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Effect of β-Carotene on the Transformation of Tyrosine by Nitrogen Dioxide and Peroxynitrous Acid

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In the NO_2-exposure of tyrosine in 70% dioxane/phosphate buffer (pH 7.4), β-carotene enhanced the degradation of tyrosine and/or 3-nitrotyrosine produced, whereas α-tocopherol and ascorbyl palmitate inhibited the transformation of tyrosine into 3-nitrotyrosine. Generation of certain active species in the interaction of β-carotene with NO_2 was suggested. Ascorbyl palmitate effectively and α-tocopherol slightly inhibited the transformation of tyrosine in the NO_2-exposure in the presence of β-carotene. In the reaction of tyrosine with $ONOO^-$/ONOOH, β-carotene enhanced the degradation of 3-nitrotyrosine produced suggesting generation of certain active species, whereas α-tocopherol and ascorbyl palmitate completely suppressed the transformation of tyrosine into 3-nitrotyrosine.

Keywords: β-Carotene, nitrogen dioxide, peroxynitrite, tyrosine

INTRODUCTION

Reactive nitrogen species (RNS) including nitrogen dioxide (NO_2) and peroxynitrous acid (ONOOH) are produced from nitrogen monoxide (NO) synthesized by endothelial cells, nerve cells and many types of cells[1] in contact with oxygen[2] and superoxide,[3] respectively. NO_2 and ONOOH are considered to be an undesirable mediator for tissue injury. NO_2 is also a pollutant in urban air and cigarette smoke.[4] Most serious injury caused by RNS is the modification of proteins especially tyrosine and tryptophan residues. Thus, tyrosine is converted into 3-nitrotyrosine by NO_2[5,6] and ONOOH,[7,8] and tryptophan is degraded by NO_2[6] and ONOOH.[8,9] 3-Nitrotyrosine has been considered to be a biomarker of generation of NO_2 and ONOOH, and detected in many biological samples.[10–17]

Attention has been paid to β-carotene as an effective scavenger for reactive oxygen species (ROS) including singlet oxygen.[18] There have been few reports demonstrating chemical interaction of β-carotene with RNS. Reaction of β-carotene with NO_2 yields a nitrosating agent in the dark[19] or β-carotene cation radical and

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HNO_2.[20] Our previous paper has shown that β-carotene scavenges NO_2 and ONOOH more effectively than other antioxidants.[21] In the present paper, effect of β-carotene on the transformation of tyrosine into 3-nitrotyrosine in the reaction with NO_2 or ONOOH was investigated. It was found that β-carotene effectively enhanced the degradation of tyrosine and/or 3-nitrotyrosine by NO_2 and ONOOH, whereas other antioxidants inhibited the transformation of tyrosine into 3-nitrotyrosine.

MATERIALS AND METHODS

Materials

NO_2 in air (about 100 ppm) was obtained from Nippon Sanso Ltd. (Tokyo, Japan). Concentration of NO_2 in a 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.[21] Peroxynitrite ($ONOO^-$) was prepared according to the method previously described.[22] Strongly alkaline solution containing 0.5 M $ONOO^-$ was used. $ONOO^-$ is in turn transformed into chemically reactive ONOOH by protonation at p*Ka* 6.8[3] in the reaction buffer system. β-Carotene (purity about 95%) and 3-nitrotyrosine were obtained from Sigma Chemical Company (St. Louis, MO, USA). *dl*-α-Tocopherol (α-Toc) (purity; above 98%) and L-ascorbyl-6-palmitate (ascorbyl palmitate) (purity above 95%) were obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). 3,3′-Dityrosine (dityrosine) was prepared according to the method previously described.[6]

Analysis

Tyrosine, 3-nitrotyrosine and dityrosine were determined by high performance liquid chromatography (HPLC) using a Shimadzu LC-6A liquid chromatograph (Osaka, Japan) equipped with a column (4.6 mm i.d × 250 mm) of Inertsil ODS-2 (GL Science Inc., Tokyo, Japan). The column was eluted with a mobile phase composed of 0.5% acetic acid/methanol (29 : 1, v/v) at a flow rate of 0.8 ml/min.[6] The fractions were monitored by a Shimadzu SPD-6A UV spectrometric detector at 280 nm for tyrosine and 3-nitrotyrosine and by a Shimadzu AF-535 fluorescence HPLC monitor at excitation 285 nm/emission 410 nm for dityrosine. Tyrosine and 3-nitrotyrosine were eluted at retention times of 9 and 32 min, respectively. Dityrosine was eluted at a retention time of 13 min. Concentration of each compound in sample solutions was determined by comparing the corresponding peak height with that obtained from the standard solution of each compound.

Reaction of Tyrosine with NO_2

NO_2 in air (50 ppm) was introduced into a 5 ml mixture composed of 3.5 ml of dioxane containing β-carotene, α-Toc or ascorbyl palmitate, or combined antioxidants, 0.5 ml of 50 mM phosphate buffer (pH 7.5) and 1.0 ml of a solution of tyrosine, 3-nitrotyrosine or tryptophan in water, at a flow rate of 10 ml/min for 60 min. The pH of the reaction mixture was unchanged by the NO_2-exposure during the period. The reaction mixture was then subjected to the product analysis.

Reaction of Tyrosine with $ONOO^-$/ONOOH

Reaction of $ONOO^-$/ONOOH was conducted by final addition of the alkaline solution of $ONOO^-$ to the reaction mixture. To 5 ml of a solution composed of 3.5 ml of a solution of β-carotene, α-Toc or ascorbyl palmitate in dioxane, 0.5 ml of 50 mM phosphate buffer (pH 7.0) with 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 1 ml of a solution of tyrosine or 3-nitrotyrosine in water, an aliquot (about 20 μl) of the alkaline solution of $ONOO^-$ was added to make a final concentration of $ONOO^-$/ONOOH at 2 mM.

The pH value of the buffer after the addition of $ONOO^-$ was checked, and it was maintained between 7.3 and 7.7. DTPA was added to the reaction mixture to avoid the metal-dependent generation of hydroxyl radical from the preparation of $ONOO^-$/ONOOH.[22] The reaction mixture was immediately subjected to the product analysis.

RESULTS

When NO_2 in air (50 ppm) was introduced into a solution of 10 μM β-carotene in 70% dioxane/phosphate buffer (pH 7.5) for 1 h, β-carotene was completely destroyed after introduction of 2 equimolar amounts of NO_2 (Figure 1, closed circle). The results were consistent with those obtained using *n*-hexane as a solvent.[21] Under the same conditions introduction of air alone did not affect the stability of β-carotene (data not shown). In the presence of 20 equimolar amounts (0.2 mM) of tyrosine (Figure 1, open circle) β-carotene was destroyed at the similar rate, indicating that tyrosine was not effective to prevent β-carotene destruction. In the presence of 50 equimolar amounts (0.5 mM) of α-Toc (Figure 1 closed square) destruction of β-carotene was only partially prevented, indicating that α-Toc is a weak scavenger for NO_2. In the presence of 50 equimolar amounts (0.5 mM) of ascorbyl palmitate (Figure 1, open square) destruction of β-carotene was completely prevented, indicating that ascorbyl palmitate is an effective scavenger for NO_2.

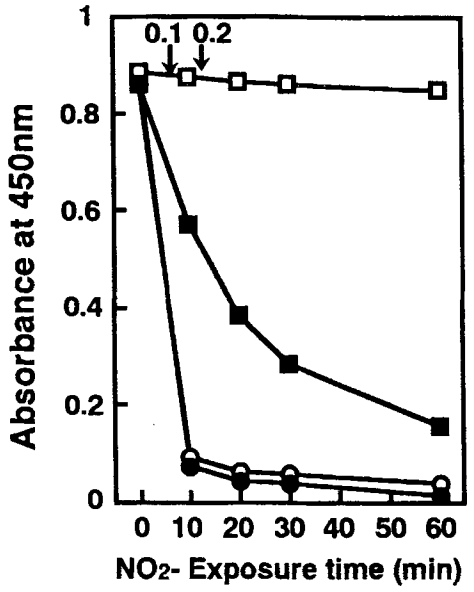

FIGURE 1 Time-course of decrease of β-carotene by the NO_2-exposure in the presence of tyrosine, α-Toc and ascorbyl palmitate in 70% dioxane/phosphate buffer (pH 7.5). NO_2 in air (50 ppm) was introduced into a 5 ml solution of 10 μM (50 nmol) β-carotene in 70% dioxane/phosphate buffer (pH 7.5) containing none (●), 0.2 mM (1.0 μmol) tyrosine (○), 0.5 mM (2.5 μmol) α-Toc (■) and 0.5 mM (2.5 μmol) ascorbyl palmitate (□) at a flow rate of 10 ml/min. Decrease of β-carotene was followed by maximum absorbance of β-carotene at 450 nm. The numbers indicated by arrows are μmols of NO_2 introduced. The data shown are the mean values of duplicate experiments.

When NO_2 in air (1.35 μmol) was introduced into 70% dioxane/phosphate buffer (pH 7.5) containing 0.2 mM tyrosine for 1 h, about 22%, of tyrosine was lost and 16% of 3-nitrotyrosine was produced. The amount of dityrosine was less than 0.2%. A significant amount of tyrosine was missing (Table I, lane 1). Addition of β-carotene at the concentrations between 10 and 50 μM, the amounts much smaller than that of NO_2 introduced, to the solution of tyrosine before the NO_2-exposure caused the enhancement of tyrosine destruction and the decrease in 3-nitrotyrosine formation depending on the dose of β-carotene (Figure 2(A)). Thus, 27.5% of tyrosine was lost and 7.5% of 3-nitrotyrosine was produced in the presence of 50 μM of β-carotene, the amount of missing tyrosine being increased to 20% (Table I, lane 2). The amount of dityrosine produced was negligible.

Because 3-nitrotyrosine is destroyed by the NO_2-exposure,[6] the effect of β-carotene on the 3-nitrotyrosine destruction by the NO_2-exposure was investigated. When NO_2 in air (1.25 μmol) was exposed to a solution of 50 μM 3-nitrotyrosine, 24% of 3-nitrotyrosine was lost, and when the same amount of NO_2 was exposed to the solution in the presence of 50 μM β-carotene,

TABLE I Effect of β-carotene, α-Toc and ascorbyl palmitate on the loss of tyrosine and 3-nitrotyrosine caused by NO_2-exposure

Antioxidant	Amounts %		
	Tyrosine	3-Nitrotyrosine	Missing tyrosine
1. None	78.0 ± 2.0	16.0 ± 2.0	6.0 ± 1.5
2. 50 μM β-carotene	72.5 ± 3.0	7.5 ± 2.6	20.0 ± 2.5
3. 0.5 mM α-Toc	100	0	0
4. 50 μM β-carotene + 0.5 mM α-Toc	91.0 ± 4.0	0	9.0 ± 1.5
5. 50 μM β-carotene + 1 mM α-Toc	91.0 ± 3.8	0	9.0 ± 2.1
6. 0.5 mM ascorbyl palmitate	100	0	0
7. 50 μM β-carotene + 0.5 mM ascorbyl palmitate	100	0	0
8. 50 μM β-carotene + 1 mM ascorbyl palmitate	100	0	0

NO_2 in air (50 ppm) (1.35 μmol) was introduced into a 5 ml solution of 0.2 mM (1 μmol) tyrosine in the presence of β-carotene, α-Toc ascorbyl palmitate, β-carotene + α-Toc and β-carotene + ascorbyl palmitate, at a flow rate of 10 ml/min for 1 h. The amounts of tyrosine and 3-nitrotyrosine were determined by HPLC. The data shown are mean values ± SD obtained from triplicate experiments.

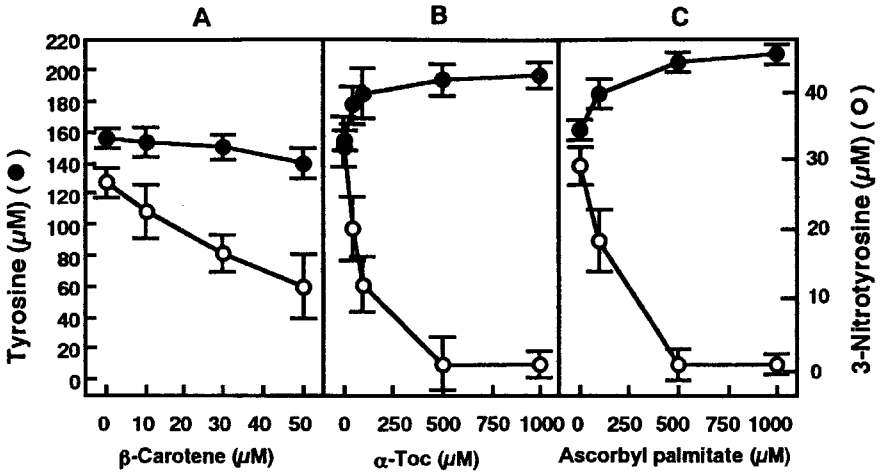

FIGURE 2 Effect of β-carotene (A), α-Toc (B) and ascorbyl palmitate (C) on the decrease of tyrosine and formation of 3-nitrotyrosine caused by the NO_2-exposure. NO_2 in air (50 ppm) (1.35 μmol) was introduced into a 5 ml solution of 0.2 mM (1.0 μmol) tyrosine in 70% dioxane/phosphate buffer (pH 7.5) containing each of the antioxidants at the indicated final concentration at a flow rate of 10 ml/min for 60 min. The concentrations of tyrosine (●) and 3-nitrotyrosine (○) were determined by HPLC. The data shown are mean values ± SD obtained from triplicate experiments.

46% of 3-nitrotyrosine was lost. Hence β-carotene enhanced loss of both tyrosine and 3-nitrotyrosine by the NO_2-exposure. It is likely that certain active species that enhanced the destruction of tyrosine and 3-nitrotyrosine may be generated in the interaction of β-carotene and NO_2.

Effect of α-Toc (Figure 2(B)) or ascorbyl palmitate (Figure 2(C)) on the destruction of tyrosine and the formation of 3-nitrotyrosine by the NO_2-exposure was studied. Decrease of tyrosine and formation of 3-nitrotyrosine by the NO_2-exposure were inhibited depending on the concentrations of α-Toc and ascorbyl palmitate and completely inhibited by more than 0.5 mM of α-Toc (Figure 2(B)) and ascorbyl palmitate (Figure 2(C)). The amount of missing tyrosine was negligible (Table I, lanes 3 and 6). The concentrations of these antioxidants required for complete prevention of tyrosine transformation were more than 2 equivalents to the dose of NO_2.

Effect of β-carotene on the tyrosine destruction by the NO_2-exposure (Figure 2(A)) was apparently different from those of other antioxidants (Figures 2(B) and (C)).

Effect of α-Toc and ascorbyl palmitate on the loss of tyrosine and 3-nitrotyrosine caused by the combination of β-carotene and NO_2 was examined. The loss of 0.2 mM tyrosine and the formation of 3-nitrotyrosine by NO_2 (1.25 μmol)-exposure in the presence of 50 μM β-carotene (Table I, lane 2) were inhibited partially by 0.5 or 1 mM α-Toc (Table I, lanes 4 and 5) and completely by 0.5 or 1 mM ascorbyl palmitate (Table I, lanes 7 and 8). Ascorbyl palmitate effectively scavenged NO_2 to prevent the interaction with β-carotene and thus prevented the loss of tyrosine, whereas α-Toc slightly scavenged NO_2 before the interaction with β-carotene.

When 2 mM $ONOO^-$/ONOOH was added to a solution of 0.5 mM tyrosine in 70% dioxane/phosphate buffer (pH 7.3–7.7), about 40% of tyrosine was lost and 16% of 3-nitrotyrosine was produced. The amount of dityrosine was less than 0.2%. Twenty four percentages of tyrosine were missing. Addition of β-carotene in the concentrations between 10 and 50 μM much smaller than that of $ONOO^-$/ONOOH, to the solution of tyrosine did not affect tyrosine destruction but caused decrease in 3-nitrotyrosine formation depending on the dose of β-carotene (Figure 3(A)). The amount of 3-nitrotyrosine produced in the presence of 50 μM β-carotene was 11%, and the amount of missing tyrosine was increased to 35% by β-carotene. Under the same conditions, 3-nitrotyrosine was destroyed by $ONOO^-$/ONOOH, but the loss was not enhanced by addition of β-carotene. Thus, when 2 mM $ONOO^-$/ONOOH was added to a solution of 0.2 mM 3-nitrotyrosine in the buffer in the absence and the presence of 50 μM β-carotene, the losses of 3-nitrotyrosine were 25% and 27%, respectively. β-Carotene appeared to prevent the formation of 3-nitrotyrosine by $ONOO^-$/ONOOH, but it is likely that certain active species that destroyed tyrosine was produced in the interaction of β-carotene and $ONOO^-$/ONOOH.

Effect of α-Toc (Figure 3(B)) and ascorbyl palmitate (Figure 3(C)) on the loss of tyrosine and the formation of 3-nitrotyrosine caused by $ONOO^-$/ONOOH was examined. α-Toc and

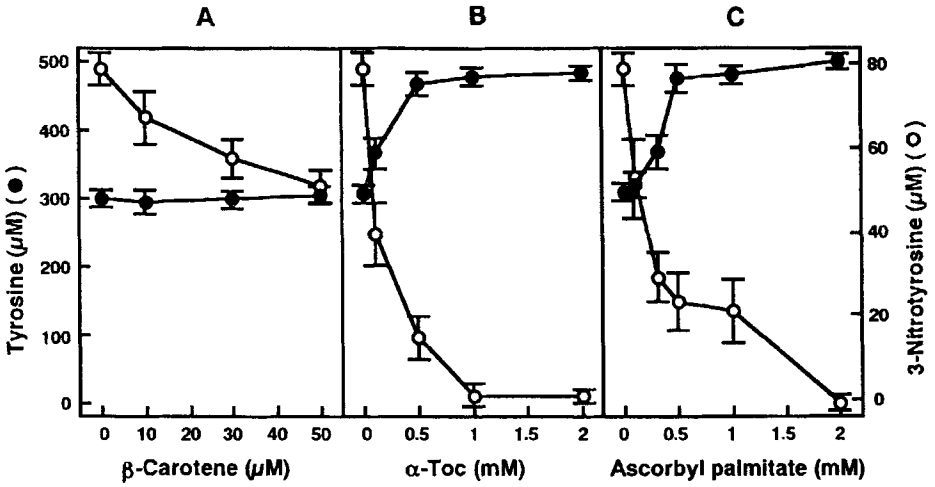

FIGURE 3 Effect of β-carotene (A), α-Toc (B) and ascorbyl palmitate (C) on the decrease of tyrosine and formation of 3-nitrotyrosine caused by $ONOO^-$/ONOOH. $ONOO^-$/ONOOH (10 μmol, 2 mM) was added to a 5 ml solution of 0.5 mM (2.5 μmol) tyrosine in 70% dioxane/phosphate buffer (pH 7.3–7.7) containing each of the antioxidants at the indicated final concentration. The concentrations of tyrosine (●) and 3-nitrotyrosine (○) were immediately determined by HPLC. The data shown are mean values ± SD obtained from triplicate experiments.

ascorbyl palmitate inhibited the loss of tyrosine and the formation of 3-nitrotyrosine depending on the concentrations of these antioxidants, and completely inhibited at the concentrations between 1 and 2 mM. Hence, the interaction of β-carotene with $ONOO^-$/ONOOH was different from those of α-Toc and ascorbyl palmitate with $ONOO^-$/ONOOH.

DISCUSSION

Reaction of β-carotene with NO_2 or $ONOO^-$/ONOOH is a complicated chemistry. It has been shown that β-carotene scavenges NO_2 and $ONOO^-$/ONOOH more effectively than other antioxidants.[21] Most of NO_2 are tightly bound to the β-carotene molecule.[21] Reaction of β-carotene with NO_2 gives a small amount of nitrosating agent in the dark and NO in the light,[19] and β-carotene cation radical together with HNO_2.[20] It was suggested in the present study that certain active species that destroy tyrosine and/or 3-nitrotyrosine produced by the reaction with NO_2 or ONOOH are generated by the interaction of β-carotene with NO_2 or $ONOO^-$/ONOOH. Detection of radical species including β-carotene cation radical in the present reaction mixture was attempted but unsuccessful. It is not known what active species to destroy tyrosine or 3-nitrotyrosine are produced in the interaction of β-carotene with NO_2 or $ONOO^-$/ONOOH. More detailed study may be necessary for the active species that destroy tyrosine and/or 3-nitrotyrosine.

α-Toc and ascorbyl palmitate may inactivate NO_2 and $ONOO^-$/ONOOH to prevent tyrosine loss and 3-nitrotyrosine formation. Reaction of α-Toc with NO_2 produces α-tocopherylquinone nitrite ester with nitrosating activity,[23,24] and reaction of α-Toc with $ONOO^-$/ONOOH produces α-tocopherone cation by two electron process which is in turn hydrolyzed into α-tocopherylquinone.[25,26] Reaction of ascorbyl palmitate with NO_2 and $ONOO^-$/ONOOH may involve the reduction of these nitrogen species. These reactions may be involved in the prevention of tyrosine loss and 3-nitrotyrosine formation. The doses of these antioxidants to give effective influence on the tyrosine transformation were, however, relatively high.

3-Nitrotyrosine is considered to be a biomarker for generation of NO_2 or $ONOO^-$/ONOOH in biological samples.[10–17] It was found in the present investigation that β-carotene, α-Toc and ascorbyl palmitate affected the tyrosine transformation by NO_2 and $ONOO^-$/ONOOH. Effect of β-carotene may be important, because β-carotene affected the transformation at relatively low doses. 3-Nitrotyrosine formation in biological samples may be regulated by β-carotene at low concentrations and α-Toc and ascorbyl palmitate at high concentrations or by combination of them. Effect of β-carotene on the degradation of tryptophan induced by NO_2 and ONOOH[6,8,9] was also investigated in the present study, but the effect of β-carotene at the low doses was small.

Acknowledgements

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