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β-Carotene effectively scavenges toxic nitrogen oxides: nitrogen dioxide and peroxynitrous acid

Kiyomi Kikugawa*, Kazuyuki Hiramoto, Susumu Tomiyama, Yoshiko Asano

School of Pharmacy, Tokyo University of Pharmacy and Life Science, (Formerly Tokyo College of Pharmacy), 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

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Abstract **β-Carotene absorbed 2 equimolar amounts of NO_2 accompanying the complete destruction of β-carotene. Electron spin resonance study using 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl revealed that no significant amounts of NO were released by the interaction. Nitrogen atoms derived from NO_2 were tightly bound to the β-carotene molecules. Destruction of β-carotene was inhibited little by α-tocopherol and polyunsaturated fatty esters, and slightly by ascorbyl palmitate, indicating that β-carotene was a more effective scavenger of NO_2. ONOOH/$ONOO^-$ and 3-morpholinosydononimine similarly destroyed β-carotene. The results suggest that β-carotene contributes to the prevention of cytotoxicity and genotoxicity of NO_2 and ONOOH/$ONOO^-$ derived from NO.**

Key words: β-Carotene; Nitrogen monoxide; Nitrogen dioxide; Peroxynitrous acid

1. Introduction

It is well-known that nitrogen monoxide (NO) is synthesized by endothelial cells [1] and nerve cells [2] as an intercellular messenger, which has important roles in vasoregulation and synaptic plasticity. Furthermore, pathological conditions can greatly stimulate the synthesis of NO in many types of tissues [3]. In contrast, NO can also be considered an undesirable mediator of tissue injury. NO is readily converted into chemically reactive nitrogen dioxide (NO_2) in contact with molecular oxygen [4]. NO is converted into peroxynitrite ($ONOO^-$) in contact with superoxide, which is in turn transformed into chemically reactive peroxynitrous acid (ONOOH) by protonation at pK_a 6.8 [5,6].

Much literature has shown that β-carotene is an effective scavenger for reactive oxygen species and protects against oxygen-mediated cytotoxicity and genotoxicity by scavenging singlet oxygen and other reactive oxygen species [7]. A recent study by Cooney et al. [8] has shown that NO_2 is converted by β-carotene into nitrosating agents in the dark and into NO in the light. Everett et al. [9] have shown that reaction of β-carotene with NO_2 generated by pulse radiolysis gives β-carotene radical cation and HNO_2. In the present study, the reactivity of β-carotene toward NO, NO_2, ONOOH/$ONOO^-$ and 3-morpholinosydononimine (SIN-1) that generates ONOOH/$ONOO^-$ was examined. It was found that β-carotene had a strong scavenging activity for NO_2, ONOOH/$ONOO^-$ and SIN-1, but not for NO.

2. Materials and methods

2.1. Materials

NO_2 (103 ppm) in air, NO_2 (101 ppm) in nitrogen gas and NO (100 ppm) in nitrogen gas were obtained from Nippon Sanso Ltd. (Tokyo, Japan). The concentrations were determined by the chemiluminescence method [10]. Peroxynitrite ($ONOO^-$) was prepared according to the method previously described [11]. In brief, a solution of $NaNO_2$, a solution of H_2O_2 in HCl and a solution of NaOH were mixed in a quenched flow reactor with two T-junctions at a flow rate of 40 ml/min. The alkaline $ONOO^-$ solution obtained in a collection chamber was stored in a −20° freezer to concentrate $ONOO^-$ into a yellow-colored liquid layer on the top of the ice crystal. The liquid layer obtained contained about 0.5 M $ONOO^-$ when determined using an extinction coefficient of 1670 at 302 nm [11] in 1.0 M NaOH.

β-Carotene (purity about 95%) was obtained from Sigma Chemical Company (St. Louis, MO, USA). DL-α-Tocopherol (α-toc) (purity >98%), L-ascorbyl 6-palmitate (ascorbyl palmitate) (purity >95%), methyl linoleate (ML) (purity >95%) and 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Ethyl icosapentaenoic acid (EIPA) (purity about 90%) and ethyl docosahexaenoic acid (EDHA) (purity about 90%) were obtained from Nippon Oil and Fats Company (Tokyo, Japan). SIN-1 was a product of Dojindo Laboratories (Kumamoto, Japan).

2.2. Analysis

In order to determine the effective concentrations of NO_2 and NO after passage through the tubing, their concentrations were determined each time according to the method of Saltzman [12] using a conversion coefficient of $NO_2^-/NO_2 = 0.84$, where the measured NO_2^- concentration was divided by 0.84 to convert to the concentration of NO_2. NO_2 in air or in nitrogen was bubbled directly into 10 ml of the Saltzman solution composed of sulfanilic acid, *N*-(1-naphthyl)ethylenediamine and acetic acid placed in a tube (15 mm i.d. and 105 mm) at a flow rate of 10 ml/min for exactly 10 min. The solution was made up to 25.0 ml with Saltzman solution, and its absorbance at 545 nm was determined. The concentration of NO_2^- was determined by comparing the absorbance with that obtained from 25.0 ml of the standard solution containing 0.375 μmol $NaNO_2$. The concentration of NO in nitrogen was determined after passing through 20 ml of the Saltzman oxidizing solution containing $KMnO_4$ and H_2SO_4 in order to convert NO into NO_2. NO_2 and NO in *n*-hexane could be purged out completely into the above solution with purified nitrogen gas stream at a flow rate of 10 ml/min for 30 min.

Electron spin resonance (ESR) spectra were measured using an X-band JES-RE1X spectrometer (JEOL, Tokyo, Japan) with a Mn^{2+} marker at room temperature using a capillary tube. The instrumental conditions were: field setting at 336.0 mT, scan range of 10 mT, modulation frequency of 100 kHz, microwave power of 10 mW and modulation amplitude of 0.1 mT.

2.3. Reaction of ONOOH/$ONOO^-$ with β-carotene

The reaction of ONOOH/$ONOO^-$ was conducted by final addition of the alkaline solution of $ONOO^-$ to the reaction mixture. Thus, to 5.0 ml of a solution composed of 0.5 ml of a solution of 100 μM β-carotene in dioxane, 3.0 ml of dioxane, 0.5 ml of 50 mM phosphate buffer (pH 7.0) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1.0 ml of water, an aliquot of less than 6.25 μl of the alkaline solution of $ONOO^-$ was added to make a final concentration of ONOOH/$ONOO^-$ of 0–500 μM. For investigation of the effect of α-toc and ascorbyl palmitate, 1.0 ml of dioxane was replaced by 1.0

*Corresponding author. Fax: (81) (426) 764508.

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ml of a solution of 50 mM α-toc or ascorbyl palmitate in dioxane. The pH value of the buffer after the addition of the indicated amount of the alkaline $ONOO^-$ solution was checked, and it was maintained between 7.2 and 7.7. DTPA was added to the reaction mixture to avoid the metal-dependent generation of hydroxyl radical from the preparation of $ONOO^-$ [11]. A reverse-order-of-addition control experiment [11] was performed: first addition of 2 mM $ONOO^-$ to the solution without β-carotene and subsequent addition of β-carotene to the solution containing the decomposed ONOOH.

3. Results

NO (285 nmol) in nitrogen was introduced into 5.0 ml of *n*-hexane alone or *n*-hexane containing 50 nmol β-carotene for 10 min. The whole gas emitted from the solution during the exposure and after purging the solution with purified nitrogen gas was introduced into the Saltzman oxidizing solution and successively into the Saltzman solution to determine the amount of the diazotizing species. More than 96% of NO introduced was recovered from both solutions. The absorption spectrum of the solution of β-carotene in the solvent was unchanged during the NO exposure. The results indicate that NO did not react with β-carotene.

NO_2 (average 240 nmol) in air was introduced into *n*-hexane containing 50 nmol β-carotene, α-toc, ML, EIPA or EDHA for 10 min (Table 1). The whole gas was directly introduced into the Saltzman solution to determine the amount of the diazotizing species. The value obtained as the amount of NO_2 may reflect both the remaining NO_2 and the released HNO_2. β-Carotene was most effective in absorbing NO_2 ($+HNO_2$), and the percent decrease of NO_2 was about 43%. The result indicates that β-carotene absorbed 2 equimolar amounts of NO_2 ($+HNO_2$). In contrast, α-toc, ML, EIPA and EDHA caused the loss of much less NO_2 ($+HNO_2$). Because it has been shown that α-toc [13] and the polyunsaturated fatty esters [14] can convert NO_2 into HNO_2, these compounds may have converted some of the NO_2 into HNO_2 under the present conditions.

When NO_2 in air was introduced into *n*-hexane containing 50 nmol β-carotene during a period of 10 min, the characteristic absorption spectrum of the solution with a maximum at 450 nm rapidly disappeared (Fig. 1A-1), and the time course of the decrease of the absorbance showed that β-carotene was completely destroyed by about 2 equimolar amounts of NO_2 (Fig. 1A-2). Hence, the molar ratio of the reaction of β-carotene and NO_2 may be 1:2. When NO_2 in nitrogen was introduced into the solution, β-carotene was similarly destroyed. When the purified air was introduced into the solution, β-carotene was not destroyed. When NO_2 in air was introduced into methanol containing 50 nmol β-carotene during a period of 30 min, the spectral change of the solution was more gradual (Fig. 1B-1) and 6 equimolar amounts of NO_2 were required for the complete destruction of β-carotene (Fig. 1B-2). The high reactivity of β-carotene toward NO_2 in *n*-hexane may be due to the unreactivity of NO_2 toward the solvent.

Table 1
Decrease of NO_2 by β-carotene, α-toc and polyunsaturated fatty esters

Substrate	Percent recovered NO_2 ($+HNO_2$) ± SD
β-Carotene	57 ± 6.5
α-Toc	93 ± 2.6
ML	89 ± 6.3
EIPA	90 ± 3.6
EDHA	88 ± 3.5

NO_2 (52–67 ppm) in air (total amounts 210–270 nmol) was introduced into 5.0 ml of *n*-hexane containing 50 nmol of the substrate at a flow rate of 10 ml/min for 10 min. NO_2 introduced into the solvent alone was completely recovered by purging with nitrogen gas. The number of experiments was 5. Percent recovered NO_2 ($+HNO_2$) is expressed by the average value ± SD.

It is known that NO specifically reduces PTIO into a different nitroxide radical, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTI), and the conversion of the ESR signals of PTIO into those of PTI indicates generation of NO [15]. Thus, NO_2 (40 nmol) in air was introduced into chloroform containing 40 nmol PTIO and 7.2 nmol β-carotene. In the control chloroform without β-carotene, 5-line ESR signals of PTIO with hyperfine splitting constants of $a_N^1 = a_N^2 = 0.77$ mT were unchanged by the exposure (Fig. 2A). In the test solution with β-carotene, the signals of PTIO were unchanged by the exposure and no 9-line signals of PTI appeared (Fig. 2B). In con-

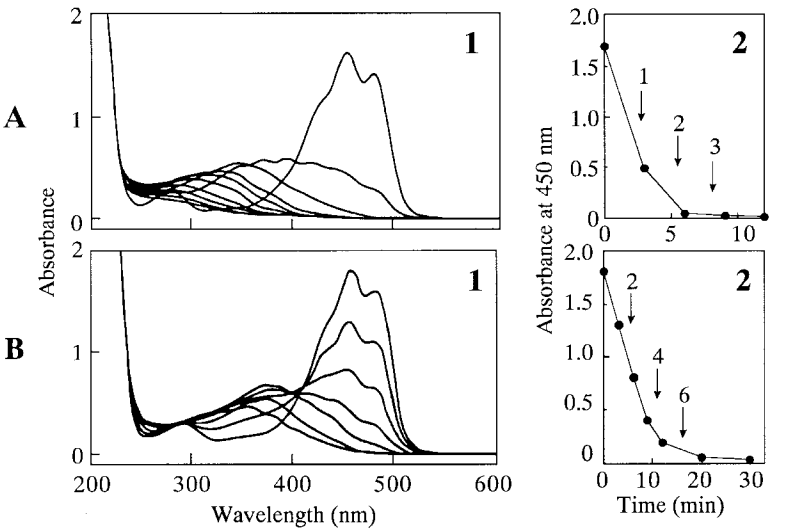

Fig. 1. Decrease of β-carotene in *n*-hexane (A) and in methanol (B) by NO_2 exposure. NO_2 (50 ppm) in air was introduced into 5.0 ml of a solution containing 50 nmol β-carotene at a flow rate of 10 ml/min. 1: Spectral change of the solution. 2: Time course of the decrease of the absorbance of the solution at 450 nm. The numbers indicated by arrows show equimolar amounts of NO_2 introduced.

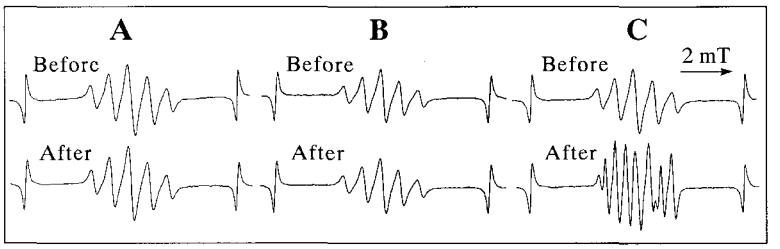

Fig. 2. ESR spectra of PTIO (A) and PTIO+β-carotene (B) exposed to NO_2 in air, and PTIO exposed to NO in nitrogen (C). NO_2 (50 ppm) in air (40 nmol) was introduced into 4.0 ml chloroform containing 40 nmol PTIO in the absence (A) and the presence of 7.2 μmol β-carotene (B) at a flow rate of 10 ml/min for 1.7 min. An equimolar amount of NO in nitrogen gas was introduced into the PTIO solution (C). ESR spectra with a receiver gain set at 790 were obtained before and after the exposure.

trast, when an equimolar amount of NO in nitrogen was introduced into the PTIO solution, the characteristic 9-line ESR signals of PTI with hyperfine splitting constants of $a_N{}^1 = 0.93$ mT and $a_N{}^2 = 0.44$ mT appeared (Fig. 2C). It was concluded that β-carotene released no significant amounts of NO in contact with NO_2.

NO_2 (30 μmol) in air was introduced into 30 ml of *n*-hexane containing 14 μmol β-carotene during the longer period of 19 h until the orange-yellow color of the solution completely disappeared. Elemental analysis of the resulting powder obtained by evaporation of the solvent showed C: 66.31, H: 10.49, N: 3.19%. Considering the elemental composition of β-carotene $C_{40}H_{56}$ (C: 89.49, H: 10.51%), 1.65 nitrogen atoms were introduced into a β-carotene molecule with 40 carbon atoms. About 80% of 2 equimolar amounts of NO_2 introduced was trapped in the β-carotene molecule by the exposure.

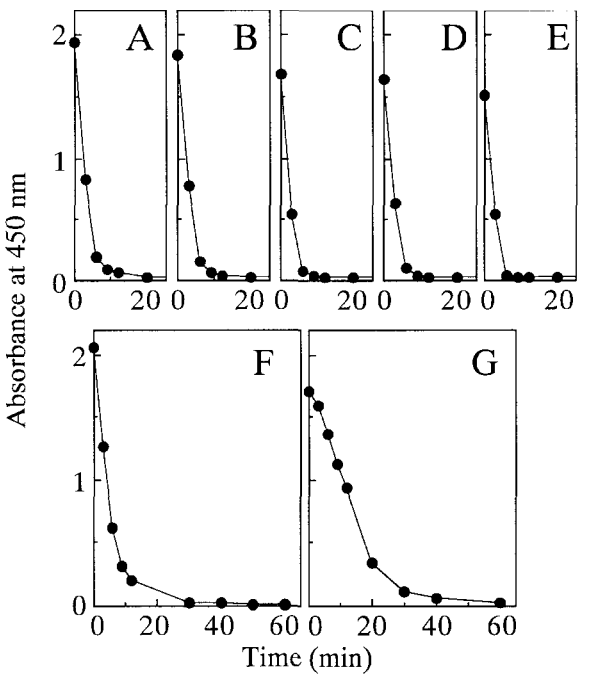

Fig. 3. Decrease of β-carotene by NO_2 exposure in the presence of additives. NO_2 (43 ppm) in air was introduced into 5.0 ml of a solution of 50 nmol β-carotene in *n*-hexane containing no addition (A), 50 nmol α-toc (B), ML (C), EIPA (D), EDHA (E), or into 5.0 ml of a solution of 50 nmol β-carotene containing no addition (F) or 50 nmol ascorbyl palmitate (G), at a flow rate of 10 ml/min. The time course of the decrease of the absorbance of the solution at 450 nm was followed.

In order to compare the reactivity of β-carotene toward NO_2 with that of α-toc, ML, EIPA, EDHA or ascorbyl palmitate, the decrease of β-carotene in *n*-hexane (Fig. 3A–E) or in methanol (Fig. 3F,G) in the presence of each of these compounds by NO_2 exposure was monitored. It was found that the destruction of β-carotene was prevented little by α-toc, ML, EIPA or EDHA and only slightly by ascorbyl palmitate. Hence, the reactivity of β-carotene toward NO_2 was much higher than that of those compounds.

The reactivity of β-carotene toward $ONOOH/ONOO^-$ prepared from H_2O_2 and NO_2^- was examined. 5.0 ml of a solution of 50 nmol β-carotene in 70% dioxane/phosphate buffer (pH 7.2–7.7) was treated with 0–2.5 μmol $ONOOH/ONOO^-$. The characteristic absorption of β-carotene disappeared on treatment with $ONOOH/ONOO^-$ in a dose-dependent manner (Fig. 4A,B). In contrast, when the solution was treated with the decomposition products of 10 μmol $ONOOH/ONOO^-$ by the reverse-order-of-addition control [11], the absorption spectrum of β-carotene was unchanged (data not shown). Hence, β-carotene was reactive toward $ONOOH/ONOO^-$ and not toward contaminating H_2O_2, NO_2^- ion, NO_3^- ion and other species. In order to compare the reactivity of β-carotene toward $ONOOH/ONOO^-$ with that of α-toc or ascorbyl palmitate, the destruction of 50 nmol β-carotene by 0.5 μmol $ONOOH/ONOO^-$ in the presence of 50 nmol α-toc or ascorbyl palmitate was examined. It was found that α-toc and ascorbyl palmitate inhibited the destruction of β-carotene partially by 50 and 70%, respectively. β-Carotene was effective in scavenging $ONOOH/ONOO^-$ in the presence of α-toc and ascorbyl palmitate.

The reaction of β-carotene toward SIN-1, which may generate $ONOOH/ONOO^-$ [16], was examined (Fig. 5). When β-carotene was incubated with an excess amount of SIN-1 at 37°C in 70% dioxane/phosphate buffer (pH 7.5), β-carotene was gradually lost in a time-dependent manner and almost completely after 120 min. This result indicates that β-carotene was reactive toward SIN-1 and thus $ONOOH/ONOO^-$.

4. Discussion

It was found in the present study that β-carotene absorbed 2 equimolar amounts of NO_2, during which β-carotene was completely destroyed. The destruction of β-carotene in the NO_2 exposure was inhibited little by α-toc or the polyunsaturated fatty esters, and slightly by ascorbyl palmitate, indicating that the reactivity of β-carotene toward NO_2 is higher than that of these biomaterials which are known to scavenge NO_2 and consequently release HNO_2 [13,14]. Interaction of β-

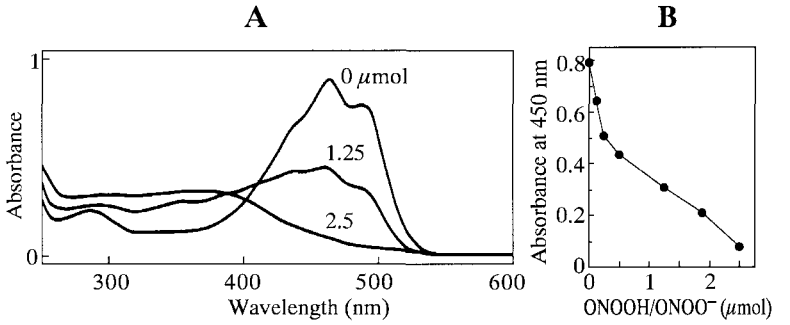

Fig. 4. Decrease of β-carotene by ONOOH/ONOO⁻. To 5.0 ml of a solution of 50 nmol β-carotene in 70% dioxane/5 mM phosphate buffer (pH 7.2–7.7) and 10 μM DTPA, 0–2.5 μmol alkaline ONOO⁻ was added. The pH value of the buffer after the addition of the alkaline ONOO⁻ was maintained at pH 7.2–7.7. The absorption spectrum of the solution was recorded immediately. A: Spectral changes of the solutions treated with 0, 1.25 and 2.5 μmol ONOOH/ONOO⁻. B: Decrease of absorbance at 450 nm versus the amount of ONOOH/ONOO⁻.

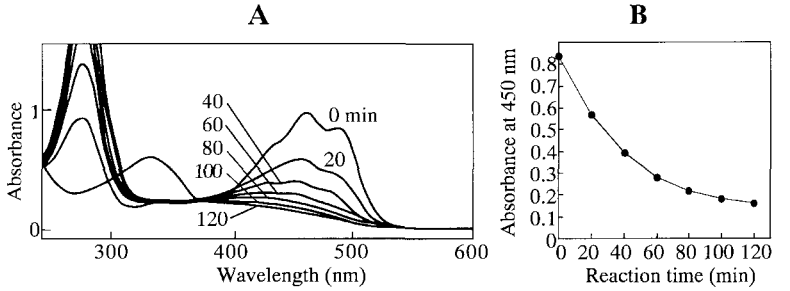

Fig. 5. Decrease of β-carotene by SIN-1 in 70% dioxane. 5 ml of a solution of 50 μmol β-carotene and 0.5 mmol SIN-1 in 70% dioxane/phosphate buffer (pH 7.5) was incubated at 37°C for 120 min. A: Spectral change of the mixture at the indicated period. B: Time course of the decrease in absorbance at 450 nm of the mixture.

carotene with NO_2 released little HNO_2 or NO under the present conditions and nitrogen atoms from NO_2 might be tightly bound to the β-carotene molecule. The present results are somewhat different from those with respect to the release of nitrogen species [8,9]. It has been shown that NO_2 is converted by β-carotene into nitrosating agents in the dark and into NO in the light [8], and that NO_2 generated by pulse radiolysis is converted into HNO_2 by β-carotene [9].

It was found that ONOOH/ONOO⁻ destroyed β-carotene. β-Carotene showed reactivity for ONOOH/ONOO⁻ even in the presence of α-toc, a known scavenger of ONOOH/ONOO⁻ [17], and ascorbyl palmitate. SIN-1, which generates both NO and superoxide and thus ONOOH/ONOO⁻ [16], similarly destroyed β-carotene.

While β-carotene appeared unreactive towards NO, it was highly reactive towards NO_2, ONOOH and SIN-1. β-Carotene may be a better scavenger for these toxic nitric oxides than α-toc and ascorbyl palmitate, and may contribute in protection against the NO-induced cytotoxicity and genotoxicity.

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