TOXICITY OF β-CAROTENE AND ITS EXACERBATION BY ACETALDEHYDE IN HEPG2 CELLS

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Abstract — In rats and baboons, the hepatotoxicity of chronic ethanol consumption is exacerbated by β-carotene feeding, but the mechanism of this adverse effect is unknown. In this study, the toxicity of β-carotene and acetaldehyde was documented by the MTT test (an assay of reduction of tetrazolium to formazan) and by lactate dehydrogenase (LDH) leakage. In HepG2 cells, β-carotene or acetaldehyde inhibited mitochondrial reduction function as indicated by a decrease of the MTT test. β-Carotene was inhibitory at very low concentration, in a dose-dependent manner. The combination of these two compounds resulted in an additive effect. Acetaldehyde increased LDH leakage from the HepG2 cells into the medium, whereas β-carotene by itself did not show such an effect, but it exacerbated the toxicity of acetaldehyde when combined. In addition, this study showed that acetaldehyde and β-carotene inhibited each other’s clearance from the medium, which suggests that these two chemicals may share, at least in part, a common metabolic pathway (possibly via aldehyde dehydrogenase) in the cells, and that a competitive inhibition may exist. In conclusion, this preliminary study indicates that β-carotene is toxic to hepatocytes, especially when combined with acetaldehyde, the metabolite of ethanol.

INTRODUCTION

In view of the overlapping metabolic pathways between ethanol and retinol (the main biological compound with vitamin A function), it is not surprising that multiple interactions between retinol and ethanol occur (Leo and Lieber, 1999). Such interactions also involve the principal retinol precursor β-carotene. β-Carotene can be converted oxidatively to vitamin A in animals by several possible routes (Barua and Olson, 2000). Alcohol consumption results in a striking depletion of hepatic vitamin A, both in experimental animals (Sato and Lieber, 1981) and in humans (Leo and Lieber, 1982). The combination of ethanol and low vitamin A results in lysosomal abnormalities (Adhikari and Vakil, 1980; Mak et al., 1987) and increased severity of squamous metaplasia of the trachea (Mak et al., 1984; Leo et al., 1986). Vitamin A deficiency has also been shown to be associated with the formation of various types of tumours (Sporn, 1977; Wald et al., 1980).

Retinol supplementation has been recommended for correction of vitamin A deficiency in alcoholics. However, vitamin A supplementation is complicated by its intrinsic hepatotoxicity, which is potentiated by concomitant alcohol consumption (Leo et al., 1982). Unlike retinoids, carotenoids (β-carotene) are not known to produce toxic manifestations. Furthermore, as β-carotene was shown to be an antioxidant, it was viewed as an effective, but less toxic, substitute for retinol. Nevertheless, in baboons, consumption of ethanol together with β-carotene resulted in striking hepatic injury (Leo et al., 1992). The well-known hepatotoxicity of ethanol was potentiated by large amounts of β-carotene and, in rats, the concomitant administration of both β-carotene and alcohol also resulted in striking liver lesions (Leo et al., 1997).

Thus far, no reports have been published on the effect of the combination of β-carotene and ethanol on liver cells in vitro. Accordingly, we investigated the effects of β-carotene and acetaldehyde (the toxic metabolite of ethanol) on liver cells in culture. HepG2 cells have served as a good model to study the hepatotoxicity of different chemicals or drugs. Although these cells have lost most of their ability to express some enzymes related to ethanol metabolism, such as alcohol dehydrogenase (ADH) (Clemens et al., 1995) and cytochrome P4502E1 (Dai et al., 1993), which are needed to metabolize ethanol to acetaldehyde, they still retain aldehyde dehydrogenase (ALDH) activity (Clemens et al., 1995). Acetaldehyde has been shown to mediate many of the biological effects of ethanol (Lieber, 1992) and plays an important role in the pathogenesis of alcoholic liver injury. In the present experiment, acetaldehyde was added directly to the HepG2 cell culture medium in view of the deficient conversion of ethanol to acetaldehyde in these cells.

MATERIALS AND METHODS

Cell line and culture conditions

The HepG2 cell line (passage 77) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), and passages 80–90 were used for all experiments. HepG2 cells were grown in complete MEM (minimum essential medium) with 2 mM l-glutamine, supplemented with 1.5 g/l sodium bicarbonate, 10% fetal bovine serum, and a 1% penicillin/streptomycin (PS) mixture (Life Technologies, Grand Island, NY, USA). Cells were maintained in a humidified atmosphere of 95% air with 5% CO2, at 37°C, and sub-cultured at a 1:10 ratio approximately once a week. MEM used in all experiments was supplemented with 10 mM HEPES, 1% PS mixture, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, leaving out fetal bovine serum and Phenol Red. The cell counts were monitored with a Coulter counter (Coulter® Z1; Coulter Electronics Limited, Luton, Beds, UK). Before the experiment, the cells were sub-cultured in complete MEM at 0.5 × 106 per 12.5 cm2 flask for 3 days. The following day, the media were replaced with the MEM without Phenol Red, and acetaldehyde and/or β-carotene was added. In some...
experiments, the concentration of acetaldehyde was monitored and maintained by compensation for losses (initially 180 µM with three or four additions in 24 h); the concentration of β-carotene was 1.5 µM. The flasks were tightly sealed with a rubber stopper. The media and cells were harvested after 24 h, the MTT test was performed, and lactate dehydrogenase (LDH) and protein were measured.

β-Carotene-liposome preparation

β-Carotene liposomes were prepared according to Liebler et al. (1997) with the following modifications: β-carotene (type II, synthetic; Sigma, St Louis, MO, USA) was dissolved in hexane (0.147 mg/ml, as measured at 450 nm with a spectrophotometer); it was mixed with 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC) dissolved in ethanol (2.5 mg/ml) in a glass tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in cyclohexane and evaporated again. It was then suspended in MEM and sonicated for 8 s. The β-carotene-liposome mixture was added directly to the culture medium. DLPC liposomes alone, processed as described above, were used as liposomal control.

Measurement of β-carotene and acetaldehyde in cells and medium

β-Carotene in the cells and medium was measured by high-performance liquid chromatography (HPLC) as previously described (Leo et al., 1992), with minor modifications: an aliquot of medium or cell suspension was mixed with 2 volumes of ethanol and extracted twice with 3 volumes of n-hexane. The hexane was removed under a stream of nitrogen, and the extract was dissolved in the mobile phase and injected onto a Zorbax C_18 column. All HPLC analyses were carried out with an HP-1090 liquid chromatograph equipped with a photo diode-array spectrophotometric detector and HPLC chromatography (Hewlett Packard, Palo Alto, CA, USA). Chromatograms at 295, 325 and 450 nm were recorded to assess tocopherols, retinols and carotenoids, respectively. β-Carotene was quantified by comparing the peak height in the known with the peak height in the known amount of the standard (Leo et al., 1992). Acetaldehyde was measured by head-space gas chromatography as described before (Hernandez-Munoz et al., 1992).

Mitochondrial activity

The effects of acetaldehyde and β-carotene on mitochondrial reductive activity were measured by the MTT test (Scudiero et al., 1988) modified as follows: before each treatment, the media were removed from the flasks, and the flasks were washed twice with Hanks’ solution; MEM with different concentrations of β-carotene and/or acetaldehyde was added, and the flasks were sealed with rubber stoppers; 24 h thereafter, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each flask at a final concentration of 0.2 mg/ml. The MTT was reduced to formazan (a product with a blue colour) by the active mitochondria. After a 2-h incubation at 37°C, the medium was removed, and 1 ml 100% dimethyl sulphoxide was added to lyse the cells and dissolve the reduced MTT. The absorbance by the reduced MTT was determined at 570 nm. The A_{50%} was taken as an index of the activity of mitochondria. The net absorbance from the flasks of cells cultured with the control medium was considered as 100% of the mitochondrial activity.

Cell membrane damage

Leakage of LDH was measured as an index of cell-membrane damage (Thomas et al., 1993; Yildiz et al., 1999). HepG2 cells were sub-cultured in 12.5 cm² flasks and grown for 3 days before treatment with acetaldehyde and β-carotene. At the end of the experiment, the media were collected, and the cells were harvested by scraping and suspended in 0.5 ml of MEM, then sonicated with an ultrasonicator (Branson Sonifier 450) for 8 s on ice. The LDH activities in the medium and in the cells were measured with an LDH assay kit (Boehringer Mannheim, Indianapolis, IN, USA). To that effect, 200 µl aliquots of culture medium or 10 µl of cell lysate were added to the LDH assay system, and the absorbance at 492 nm was recorded.

RESULTS

Effect of β-carotene and acetaldehyde on mitochondrial reducing activity

Mitochondrial reducing activity, assessed with the MTT test, was inhibited by β-carotene and acetaldehyde (Fig. 1). The combination of the two chemicals had an additive effect (Fig. 1). This β-carotene effect occurred already at very low concentrations (50 nM), and was dose-dependent (Fig. 2).

Membrane toxicity of β-carotene and acetaldehyde

Acetaldehyde alone increased LDH leakage from the cells into the medium (P < 0.05), whereas this was not the case with β-carotene alone (Fig. 3). The combination of β-carotene with
acetaldehyde significantly enhanced the LDH leakage compared to β-carotene alone (P < 0.01) and to control liposomes (P < 0.001). Liposomes alone did not show any toxicity to HepG2 cells. β-Carotene was also taken up by the cells and resulted in a concentration of 0.34 ± 0.06 nmol/mg of protein at 24 h of incubation. There were no detectable levels of retinol or retinal in the cells or in the medium, small amounts of carotenals were detected but not quantified (data not shown).

Utilization of acetaldehyde and β-carotene from the medium of HepG2 cells

To study the effect of acetaldehyde on β-carotene metabolism and the effect of β-carotene on acetaldehyde, the concentrations of acetaldehyde and β-carotene in the medium were measured by gas chromatography and HPLC, respectively, at the end of 24 h of incubation. In the group treated with acetaldehyde plus β-carotene, the concentration of acetaldehyde in the medium was higher than in the absence of β-carotene (Fig. 4), which indicates that β-carotene may inhibit acetaldehyde metabolism in HepG2 cells. In the group treated with β-carotene plus acetaldehyde, β-carotene level in the medium was higher than that without added acetaldehyde (Fig. 5), which indicates that acetaldehyde may also inhibit β-carotene metabolism in HepG2 cells.

DISCUSSION

This study shows that, in HepG2 cells, β-carotene or acetaldehyde inhibit mitochondrial reductive activity, with a dose-dependent β-carotene effect. The combination of these two compounds resulted in an additive effect. Acetaldehyde
also caused LDH leakage from the cells into the medium, and the effect was exacerbated by β-carotene. LDH is a stable cytoplasmic enzyme present in all cells. Its release into the cell culture supernatant is a sensitive indicator of cell membrane injury. The acetaldehyde-mediated increase in LDH leakage observed here (Fig. 3) indicates that cell membranes were damaged, possibly due to oxidative injury (Thomas et al., 1993; Olivares et al., 1997; Yildiz et al., 1999). β-Carotene exacerbated this effect, possibly by enhancing oxidative stress (Ni et al., 2000).

The MTT test has been used extensively to assess the toxicity of cancer chemotherapeutic drugs and to examine their impact on the mitochondrial activity of cells. In this test, soluble tetrazolium salt is metabolically reduced to a coloured formazan. To reduce MTT to formazan, dehydrogenase activity is needed to produce NADH + H⁺ (Scudiero et al., 1988). Any factor that inhibits dehydrogenase activity will affect the associated colour reaction. β-Carotene inhibited the reducing function of mitochondria (Fig. 1). This inhibition of mitochondrial activity appeared already at a concentration (50 nM) (Fig. 2) lower than that observed in human plasma (Leo et al., 1995). The effect was exaggerated by combination with acetaldehyde (Fig. 1). Its mechanism needs to be further investigated. The effect of β-carotene alone was not accompanied by LDH leakage (Fig. 3). The metabolism of β-carotene involves the activities of a dioxygenase, ALDHs (Duester, 2000), cytosolic retinol dehydrogenase (CRD), microsomal retinol dehydrogenase (MRD), and CYP450 enzymes (Leo and Libeer, 1999). ALDHs have wide substrate specificity, metabolizing acetaldehyde, retinoids, and many other physiologically important aldehydes to the corresponding carboxylic acids. These enzymes are shared by the metabolism of retinol and acetaldehyde, and thus might be the site of acetaldehyde and β-carotene interactions, such as competitive inhibition. Indeed, the effect by β-carotene or acetaldehyde on each other’s disappearance from the medium suggests that these two chemicals share a common metabolic pathway and that a competitive interaction occurs. This decrease of each other’s metabolism may contribute to the exaggerated toxicity when the two are combined.

In the Alpha-Tocopherol, Beta-Carotene and Cancer Prevention Study (ATBC) (The Alpha-Tocopherol, Beta-Carotene and Cancer Prevention Study Group, 1994), it was noted that, in smokers, β-carotene supplementation increased death from coronary artery disease and the incidence of pulmonary cancer. It has previously been shown that while promoting deficiency of vitamin A (Sato and Lieber, 1981; Leo and Lieber, 1982), ethanol also enhances its toxicity (Mak et al., 1984, 1987) as well as that of β-carotene (Leo et al., 1992). Because heavy smokers are commonly heavy drinkers, we raised the possibility that alcohol abuse was contributory (Leo and Lieber, 1994), in part because alcohol is known to act as a co-carcinogen and to exacerbate the carcinogenicity of other xenobiotics, especially those of tobacco smoke (Garro et al., 1992), and also because of the toxic interaction between β-carotene and alcohol we had observed in the liver (Leo et al., 1992). Subsequently, analysis of the data of the ATBC (Albanes et al., 1997) and the Carotene and Retinol Efficacy Trial (CARET) (Ommen et al., 1996a,b) studies showed that the increased incidence of pulmonary cancer was related to the alcohol consumed by the participants.

Many observations show that β-carotene is not a conventional antioxidant. Under certain conditions, such as low oxygen pressure, β-carotene can behave as a very effective antioxidant, but under other conditions, such as at elevated oxygen pressures, β-carotene and related compounds may act as prooxidants (Burton and Ingold, 1984). Independent of oxygen pressure, at high concentrations, β-carotene can act as a prooxidant, but at low concentrations, it becomes an antioxidant (Woods et al., 1999). There are two broad classes of antioxidants in living organisms, referred to as preventive antioxidants and chain-breaking antioxidants (McBrien and Slater, 1983). Previous experimental results indicate that β-carotene is neither a peroxide-decomposing preventive antioxidant nor a conventional chain-breaking antioxidant (McBrien and Slater, 1983). Thus, β-carotene belongs to a previously unrecognized class of biological antioxidants.

Our present study supports the hypothesis that β-carotene exerts toxicity to liver cells, especially when combined with acetaldehyde. Oxidative stress and lipid peroxidation have been reported to be one of the main aspects of acetaldehyde-mediated toxicity in the liver (Lieber, 1992; Olivares et al., 1997; Ni et al., 2000). The mechanism by which β-carotene enhances the acetaldehyde-induced damage in vitro could be explained by β-carotene acting as a prooxidant and potentiating the corresponding effect of acetaldehyde. The mechanism by which β-carotene directly inhibits the reducing activity of mitochondria is presently not understood, but will be the subject of further study.

In conclusion, the experimental results of this preliminary study demonstrate that β-carotene, even at concentrations lower than those observed in vivo, inhibits the activity of mitochondria in human liver tumour cells, and that acetaldehyde enhances this effect. The toxicity of acetaldehyde to cell membranes was also exaggerated by β-carotene. Exacerbation of each other’s toxicity by these two chemicals might involve a mechanism of competition in partially common metabolic pathways and their prooxidant property. This potentiation might be responsible for the exaggeration of the toxicity of β-carotene by ethanol observed in vivo in baboons (Leo et al., 1992) and in rats (Leo et al., 1997) as well as in man in clinical trials (Albanes et al., 1997).

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REFERENCES


