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β-Carotene Generates Thiobarbituric Acid-Reactive Substances by Interaction with Nitrogen Dioxide in Air

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Generation of thiobarbituric acid-reactive substances (TBARS) from methyl linoleate in the exposure of nitrogen dioxide/air was inhibited by β-carotene in a dose-dependent manner. However, introduction of nitrogen dioxide/air or oxygen into a solution of β-carotene generated a significant amount of TBARS accompanying loss of its characteristic yellow color. Storing β-carotene in a solid state at ambient temperatures in air generated a large amount of TBARS accompanying loss of its yellow color. TBARS from β-carotene may interfere the measurement of TBARS from polyunsaturated fatty acids, and may give undesirable effects on biomaterials.

Keywords: β-carotene, nitrogen dioxide, thiobarbituric acid-reactive substance

INTRODUCTION

Attention has been paid to β-carotene as an effective scavenger for reactive oxygen species and lipid peroxy radicals.[1–3] In addition to the scavenging activity of β-carotene against singlet oxygen, the activity as an antioxidant or a prooxidant in lipid peroxidation has been shown. Burton and Ingold[4] have shown that β-carotene belongs to a previously unknown class of biological antioxidants especially effective at low oxygen pressures in the radical-induced oxidation of methyl linoleate as assessed by oxygen consumption. The antioxidant effectiveness of β-carotene at low oxygen pressures has been shown by measurement of lipid hydroperoxide levels.[5] Many papers have demonstrated the antioxidant effectiveness of β-carotene against lipid peroxidation (references cited in Refs. [2,6–9]) and the prooxidant activity at higher oxygen pressure[10,11] by measurement of the degree of lipid peroxidation by thiobarbituric acid-reactive substances (TBARS).

Nitrogen dioxide (NO_2) produced from nitrogen monooxide (NO) *in vivo*[12] and present as a pollutant in urban air and cigarette smoke[13] induces lipid peroxidation.[14,15] While β-carotene can protect cell membrane damage induced by

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NO_2-exposure,[16] prooxidant activity of β-carotene against NO_2/air-induced lipid peroxidation as assessed by TBARS has been demonstrated.[17] It is strange why prooxidant activity of β-carotene has been observed when the degree of lipid peroxidation is measured by TBARS.

TBARS are measured as red pigment produced in the heated acidic medium containing thiobarbituric acid (TBA).[18,19] Introduction of an antioxidant, i.e., butylated hydroxytoluene (BHT), into the assay medium is a requisite to avoid lipid peroxidation during the assay. TBARS composed of malonaldehyde derivatives and alkadienal/alkenal derivatives that liberate malonaldehyde and alkadienals/alkenals in the heated acidic medium are measured as red pigment.[18,19] The whole TBARS are measured at pH 3.5, and malonaldehyde derivatives are almost selectively measured at pH 3.5 in the presence of ethylenediaminetetraacetic acid (EDTA)[20–22] or in a strongly acidic medium.[23] Furthermore, separation of red pigment by HPLC is necessary when colored interfering substances are present.[24]

The present study was undertaken to clarify the effect of the interaction of β-carotene with NO_2/air, oxygen or air on the measurement of TBARS from polyunsaturated fatty acids. It was found that as unsaturated fatty acids β-carotene as well generated TBARS in contact with NO_2/air, oxygen and air. The results suggest that the prooxidant activity of β-carotene hitherto reported is due to the TBARS released from β-carotene itself.

MATERIALS AND METHODS

Materials

NO_2 (about 100 ppm)/air was obtained from Nippon Sanso Ltd. (Tokyo, Japan). Oxygen was obtained from Taiyo-Toyo Sanso Ltd. (Kawasaki, Japan). Concentration of NO_2 in the gas bomb was determined by the chemiluminescence method, and effective concentration after passing through the tubing (about 50 ppm) was determined by the method of Saltzman as described previously.[25] Methyl linoleate and tetramethoxypropane were obtained from Tokyo Chemical Industry Company Ltd. (Tokyo, Japan). β-Carotene (purity about 95%) was obtained from Sigma Chemical Company (St. Louis, MO, USA).

Thiobarbituric Acid (TBA) Assay

TBARS in the sample were determined according to the method previously described.[21,24] To the sample, 1.6 ml of water with or without 16.2 μmol EDTA, 0.1 ml of 8.8% BHT solution in glacial acetic acid, 0.4 ml of 8.1% sodium dodecylsulfate solution, 3.0 ml of 20% acetate buffer (pH 3.5) and 3.0 ml of 0.6% TBA (Merck) solution were added in this order. The solution was transferred into a test tube with a screw cap. The mixture was kept at 5°C for 60 min and then heated at 100°C for 60 min. After cooling, the mixture was extracted with 1.5 ml of chloroform. A 10 μl aliquot of the aqueous phase was subjected to HPLC on a Hitachi L-600 liquid chromatograph using a column (4.6 mm i.d. × 250 nm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan). The column was eluted with a mobile phase composed of 0.04 M acetate buffer (pH 5.5)–methanol (6 : 4, v/v) at a flow rate of 0.8 ml/min. Peaks were detected at 532 nm. Red pigment from standard tetramethoxypropane appeared at a retention time of 12.0 min. The amount of red pigment reflecting TBARS was obtained by comparing the peak area of red pigment with that obtained from the standard tetramethoxypropane.

NO_2/air- or Oxygen-exposure to Methyl Linoleate and β-carotene

NO_2 (50–100 ppm)/air or oxygen was introduced into 5.0 ml *n*-hexane containing 50 μmol methyl linoleate and/or 0–1 μmol β-carotene at a flow rate of 10 or 20 ml/min for 80 min–10 h. The total amount of NO_2 introduced was between 1.8 and

35 μmol, and the volume of oxygen introduced was upto 9 l. Absorption spectrum of the mixture was measured after the solution was made up to 5.0 ml and diluted 40-fold by addition of *n*-hexane. The whole mixture was evaporated to dryness and subjected to the TBA assay.

RESULTS

NO_2 (50 ppm) (total amount 1.8 μmol)/air was introduced into 5.0 ml *n*-hexane containing 50 μmol methyl linoleate. The amount of TBARS was carefully estimated.[21,24] The TBA assay was conducted in acetate buffer (pH 3.5) with BHT and without EDTA, and red pigment produced was separated by HPLC. Under the assay conditions both malonaldehyde derivatives and alkadienal/alkenal derivatives were measured as TBARS.[21] The amount of TBARS in the solution was increased from 9 to 143 nmol (corresponding to 0.3% of methyl linoleate) by the NO_2/air-exposure. The increased amount of TBARS may be due mainly to alkadienal/alkenal derivatives, because TBARS from oxidized methyl linoleate are composed mainly of alkadienal/alkenal derivatives and to a lesser extent of malonaldehyde derivatives.[22] When 1.8 μmol of NO_2/air was introduced into 5.0 ml *n*-hexane containing 50 μmol methyl linoleate and 0–1 μmol β-carotene, the amount of TBARS was decreased in a dose-dependent manner of β-carotene (Figure 1A). β-Carotene at 1 μmol decreased the amount of TBARS to less than 25 nmol (17% of that obtained in the absence of β-carotene). During the reaction, β-carotene was completely destroyed as assessed by maximum absorbance of β-carotene at 455 nm (Figure 1B). The result indicates that β-carotene scavenged NO_2 and inhibited the NO_2/air-induced peroxidation of methyl linoleate.

Different amounts of NO_2/air were introduced into 5.0 ml *n*-hexane containing 1 μmol β-carotene alone (Figure 2). During the exposure of more than 1.6 μmol NO_2/air, characteristic absorption of β-carotene was completely lost. Instead, a

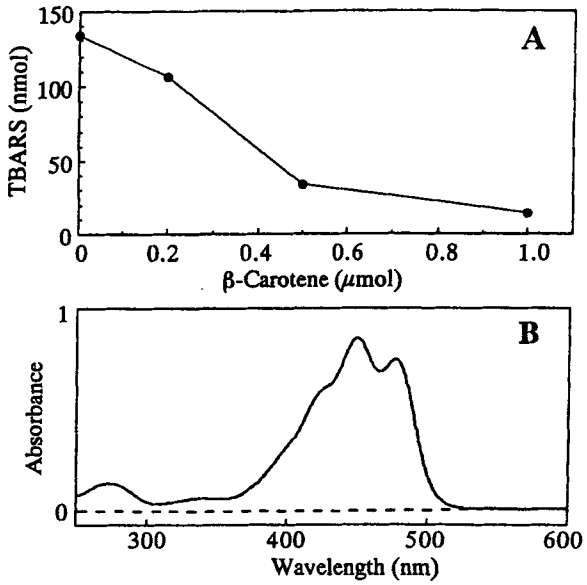

FIGURE 1 Effect of β-carotene on the formation of TBARS from methyl linoleate in the NO_2/air-exposure. NO_2 (1.7 μmol) in air (total volume of oxygen: 2.25 l) was introduced into 5.0 ml *n*-hexane containing 50 μmol methyl linoleate and 0–1 μmol β-carotene. A: TBARS of the mixture were measured as the amount of red pigment after the reaction in acetate buffer (pH 3.5) with BHT and without EDTA, and successive separation by HPLC. B: Absorption spectra of the 40-fold diluted solution of the mixture containing 1 μmol β-carotene were measured before and after the exposure.

significant amount of TBARS was generated. The amount of TBARS was increased in a dose-dependent manner of NO_2/air. The amount of TBARS generated in the exposure of 35 μmol NO_2/air was estimated to be 3.7 nmol (0.37% of β-carotene). As methyl linoleate, β-carotene as well effectively generated TBARS in the NO_2/air-exposure. Hence, the amount of TBARS obtained in the NO_2/air-exposure of methyl linoleate in the presence of β-carotene (Figure 1) may be derived from both methyl linoleate and β-carotene.

When a large amount of oxygen was introduced into 5.0 ml *n*-hexane containing 1 μmol β-carotene (Figure 3), maximum absorbance of β-carotene was decreased. Instead, a significant amount of TBARS was generated. The amount

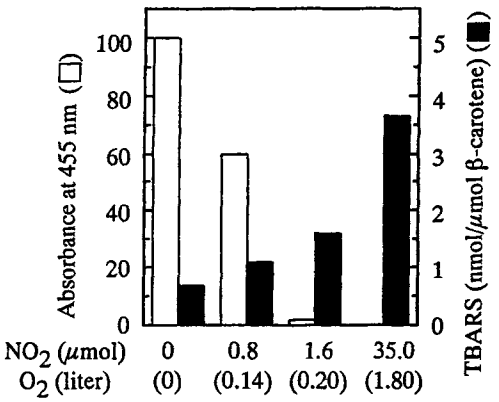

FIGURE 2 Formation of TBARS from β-carotene in *n*-hexane in the NO_2/air-exposure. NO_2 at the indicated amount in air (volume (l) of oxygen) was introduced into 5.0 ml *n*-hexane containing 1 μmol β-carotene. Absorbance at 455 nm of the 40-fold diluted solution of the mixture before the exposure was 0.79 (expressed as 100%). Absorbance of the exposed solution was measured and the percentage of the absorbance against that of the unexposed control is shown (□). TBARS of the mixture were measured as the amount of red pigment after the reaction in acetate buffer (pH 3.5) with BHT and without EDTA, and successive separation by HPLC (■).

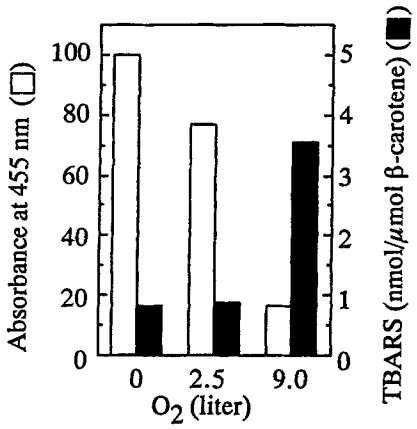

FIGURE 3 Formation of TBARS from β-carotene in *n*-hexane in the oxygen-exposure. Oxygen at the indicate volume (l) was introduced into 5.0 ml *n*-hexane containing 1 μmol β-caroten. Absorbance of the exposed solution was measured and the percentage of the absorbance against that of the unexposed control is shown (□). TBARS of the mixture were measured as the amount of red pigment after the reaction in acetate buffer (pH 3.5) with BHT and without EDTA, and successive separation by HPLC (■).

of TBARS was increased in a dose-dependent manner of oxygen. The amount of TBARS generated in the exposure of 9 l of oxygen was estimated to be 3.7 nmol (0.37% of β-carotene).

It has been claimed that great caution should be taken to preserve β-carotene powder with respect to temperature, light and air. When β-carotene powder was kept at −20°C in air in the dark, maximum absorbance at 455 nm was unchanged during 136 h, and the amount of TBARS was kept at 0.5–1 nmol during 256 h (Figure 4A). When the powder was kept at 22°C in air in the dark (Figure 4B) or in the light (Figure 4C), the absorbance was decreased to around 60% after 136 h and the amount of TBARS was increased to 1.5 nmol (0.15% of β-carotene) after 136 h and 3–4 nmol (0.3–0.4% of β-carotene) after 256 h. When the powder was kept at 37°C in air in the dark (Figure 4D), the absorbance was decreased to around 45% after 136 h, and the amount of TBARS increased to 6 nmol (0.6% of β-carotene) after 136 h and 10 nmol (1.0% of β-carotene) after 256 h. When the powder was kept at 45°C in air in the dark (Figure 4E), the absorbance was decreased to around 20% after 136 h, and the amount of TBARS increased to 7 nmol (0.7% of β-carotene) after 136 h and 14 nmol (1.4% of β-carotene) after 256 h.

The amounts of TBARS in the powder kept at 45°C under air in the dark after 256 h were estimated in the TBA assay with EDTA. The amount of TBARS without EDTA (Figure 4E ■) was slightly decreased in the assay with EDTA (Figure 4E □). Eighty-five percent of TBARS was measured in the presence of EDTA, indicating that the major TBARS from β-carotene oxidation were malonaldehyde derivatives. TBARS from β-carotene oxidation may be composed mainly of malonaldehyde derivatives and to a lesser extent of alkadienal/alkenal derivatives.

DISCUSSION

In the lipid peroxidation studies, TBARS composed of malonaldehyde derivatives and alkadienal/alkenal derivatives are measured at pH 3.5, and those composed of malonaldehyde derivatives are selectively measured at pH 3.5

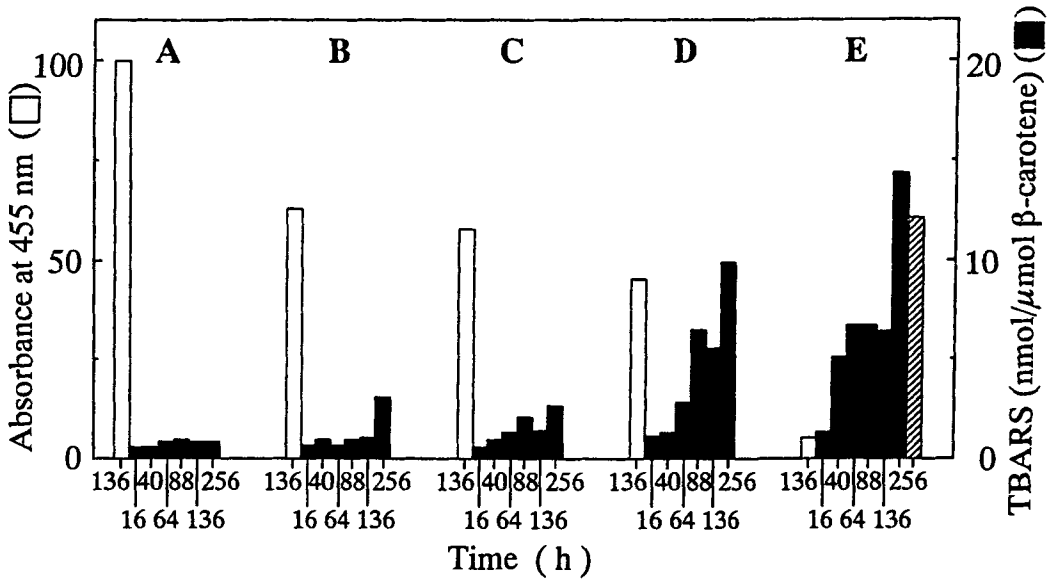

FIGURE 4 Formation of TBARS from β-carotene powder upon standing in air. β-Carotene powder was kept in air at −20°C in the dark (A), at 22°C in the dark (B), at 22°C in the light (C), at 37°C in the dark (D), and at 45°C in the dark (E). One micromolar equivalent amount of the sample was dissolved into 5.0 ml *n*-hexane. Absorbance of the solution was measured and the percentage of the absorbance against that of the unexposed sample is shown (▨). TBARS of the mixture were measured as the amount of red pigment after the reaction in acetate buffer (pH 3.5) with BHT and without EDTA (■), with BHT and EDTA (□), and successive separation by HPLC.

in the presence of EDTA because iron ion is required for the production of red pigment from alkadienal/alkenal derivatives.[20–22]

In the exposures of NO_2/air, oxygen, or air, β-carotene generated a significant amount of TBARS in the TBA assay at pH 3.5. The amount of TBARS generated from β-carotene in *n*-hexane in the exposure of NO_2/air-exposure or a large amount of oxygen was increased to 0.37% of β-carotene. The amount of TBARS generated from β-carotene in a solid state at ambient temperatures in the air-exposure was increased to 1.4% of β-carotene. The amounts of TBARS from β-carotene oxidation were high enough when compared to that of TBARS generated from methyl linoleate in *n*-hexane in the NO_2/air-exposure (0.3% of methyl linoleate).

In the lipid peroxidation studies, it has been shown that TBARS composed of malonaldehyde derivatives were reactive and those composed of alkadienals/alkenals were unreactive in the presence of EDTA. By the differences in the TBA reactivity in the absence and presence of EDTA, TBARS of oxidized plant oils and methyl linoleate are found to be composed mainly of alkadienal/alkenal derivatives,[22] those of rat tissue homogenates of both alkadienal/alkenal and malonaldehyde derivatives,[21] and those of human urine mainly of malonaldehyde derivatives.[24] TBARS from β-carotene in the present study may be composed mainly of malonaldehyde derivatives, because most of TBARS were reactive in the presence of EDTA. While the structure of the TBARS from β-carotene is not known, they may readily liberate malonaldehyde upon heating in the acidic medium of the TBA assay. While other phenolic antioxidants cannot generate TBARS under the oxidative conditions, β-carotene is different in its nature for production of TBARS under the oxidative conditions. The use of the TBA assay for lipid samples containing β-carotene may be limited, although many workers have employed the assay for evaluation of antioxidant effectiveness of β-carotene against lipid

peroxidation (references cited in Refs. [2,6–11, 17]). While it appeared that β-carotene protected methyl linoleate from the NO_2/air-induced peroxidation (Figure 1), the results may not be accurate because β-carotene generated TBARS. In this reaction, the molar ratio of β-carotene to methyl linoleate was less than 2%. In the estimation of the NO_2/air-induced peroxidation of microsomal lipids containing the higher concentration of β-carotene at 4–24% against phospholipids,[17] β-carotene may generate a considerable amount of TBARS. Prooxidant activity of β-carotene obtained as assessed by TBARS[10,11,17] may be ascribable to TBARS generated from β-carotene.

TBARS generated from β-carotene in the NO_2/air-exposure may exert some deteriorative effect on biomaterials. While epidemiological studies in humans have suggested that β-carotene aids in cancer prevention,[26] surprising reports showing that β-carotene can increase incidence of lung cancer in heavy smokers appeared.[27,28] However, the chemistry of the interaction of β-carotene with NO_2, a major free radical toxin in cigarette smoke, remains obscure. There are a few earlier observations on the interaction of β-carotene and NO_2. β-Carotene scavenges NO_2 effectively and is degraded more rapidly than polyunsaturated fatty acids,[25] it gives nitrosating agent in the dark and NO in the light,[29] it generates β-carotene cation radical,[30] and it destroys tyrosine and/or 3-nitrotyrosine.[31] TBARS generated from β-carotene by the interaction with NO_2 may give some undesirable effects on biomaterials.

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