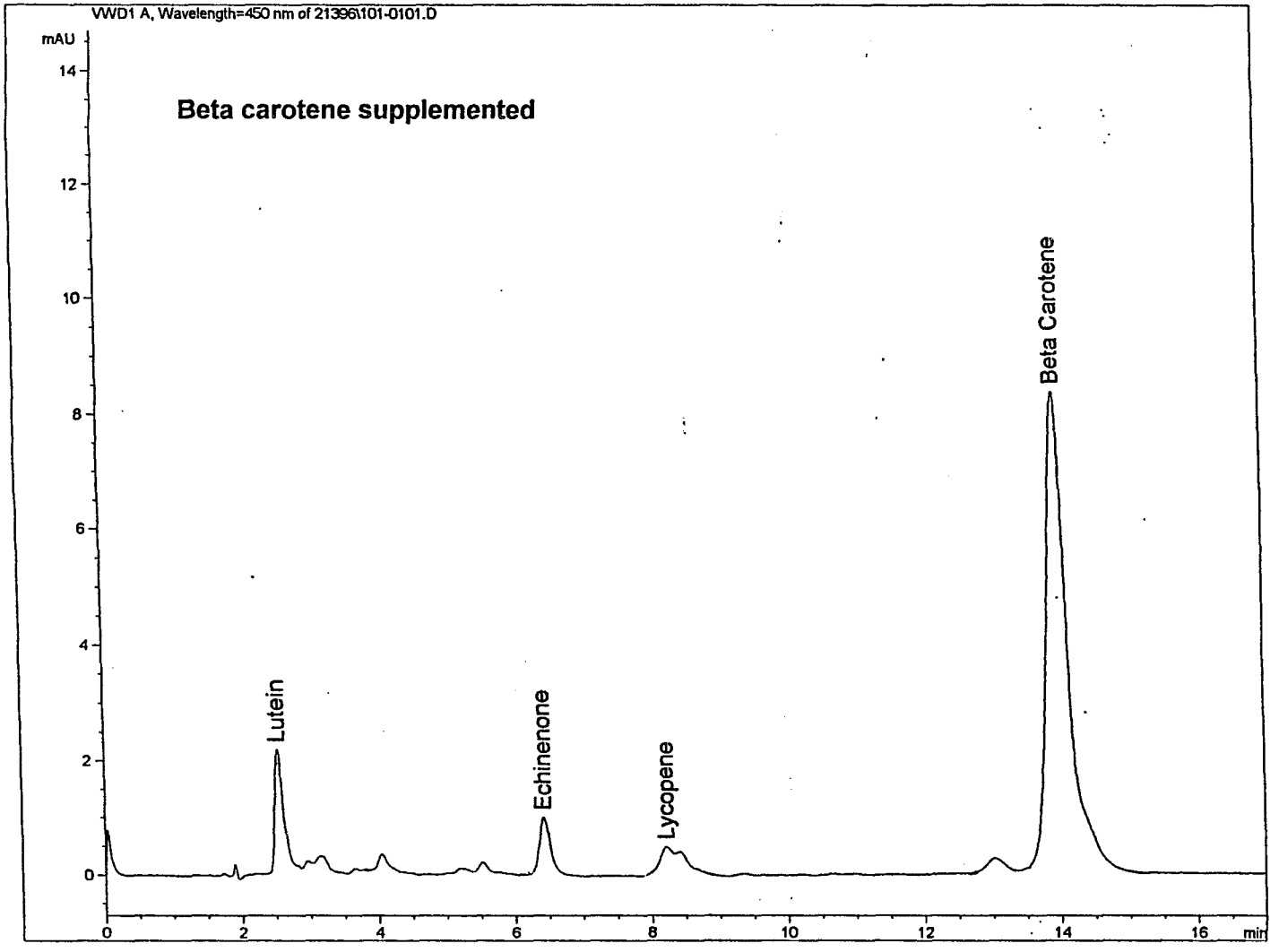

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Fig. 4.3

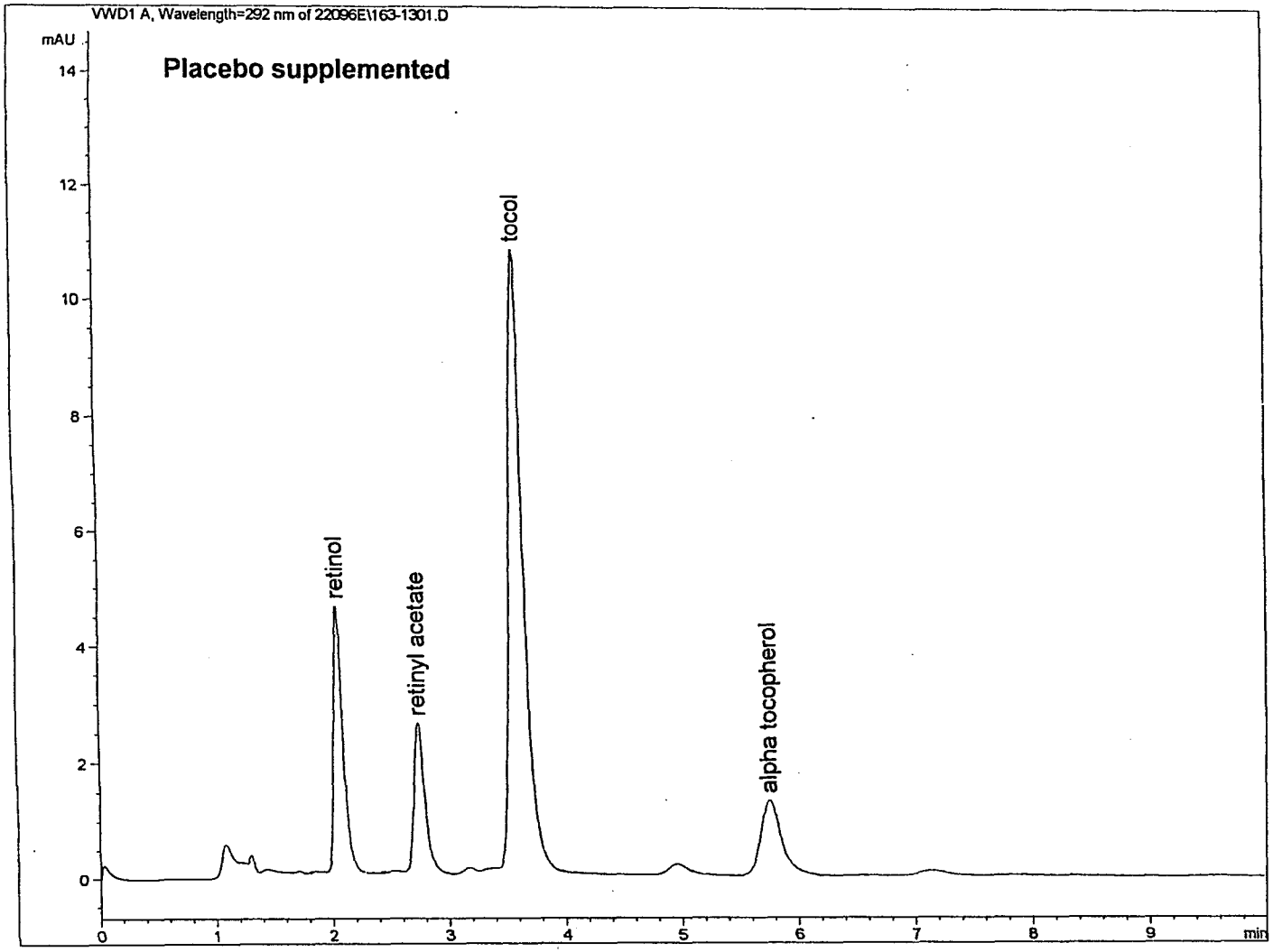

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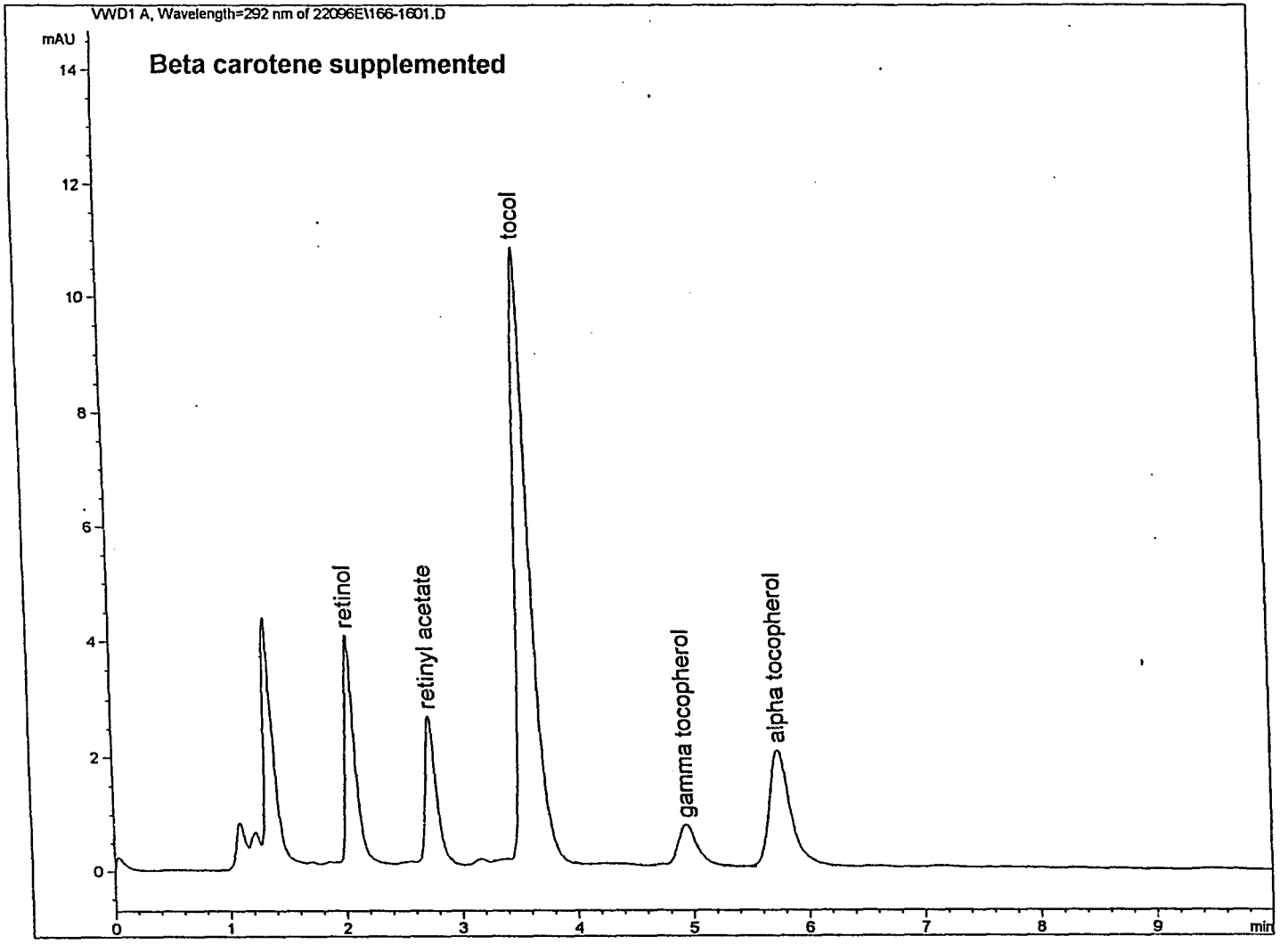
Fig. 4.4



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Figures 1-4. *Representative chromatograms of β-carotene (BC) and α-tocopherol (α-TC) in human plasma from subjects supplementing with β-carotene or placebo for 4 weeks.*

Five healthy adult males in our laboratory volunteered to ingest 60 mg/d β-carotene or placebo for 4 weeks. Aliquots of plasma from each subject were extracted and analyzed by high performance liquid chromatography (HPLC) to determine concentrations of BC and α-TC. Figures 4.1 and 4.3 are chromatograms showing the relative levels of carotenoids and retinoids and tocopherols in a sample from a subject ingesting the placebo. Figures 4.2 and 4.4 are chromatograms showing the levels of carotenoids and retinol and tocopherols in a sample from a subject ingesting the β-carotene supplement for four weeks. The relative size of the β-carotene peak is the most evident difference in the profiles.

RESULTS

Our initial aim was to generate BC-enriched lipoproteins by supplementing several subjects with 60 mg/d BC. Two independent studies were conducted with individuals assigned to ingest either the BC supplement (KM and MF) or the placebo (n=3). Because the response to the treatment was similar in both studies, data have been pooled for presentation. The concentrations of BC and α-TC in plasma and total lipoproteins of subjects at baseline and after 4 wk of supplementation with either BC or placebo are shown in Tables 4.1 and 4.2. After 4 weeks of supplementation with BC, plasma levels of the carotenoid increased more than 13-fold above the concentrations of BC in subjects receiving placebo. In the BC-supplemented individuals, the concentration of BC was significantly ($p<0.05$) increased in all lipoprotein fractions (Table 4.1). The concentration of BC in total lipoproteins of supplemented subjects after 4 wk increased 11.5-fold above that in LP of subjects receiving placebo over the same period. The magnitude of the increase in lipoprotein fractions was similar with relative distributions as follows: 67 ± 2% was in LDL, 25 ± 3% was in HDL and 8 ± 2% in VLDL in subjects at both baseline and after 4 wk supplementation with BC. BC concentrations in the plasma and lipoprotein fractions of subjects using placebo were similar ($p>0.05$) at baseline and 4 wk.

The concentrations of α-TC in plasma were not significantly altered ($p > 0.05$) by BC supplementation for 4 wk (Table 4.2). α-TC was associated primarily with LDL (43 ± 5%) with lesser amounts in HDL (36 ± 1%) and VLDL (21 ± 2%). After 4 wk of supplementation with BC, the concentration of α-TC (51.6 ± 5.1 μmol/L) in total LP (Table 4.2) remained markedly greater than the level of BC (4.7 ± 0.8 μmol/L; Table 4.1). Plasma lipids were not affected by supplementation with either BC or placebo (Table 4.3). Total plasma cholesterol and its relative

TABLE 4.1

Concentrations of β-carotene in human plasma and lipoproteins after supplementation with β-carotene or placebo for 4 weeks[1]

	Baseline	4 week supplement	
		β-carotene	placebo
	β-carotene (μmol/L)		
plasma	0.37 ± 0.04	5.12 ± 0.81*	0.39 ± 0.05
total lipoprotein	0.41 ± 0.12	4.71 ± 0.31*	0.46± 0.07
VLDL	0.03 ± 0.01	0.73 ± 0.32*	0.02 ± 0.01
LDL	0.35 ± 0.03	3.44 ± 0.81*	0.38 ± 0.03
HDL	0.12 ± 0.04	1.41 ± 0.30*	0.12 ± 0.04

[1]Distribution and concentrations of β-carotene (BC) in human plasma and among lipoprotein (LP) fractions were determined in plasma samples collected from healthy male volunteers before (n=5) and after supplementation with 60 mg/d BC (n=2) or placebo (n=3) for 4 wk. Concentrations of BC were determined by HPLC as described in Materials and Methods. Data are pooled means ± SE for BC (n=4) and placebo-supplemented subjects (n=5). The presence of an asterisk as a superscript indicates significant ($p<0.05$) differences between means at baseline and after 4 wk. VLDL=very low density lipoproteins, LDL=low density lipoproteins, HDL=high density lipoproteins.

TABLE 4.2

Concentrations of α-tocopherol in human plasma and lipoproteins after supplementation with β-carotene or placebo for 4 weeks[1]

	Baseline	4 week supplement	
		β-carotene	placebo
	α-tocopherol (μmol/L)		
plasma	48.6 ± 6.0	48.6 ± 12.6	48.2 ± 5.3
total lipoprotein	42.3 ± 10.5	51.6 ± 5.1	42.7± 7.1
VLDL	7.8 ± 2.4	10.2 ± 0.2	11.5 ± 4.9
LDL	22.0 ± 2.4	20.1 ± 0.2	17.8 ± 2.7
HDL	15.9 ± 0.4	17.2 ± 3.5	17.6 ± 5.0

[1]Distribution and concentrations of β-carotene (BC) in human plasma and among lipoprotein (LP) fractions were determined in plasma samples collected from healthy male volunteers before (n=5) and after supplementation with 60 mg/d BC (n=2) or placebo (n=3) for 4 wk. Concentrations of BC were determined by HPLC as described in Materials and Methods. Data are pooled means ± SE for BC (n=4) and placebo-supplemented subjects (n=4). Mean levels of α-TC were not significantly ($p > 0.05$) altered by supplementation. VLDL=very low density lipoproteins, LDL=low density lipoproteins, HDL=high density lipoproteins.

TABLE 4.3

Concentrations of lipids in human plasma and lipoproteins after supplementation with β-carotene or placebo for 4 weeks[1]

	Baseline	4 week supplement	
		β-carotene	placebo
		(mmol/L)	
total triglycerides	1.1 ± 0.3	1.4 ± 0.5	1.3 ± 0.3
total cholesterol	5.0 ± 0.4	4.9 ± 0.3	5.0 ± 0.3
VLDL cholesterol	0.5 ± 0.1	0.7 ± 0.3	0.6 ± 0.1
LDL cholesterol	1.9 ± 0.3	2.1 ± 0.6	2.0 ± 0.3
HDL cholesterol	2.4 ± 0.3	1.9 ± 0.1	2.0 ± 0.3

[1]Distribution and concentrations of lipids in human plasma and among lipoprotein (LP) fractions were determined in plasma samples collected from healthy male volunteers before (n=5) and after supplementation with 60 mg/d BC (n=2) or placebo (n=3) for 4 wk. Concentrations of triglycerides and cholesterol were determined as described in Materials and Methods. Data are pooled means ± SE for BC (n=4) and placebo-supplemented subjects (n=4). Mean levels of lipids were not significantly ($p > 0.05$) altered by supplementation. VLDL=very low density lipoproteins, LDL=low density lipoproteins, HDL=high density lipoproteins.

distribution were not affected by supplementation with either BC or placebo with 58 ± 3% present in LDL, 35 ± 4% in HDL and 7 ± 1% in VLDL.

We next compared the stability of BC and α-TC in serum-free tissue culture medium after their addition in lipoproteins and micelles. The preparations were incubated for 20 hr using standard tissue culture conditions, viz., humidified atmosphere of air: CO_2 (95:5) at 37°C in sterile plastic multiwell dishes without cells. After overnight incubation, only 27 and 73% of the intial concentration of BC and α-TC were present in medium containing micelles with one of the test compounds (Table 4.4). In contrast, 88 and 94% of the initial concentrations of BC and α-TC, respectively, were detected in medium containing the total LP fraction isolated from BC-supplemented subjects. Inclusion of both α-TC and BC in the micellar preparation markedly attenuated the loss of BC during the incubation, suggesting that micellar BC was oxidatively modified under standard culture conditions.

Basal levels of BC in cells that were incubated with PBS only (control) were below the level of detection by HPLC (1 pmol/mg protein); levels of α-TC in cells were 6 pmol/mg protein. To assess whether HepG2 liver cells accumulated BC and α-TC from lipoproteins, confluent cultures were incubated in MEM containing total lipoproteins (1 mg protein/mL) that had been prepared from individuals supplemented either with BC or placebo. Cellular levels of BC ranged from 1 to 130 pmol/mg protein (Fig. 4.5) and were positively correlated (r=0.94) with the concentrations of BC in medium. The cells accumulated 11 ± 2% of BC in medium. The range of concentrations of α-TC in HepG2 cells incubated in medium containing total LP from test subjects ranged from 53 to 356 pmol/mg protein (Fig. 4.6) and was positively correlated (r=0.74) with the concentration of α-TC in medium. Cells accumulated 14 ± 2% of medium α-TC.

TABLE 4.4

Effects of standard tissue culture conditions on stability of β-carotene and α-tocopherol added to medium as either micelles or lipoproteins[1]

	Micelle	Lipoprotein
	% initial concentration	
β-carotene	26.8 ± 2.3	87.7 ± 1.6
α-tocopherol	73.1 ± 2.1	94.0 ± 1.8
β-carotene plus	67.8 ± 2.0	—
α-tocopherol	70.9 ± 2.3	—

[1]Complete medium containing either micellar preparations or total lipoproteins from BC-supplemented subjects was incubated in plastic tissue culture dishes without cells in a humidifed atmosphere of 95% air: 5% CO_2 for 20 hr. The final concentrations of BC and α-TC in medium containing micelles were 1.1 and 14.1 μmol/L, respectively. The final concentrations of BC and α-TC in medium containing lipoproteins were 4.7 and 41.6 μmol/L, respectively. Aliquots of medium were extracted immediately after preparation and sterilization of medium (n=3) and after incubation for 20 hr. The concentrations of BC and α-TC were analyzed as described in Materials and Methods. Data are means ± SE for at least triplicate samples from two separate experiments.

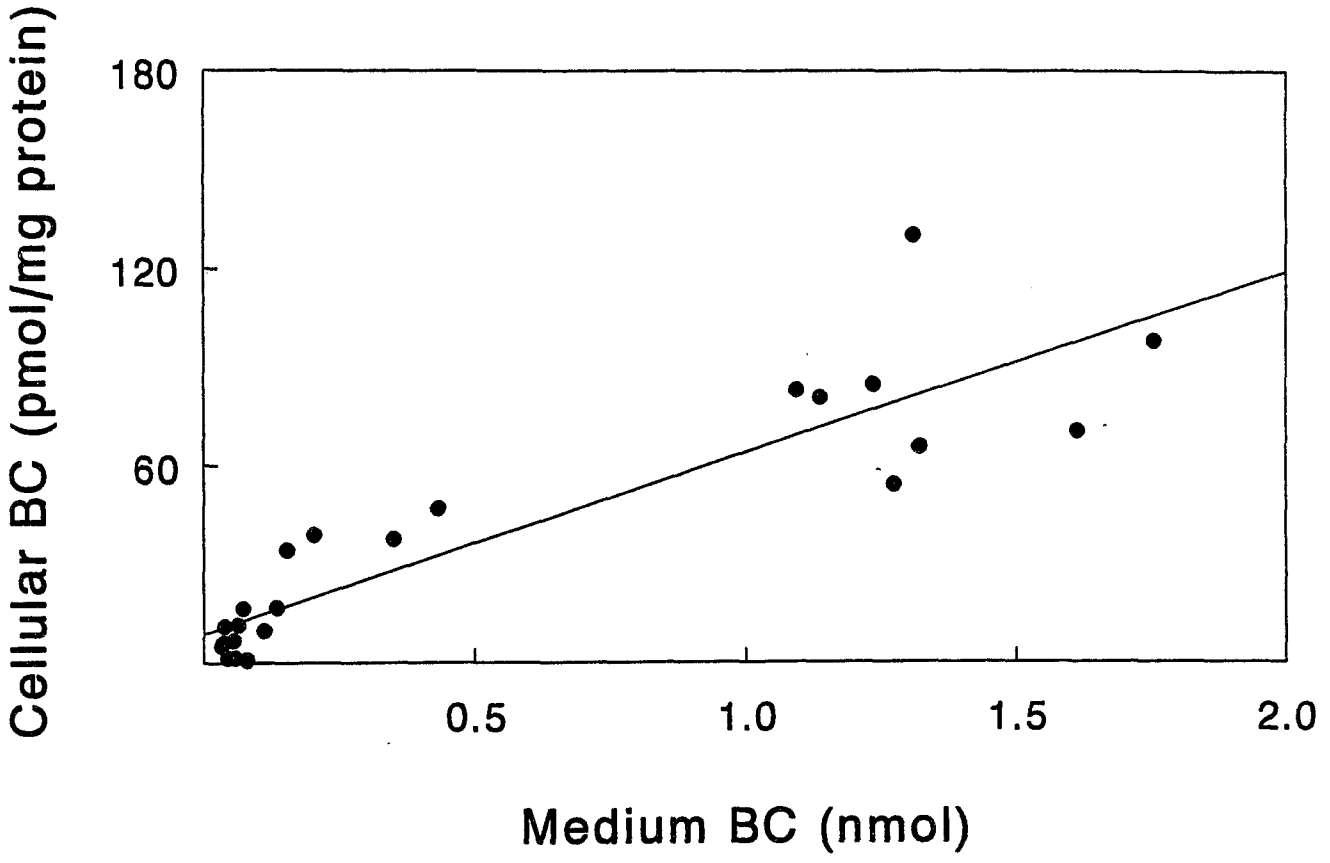

Figure 4.5. *Accumulation of β-carotene (BC) by HepG2 cultures incubated in MEM containing human total lipoproteins.*

Confluent cultures of HepG2 cells were incubated in MEM containing total lipoprotein (1 mg/mL) obtained from subjects (n=5) supplementing with either 60 mg/d BC (n=2) or placebo (n=3). Plasma samples were collected in two separate studies at the beginning and the end of the supplementation period (4 wk) and at 2 and 4 wk post-supplementation to obtain lipoproteins with different concentrations of BC. BC was extracted from fresh medium and cells incubated in medium for 20 h. BC was quantified by HPLC as described in Materials and Methods. Each point represents the mean value for a test sample that was added to three replicate wells.

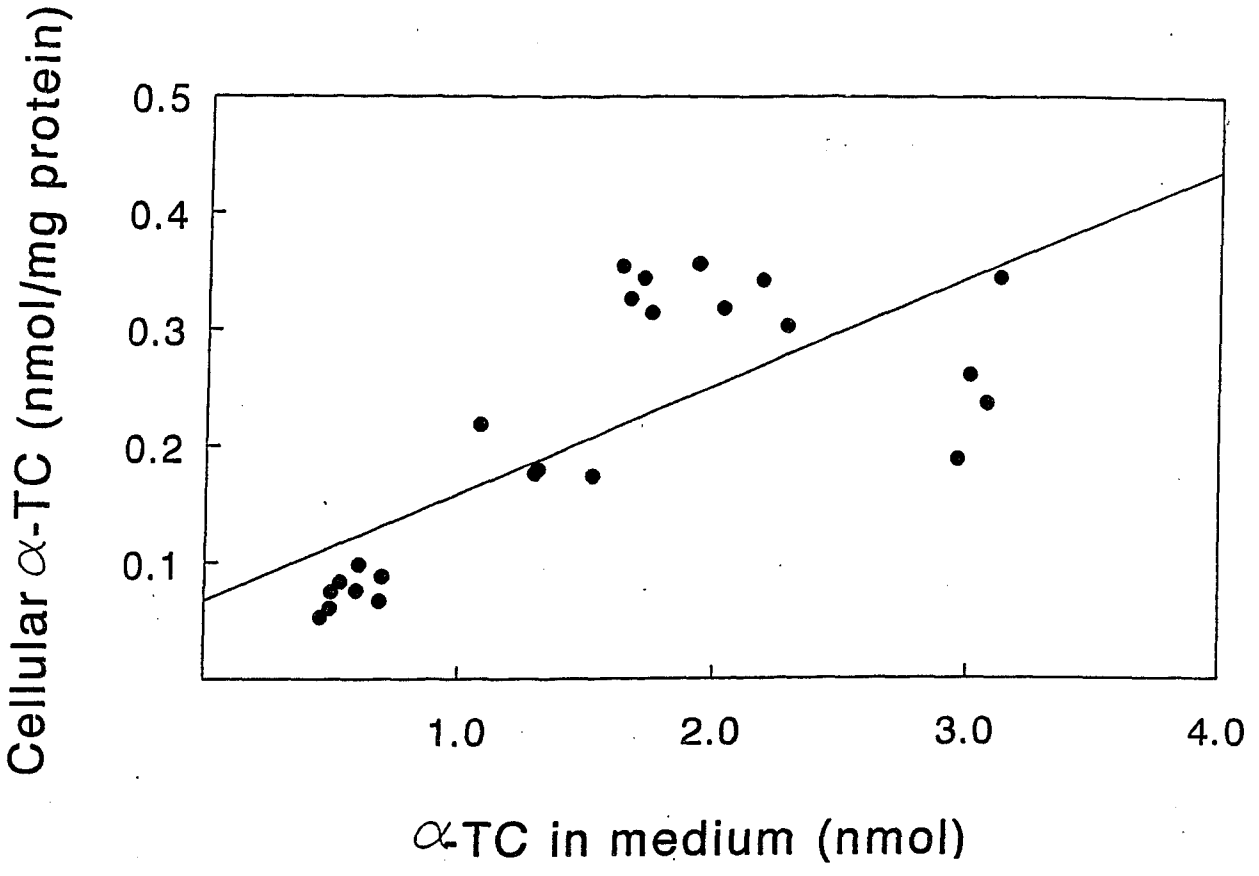

Figure 4.6. *Levels of α-tocopherol (α-TC) in HepG2 cells after overnight incubation in medium containing human total lipoproteins.*

Confluent cultures of HepG2 cells were incubated for 20 hr in medium containing human total lipoproteins (1 mg/mL) obtained from subjects supplemented with BC or placebo as described in the Figure 4.1 legend. Aliquots of fresh medium and cells incubated for 20 hr in test media were extracted and analyzed by HPLC to determine α-TC concentrations. Each data point represents the mean value for a test sample that was added to three replicate wells.

To examine if HepG2 cells preferentially accumulated BC from a specific fraction of lipoproteins, cultures were incubated overnight in medium containing either LDL (0.5 mg protein/mL) or HDL (1 mg protein/mL) isolated from plasma of subjects. The concentration of BC in HepG2 cells incubated overnight in medium with the LDL fraction was 3 to 96 pmol BC/mg protein (Fig. 4.7, upper panel) and was positively correlated (r=0.85) with the level of carotenoid in the medium. Likewise, cultures incubated in MEM with HDL contained 1 to 14 pmol BC/mg protein (Fig. 4.7, lower panel); cellular BC was positively correlated with (r=0.82) the concentration in medium. HepG2 cells accumulated 12 $\pm$ 2 and 20 $\pm$ 3% of BC introduced into medium via LDL and HDL, respectively.

α-TC levels in cells cultured with LDL also ranged from 54 to 333 pmol α-TC/mg protein (Fig. 4.8, upper panel). Similarly, the concentrations of α-TC in HepG2 cells incubated with MEM containing HDL ranged from 148 to 365 pmol α-TC/mg protein (Fig. 4.8, lower panel). The concentration of α-TC in HepG2 cells was positively correlated with that in medium when cultures were incubated with LDL (r=0.74) and HDL (r=0.66). HepG2 cells accumulated 8 $\pm$ 2 and 11 $\pm$ 1% of α-TC added as LDL and HDL to medium, respectively.

DISCUSSION

This study demonstrates that BC and α-TC in human lipoproteins are stable in tissue culture medium and readily accumulated by HepG2 human liver cells in vitro. Similar to reports by Micozzi et al. (1992), we found that supplementation with BC resulted in greater than an 11-fold increase in the concentration of this carotenoid in plasma and in all lipoprotein fractions without altering the relative distribution of BC among lipoprotein subfractions. We also found that BC was carried predominantly in the LDL fraction and to a lesser extent in HDL and VLDL, as

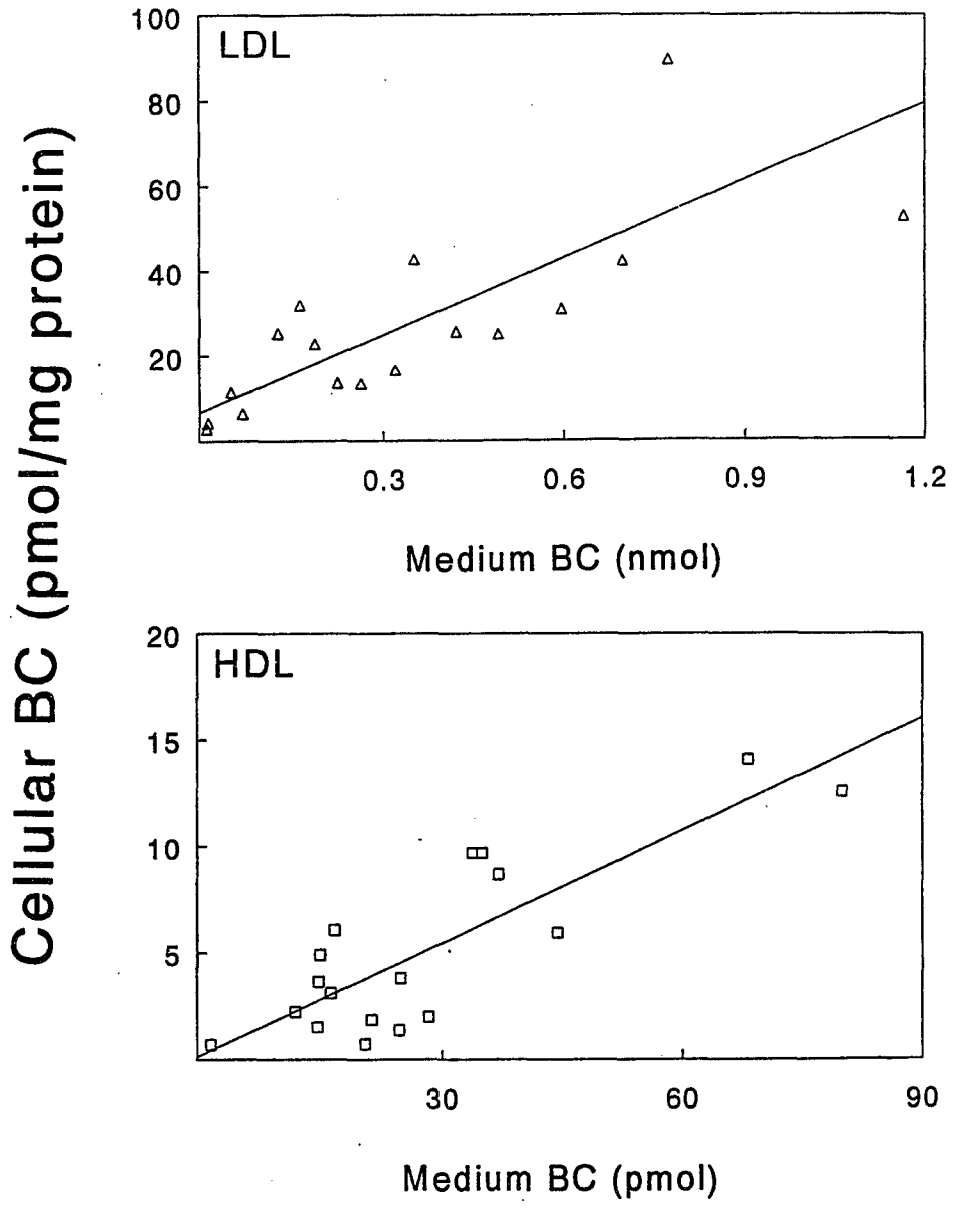

Figure 4.7. *Delivery of β-carotene (BC) to HepG2 cells by human low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions.*

Monolayers of HepG2 cells were incubated for 20 hr in medium containing either 0.5 mg/mL human LDL (upper panel) or 1 mg/mL HDL (lower panel) obtained from subjects (n=5) supplemented with either 60 mg/d BC or placebo. Plasma samples were collected at the beginning and end of the 4 wk supplementation period and at 2 and 4 wk post-supplementation to obtain lipoprotein fractions with different concentrations of BC. After removing spent medium and washing monolayers, cultures were harvested, extracted and analyzed to determine cellular BC. Each data point represents the mean value for a test sample that was added to three replicate wells.

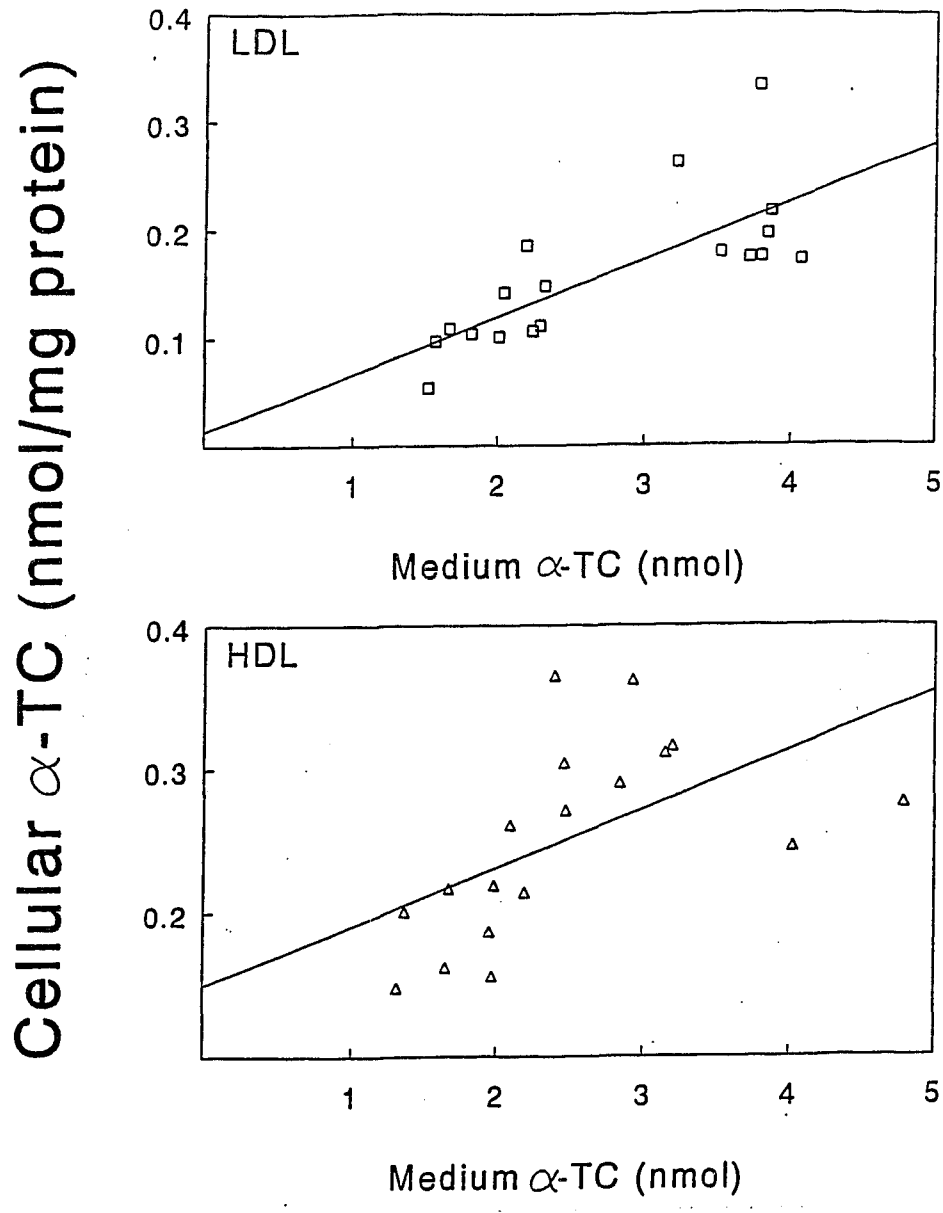

Figure 4.8. *Accumulation of α-tocopherol (α-TC) by HepG2 cells incubated in medium containing either low density lipoprotein (LDL) or high density lipoprotein (HDL) fractions.*

Confluent monolayers of HepG2 cells were incubated with medium containing either 0.5 mg/mL human LDL (upper panel) or 1.0 mg/mL HDL (lower panel) obtained from subjects supplemented with 60 mg/d BC or placebo for 4 wk as described in Figure 4.7 legend. Aliquots of fresh medium and cell sonicates prepared after 20 hr incubation were extracted and analyzed to determine the concentration of α-TC. Each data point represents the mean value for a test sample that was added to three replicate wells.

recently reported by Romanchik et al. (1995) and Johnson and Russell (1995). BC supplementation did not affect either the concentration or the relative distribution of α-TC in lipoproteins which is similar to the findings of Traber et al. (1994) and Ribaya-Mercado et al. (1995).

Our initial goal was to generate BC-enriched lipoproteins for delivery of the carotenoid to cells in culture. Because studies regarding the delivery of carotenoids to cells have failed to consider the relative stability of the test compounds in cell culture medium, we compared the stability of BC and α-TC delivered as micelles and human lipoproteins to MEM and incubated overnight using standard tissue culture procedure. Micellar preparations of both compounds, and particularly BC, were less stable in tissue culture medium than in lipoproteins. Inclusion of both α-TC and BC in the micellar preparation attenuated the loss of BC during incubation, suggesting that micellar BC is oxidized under standard tissue culture conditions. In contrast to our data, Scita (1993) reported that micellar BC was relatively stable (<4% degraded) in medium that was incubated in a humidified atmosphere of air/CO_2 (95:5 v/v) at 37^{o}C for 24 hr. Micelles were prepared by adding BC solubilized in tetrahydrofuran-dimethyl sulfoxide to culture medium or tetrahydrofuran to Tris buffer containing taurodeoxycholic acid. Cooney et al. (1993) have suggested that inclusion of tetrahydrofuran in these preparations may have solubilized and stabilized BC by forming a molecular "cage" around one or more BC molecules. It is likely that the specific composition of both the micelle and the tissue culture medium, as well as the handling of the samples prior to incubation, influences the stability of BC in tissue culture medium.

Numerous investigators have estimated the concentrations and characterized the distribution of carotenoids in human lipoproteins (Clevidence and Bieri, 1993; Johnson and

Russell, 1995; Traber et al., 1994). However, we are unaware of previous studies evaluating the ability of lipoproteins, viz., HDL and LDL, to deliver BC and other carotenoids to cultured human cells. Thus, our initial studies focused on the accumulation of BC and α-TC from total lipoproteins (LP) by HepG2 cells obtained from subjects supplemented with either BC or placebo. Since most hydrophobic compounds in human plasma are associated with LDL and HDL, the accumulation of BC and α-TC by HepG2 cultures incubated in medium containing these fractions was examined. Our data indicate that confluent cultures of cells readily accumulated BC from medium containing either LP, LDL or HDL as the carrier. Moreover, the efficiency of BC uptake from LDL and HDL were similar. Comparison of our results with several other reports examining BC levels in cells merits consideration. Oarada et al. (1993) reported that FU-5 rat hepatoma cells accumulated 70 pmol BC/mg protein when cultures were incubated for 24 hr in Ham's F-12 medium containing 3.5 μmol/L BC (tetrahydrofuran vehicle). HepG2 cells incubated in medium containing total lipoproteins (1 mg protein/mL) isolated from the plasma of subjects supplemented with BC accumulated similar quantities of BC. Peng et al. (1994) reported 2.2 pmol BC/mg protein in human buccal mucosal cells that had been collected from subjects supplemented with BC for ≤10 d. For all fractions, cellular BC was proportional to the quantity of BC in the medium added as lipoproteins.

Confluent cultures of HepG2 cells express LDL receptors and accumulate and metabolize human LDL (Javitt, 1990; Dashti and Wolfbauer, 1987). Therefore, it is likely that BC accumulation from LDL was mediated at least in part by receptor-mediated endocytosis. Although HDL receptors have been identified on the surface of HepG2 liver cells (Javitt, 1990; Perova et al., 1988; Dashti et al., 1985), the mechanism by which they facilitate the uptake of components of HDL remains unclear. The literature suggests there are two distinct processes for uptake of HDL components, viz., one involving endocytotic uptake of apoAI (and thus

presumably the HDL particle) and the other involving nonendocytotic uptake of cholesterol esters mostly without uptake of the particle (Pittman et al., 1987). The latter pathway is not limited to the uptake of cholesterol esters since a long chain dialkyl ester, sucrose octaoleate, is rapidly accumulated from HDL (Pittman et al., 1987). This raises the possibility that the nonendocytotic pathway may be important for the uptake of nonpolar compounds such as BC that are carried in HDL. Indeed, Leblond and Marcel (1993) reported that HDL cholesterol ester is taken up by HepG2 without uptake of the HDL apoprotein. The high affinity plasma membrane receptors for HDL in HepG2 cells also appear to mediate the removal of excess intracellular cholesterol, i.e., reverse cholesterol transport (McKnight et al., 1992). The cellular binding sites for HDL in intact cells is enhanced when cells are loaded with cholesterol or when the rate of cell proliferation is inhibited as often observed in confluent cultures. It is unknown if BC and other carotenoids may be transferred from the liver cell to HDL by this efflux pathway. Additionally, the extent of metabolism of BC by HepG2 cells accumulated in different lipoprotein fractions is unknown.

The possibility that accumulation of BC from LDL and HDL occurred in part by direct transfer of the hydrophobic compounds present in lipoproteins to the hydrophobic plasma membrane of HepG2 cells merits further investigation. Indeed, in vitro studies using vehicles such as organic solvents, water miscible beadlets and liposomes which lack the components of human lipoproteins have resulted in significant cellular accumulation of carotenoids. In our studies (see Chapter 3), we have effectively used micelles to deliver BC and α-TC to HepG2 cells. Systematic investigation of the mechanism(s) of cellular accumulation of carotenoids from the different lipoprotein classes is merited.

In conclusion, this study has demonstrated that BC-enriched lipoprotein fractions are relatively stable in human lipoproteins in vitro. Furthermore, lipoproteins represent effective

vehicles for delivery of BC and other lipophilic compounds to HepG2 human liver cells. Together, the use of the highly differentiated human liver cell line and human lipoproteins represent a physiologically relevant system for investigating the characteristics of carotenoid uptake and metabolism.

EPILOGUE

Over 700 distinct carotenoids have been identified in nature and approximately 100 of these compounds are present in the typical United States diet. An increasing body of literature supports the potential role of carotenoids as antioxidants and has stimulated interest in this class of compounds. The results of my research strongly support the use of the HepG2 human cell line as an appropriate model for investigating the purported role of carotenoids as antioxidants in protecting various cellular structures and functions against oxidative stress. Moreover, my latter studies indicate that carotenoids are readily accumulated by HepG2 cells in vitro from human lipoproteins, as well as micelles. However, the focus of numerous studies regarding the potential antioxidant activity and metabolism of carotenoids has been limited to only a few compounds and particulary β-carotene. The use of highly differentiated human cell lines coupled with carotenoid-enrichment lipoproteins provides a physiologically relevant system for elucidation of key questions regarding the metabolic and physiologic functions of carotenoids. If time had permitted, I would have initiated several additional studies addressing topics of intense current interest regarding carotenoids. These are described in the following sections.

At least 42 well-controlled epidemiologic studies have been conducted over the past two decades and have consistently indicated an inverse correlation between the incidence of several forms of cancer in humans and the increased consumption of carotenoid-rich fruits and vegetables. A key association that has been regularly noted is the decreased incidence of lung cancer in individuals consuming relatively high levels of β-carotene. This has been of great interest and relevance particularly in the United States where smoking is prevalent. However, two recent studies have provided compelling evidence that supplementation with high levels of β-carotene actually may be associated with increased occurrence and severity of lung cancer

and particularly for those individuals who smoke. To address these conflicting observations, I propose development of a cell model to investigate the potential role of β-carotene in the exacerbation of lung pathology in individuals who smoke. Anchorage-dependent human cells of bronchiolar (i.e. CCD-14Br) or lung carcinoma (alveolar) origin (i.e. WI-38) would be obtained from ATCC and cultured on semipermeable membranes. Medium containing human lipoproteins or micelles enriched with β-carotene would be added to the basolateral compartment of confluent monolayers to simulate the in vivo delivery of carotenoids from the plasma. Since BC and α-TC are both transported in lipoproteins, enrichment of the particles with BC without altering the profile of α-tocopherol would facilitate my focus on β-carotene. After overnight incubation, medium would be removed and cultures washed rigorously before transferring cultures to a separate incubator maintained at 37^{o}C and air to mimic the partial pressure of oxygen of inhaled ambient air. Medium containing cigarette smoke extract (CSE) would be prepared using a vacuum driven glass apparatus currently used in Dr. Loo's laboratory. Smoke from cigarettes could be passed through medium allowing entrapment of contaminants of cigarette smoke in the fluid matrix which would simulate pulmonary secretions. Filter sterilized medium containing the CSE would then be added to the apical compartment and cultures would be incubated for various times. Medium from both the upper and lower compartments would be examined to assess the effects of treatment on the structural integrity and metabolic activities of the cells as described in Chapters 2 and 3. It is noteworthy that BC has been reported to act as a pro-oxidant at high oxygen tensions such as that typically encountered in the lung, whereas α-tocopherol is a potent, effective antioxidant at high oxygen tension. Therefore, I also would analyze the concentrations of these two compounds in the test cell type. The information gained in these studies would begin to provide new insights about the relationship between the two antioxidants in cells of pulmonary origin and their role in attenuating free radical-mediated damage often associated with cytotoxicity and neoplasia.

Fat soluble vitamins and carotenoids are absorbed via emulsification with pancreatic and biliary secretions in the lumen of the intestine. A major concern regarding reported human studies evaluating the differential absorption of carotenoids has been the potential for interaction of carotenoids in the intestine and the relative impact on absorption of individual carotenoids. Numerous human studies have provided conflicting evidence for interaction of specific carotenoids in the intestinal lumen. Additionally, the recent approval of Olestra^{R} (Procter & Gamble) by the Food and Drug Administration has prompted intense debate over the use of this fat substitute and similar products and the potential compromise of antioxidant status in individuals as a result of decreased absorption of fat soluble vitamins and compounds. The use of highly differentiated human intestinal cell lines, viz., Caco-2, would provide a tool to evaluate carotenoid interactions and the effects of compounds such as Olestra on the uptake and absorption of carotenoids. Caco-2 human intestinal cells would be cultured on semipermeable membranes as described in Chapter 2. Confluent differentiated monolayers would be incubated with micellar preparations of a test carotenoid alone or in combination with either other carotenoids or fat soluble compounds such as α-tocopherol and Olestra, in the apical compartment to simulate the lumenal environment of the small intestine. After various times, medium would be removed and the monolayers washed rigorously. The accumulation of the carotenoids would be analyzed by high performance liquid chromatography. Information from these studies would provide insights about the efficacy of use of synthetic macronutrient substitutes and elucidate the relative impact of disproportionate consumption of fat soluble compounds such as β-carotene supplements. Moreover, the model would facilitate the development of rational recommendations concerning the beneficial and detrimental effects of carotenoid supplementation and fat substitutes.

As an extension of studies regarding the interaction of carotenoids, the culture of Caco-2 cells on microporous inserts would also allow evaluation of metabolic modification of carotenoids since very little is currently known. Both cellular and secretory metabolites (basolateral) could possibly be identified and quantified providing important informaton on the disposition of carotenoids during absorptive and post-absorptive periods within the human gut. Moreover, the inclusion of a second cell type within the culture system, i.e., a co-culture technique, has the potential for evaluating distribution of carotenoids and their metabolites. For example, cultures of HepG2 human liver cells, adipocytes or myocytes could be grown on multiwell dishes and Caco-2 intestinal cells grown on microporous membrane inserts and placed into those wells to evaluate absorption and tissue-specific uptake, or metabolism of carotenoids.

Finally, the development of a cellular model for evaluation of carotenoids as antioxidants has also provided the means for evaluating the potential roles of other compounds as antioxidants. For instance, human lipoproteins transport lipophilic carotenoids and numerous other compounds with potential antioxidant activity, viz., ubiquinol-10. Additionally, there has been increasing interest in the role of a group of plant compounds called flavonoids as potential antioxidants. The described cell models provide a valuable screening tool for determination of antioxidant potential. Inclusion of the well-established lipophilic antioxidant α-tocopherol would allow the development of a relative index of cytoprotection. Together, these results would provide insight into the activity and potency of numerous dietary compounds and potentially facilitate recommendations for dietary practices.

The initiation of projects addressing the aforementioned questions would provide novel information about the functions, distribution and metabolism of carotenoids and other potentially beneficial dietary compounds. The challenge will be given to other students because I face new

opportunities at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

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