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Effective Inhibition by β-Carotene of Cellular DNA Breaking Induced by Peroxynitrous Acid

KAZUYUKI HIRAMOTO, SUSUMU TOMIYAMA and KIYOMI KIKUGAWA*

School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

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Peroxynitrous acid synthesized by reaction of hydrogen peroxide and nitrite and generated from 3-morpholinosydononimine (SIN-1) induced cellular DNA breaking of human promyelocytic leukemia HL-60 cells in phosphate buffer (pH 7.5) as assessed by alkaline single cell gel electrophoresis (comet) assay and quantification of comet types. Ascorbate and Trolox inhibited cellular DNA breaking induced by peroxynitrous acid, but the concentrations of these antioxidants required for effective inhibition was about 50-fold higher than that of peroxynitrous acid. β-Carotene protected DNA breaking by peroxynitrous acid in 20% tetrahydrofuran–phosphate buffer (pH 7.5) much more effectively than ascorbate and Trolox. The concentrations of β-carotene required for effective inhibition was lower than the concentration of peroxynitrous acid.

Keywords: β-Carotene, comet assay, DNA breaking, HL-60 cell, peroxynitrous acid

INTRODUCTION

Peroxynitrous acid is produced from nitrogen oxide synthesized by endothelial cells, nerve cells and many other types of cells[1] in contact with superoxide.[2] Peroxynitrous acid is considered to be an undesirable mediator for tissue injury. One of the serious injuries caused by peroxynitrous acid is the damage of DNA. Treatment of supercoiled DNA with peroxynitrous acid causes DNA single strand breaks,[3,4] where mannitol is not active to protect DNA from damage by peroxynitrous acid, and benzoate and dimethyl sulfoxide amplify the damage.[3] Selenium containing compounds, selenocystine, selenomethionine and ebselen [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] are found to be effective to protect the damage.[4] Treatment of rat thymocytes with peroxynitrous acid causes cellular DNA breaking as assessed by the fluorescence analysis of DNA unwinding assay, and this damage is also amplified by dimethyl sulfoxide and benzoate, and inhibited by Trolox.[5]

Our previous paper has shown that β-carotene scavenges peroxynitrous acid more effectively than α-tocopherol and ascorbate.[6] In the present investigation, effect of β-carotene on the cellular DNA breaking induced by peroxynitrous acid was investigated using human promyelocytic

* Corresponding author. Tel.: 81-426-4503. Fax: 81-426-4508. E-mail: kikugawa@ps.toyaku.ac.jp.

leukemia HL-60 cell line by alkaline single cell gel electrophoresis (comet) assay. The assay is sensitive, simple and rapid for visualizing and measuring DNA strand breaks in single cell preparation.[7] In the assay a small number of treated cells suspended in a thin agarose sandwich are lysed, electrophoresed at alkaline pH, and stained with a fluorescent DNA binding dye. Relaxed and broken DNA fragments migrate at different, size-related rates according to the extent of damage and form a comet tail, and the cells can be classified by the comet tails. It was found that β-carotene inhibited the DNA damage by peroxynitrous acid more effectively than Trolox and ascorbate.

MATERIALS AND METHODS

Materials

Peroxynitrite ($ONOO^-$) was prepared according to the method previously described.[8] Strongly alkaline solution containing 0.5 M $ONOO^-$ was used. In order to remove contaminated hydrogen peroxide, the preparation was treated with manganese dioxide. $ONOO^-$ is in turn transformed into chemically reactive peroxynitrous acid (ONOOH) by protonation at pK_a 6.8[2] in the reaction buffer system. 3-Morpholinosydnonimine (SIN-1) was obtained from Dojindo Laboratories (Kumamoto, Japan). β-Carotene (purity about 95%), *N*-lauroylsarcosine sodium salt (sodium sarcosinate) and Triton X-100 were obtained from Sigma Chemical Company (St. Louis, MO, USA). Trolox was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). RPMI 1640 medium and fetal calf serum (FCS) were obtained from GIBCO Laboratories (New York, USA) and Bio Whittaker (Maryland, USA), respectively.

Human promyelocytic leukemia HL-60 cell line was obtained from Japanese Collection of Research Bioresources Cell Bank of National Institute of Health Sciences. HL-60 cells were incubated in RPMI 1640 medium containing 10% FCS in CO_2 incubator (air: 95%, CO_2: 5%) to reach the cell number at 1×10^6 cells/ml.

Alkaline Single Cell Gel Electrophoresis (Comet) Assay

Alkaline single cell gel electrophoresis (comet) assay was carried out according to the method previously described.[9,10] Briefly, a fully frosted microscope slide glass was coated with normal-melting-point agarose. HL-60 cells suspended in low-melting-point agarose were overlaid on the slide glass and low-melting-point agarose alone was subsequently overlaid. Three slide glasses were used for one assay sample. Each slide glass was kept at 4°C for 1 h in a lysing solution composed of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris (pH 10.0), 1% sodium sarcosinate, 1% Triton X-100 and 10% dimethyl sulfoxide. The slide glass was kept at 4°C for 40 min the electrophoresis buffer composed of 0.3 M NaOH and 1 mM EDTA to allow the unwinding of the DNA and expression of alkali labile sites. Electrophoresis was performed in the alkaline buffer at a constant voltage of 30 V and a constant current of 300 mA for 24 min. The slide glass was neutralized with 0.4 M Tris-HCl buffer (pH 7.4) and stained with 50 µl of a solution of 50 µg/ml ethidium bromide. One hundred cells on each slide were counted under a fluorescence microscope with *G* excitation at 200× magnification, and the individual cells were classified into five types: type 1, undamaged cells without tail; type 2, cells with tiny tail; type 3, cells with a dim tail; type 4, cells with a clear tail; and type 5, tail alone (Figure 1). The number of comets of types 1–5 amounting to 100 nuclei are shown. The mean values of the

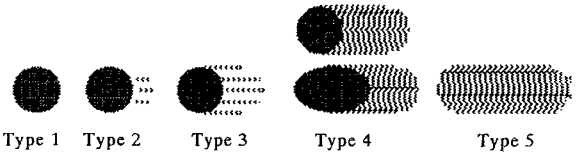

FIGURE 1 Classification of comet types.

number of comets obtained from three slides for one assay sample were obtained. Representative comet images were photographed using black and white film after 400× magnification.

Treatment of HL-60 Cells with $ONOO^-$/ONOOH or SIN-1

A 3.0-ml suspension of HL-60 cell (1×10^6 cells/ml) was washed twice with phosphate buffered saline, and the cells were resuspended in 3.0 ml of 100 mM phosphate buffer containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA). The pH of the buffer was well controlled at 7.5 throughout the experiments. To a 0.5-ml cell suspension, 0.5 ml of the buffer containing each of the antioxidants tested at the indicated final concentration, and 0–6 μl of 5 mM $ONOO^-$ in 1 M NaOH were added in this order. The buffer containing β-carotene was prepared as follows: 0.6 ml of tetrahydrofuran containing β-carotene at the indicated final concentration was added to 2.4 ml of the buffer. For reversed order addition control of $ONOO^-$,[7] to a 0.5-ml cell suspension a mixture of 0.5 ml of the buffer and 6 μl of 5 mM $ONOO^-$ solution was added. The mixture was immediately centrifuged at 350× *g* for 5 min to obtain the cell pellet (100 μl). For investigation of the effect of SIN-1, to a 0.5-ml cell suspension 0.5 ml of the buffer containing each of the antioxidants and 0–20 μl of 10 mM SIN-1 in 0.5 M HCl was added, and the cells were incubated at 37°C for 30 min. The mixture was centrifuged to obtain the cell pellet. A 10-μl cell pellet was suspended into low-melting-point agarose for the alkaline single cell gel electrophoresis assay.

RESULTS

Examples of the fluorescence microscopy images of single cell gel electrophoresis of HL-60 cells treated with none (Figure 2A) and with $ONOO^-$/ONOOH at 30 μM (Figure 2B) in phosphate buffer (pH 7.5) are shown. The comets were classified into 5 types as shown in Figure 1. HL-60 cells treated with none were abundant in type 1 comets and those treated with $ONOO^-$/ONOOH were in type 4 comets, indicating that cellular DNA of HL-60 cells was cleaved by $ONOO^-$/ONOOH. The number of each type of comets after treatment with 0–30 μM $ONOO^-$/ONOOH was counted. It was found that cellular DNA was cleaved by $ONOO^-$/ONOOH in a dose-dependent manner: the number of type 1 comets decreased and the number of type 4 comets increased with the increasing doses of $ONOO^-$/ONOOH (Figure 3A). The reversed-order addition control[7] of the agent at 30 μM did not cause increase in the number of type 4 comets indicating that the breaking was not induced (Figure 3A). Hence, the cellular DNA was cleaved by $ONOO^-$/ONOOH, and the breaking was not induced by contaminating components in the agent.

Inhibitory effect of several compounds on cellular DNA breaking induced by $ONOO^-$/ONOOH at 20 μM in the phosphate buffer was investigated. Addition of sorbic acid bearing conjugated diene structure at 0.2 and 1.0 mM did not prevent the breaking. Addition of hydroxyl radical scavenger, mannitol, at the high doses of 0.2 and 1.0 mM did not prevent the breaking, indicating DNA breaking by $ONOO^-$/ONOOH was not caused by hydroxyl radical. Addition of hydroxyl radical scavenger, dimethyl sulfoxide, at below 30% (3.8 M), did not prevent but slightly amplified the breaking: the increased number of type 4 comets was slightly enhanced as compared

FIGURE 2 Photomicrographs of the alkaline single cell gel electrophoresis (comet) assay of HL-60 cells treated with none (A) and 30 μM $ONOO^-$/ONOOH (B) at pH 7.5.

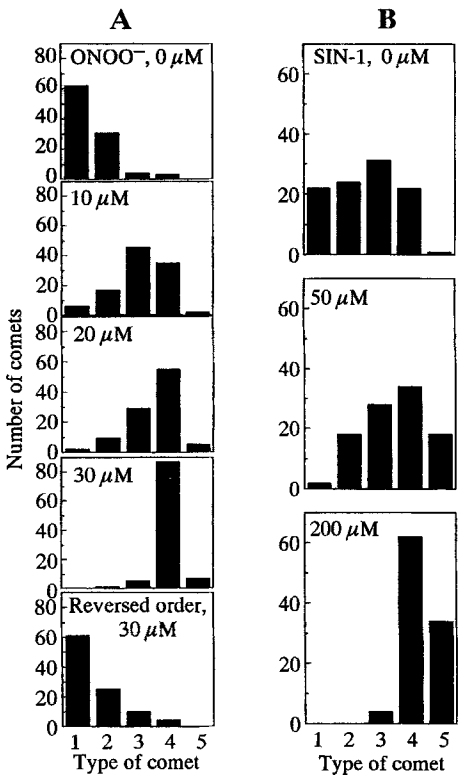

FIGURE 3 DNA breaking of HL-60 cells caused by $ONOO^-$/ONOOH (A) and SIN-1 (B). The cells were treated with $ONOO^-$/ONOOH or SIN-1 at the indicated final concentration in the phosphate buffer (pH 7.5).

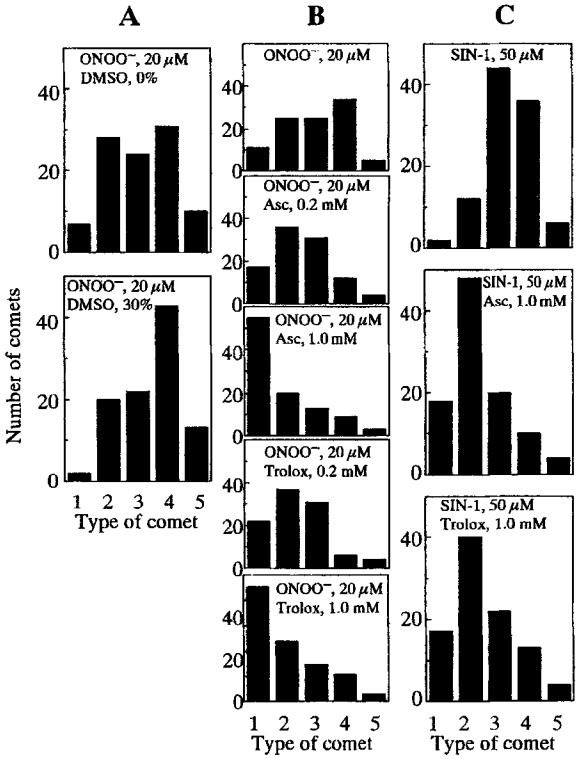

FIGURE 4 Effect of dimethyl sulfoxide, sodium ascorbate and Trolox on the DNA breaking of HL-60 cells caused by $ONOO^-$/ONOOH or SIN-1. The cells were treated with 20 μM of $ONOO^-$/ONOOH (A, B) or 50 μM SIN-1 (C) in the presence of dimethyl sulfoxide (DMSO) (A), sodium ascorbate (Asc) and Trolox (B, C) at the indicated final concentration in the phosphate buffer (pH 7.5).

with that in the absence of dimethyl sulfoxide (Figure 4A). Sodium ascorbate and Trolox at the high doses of 0.2 and 1.0 mM effectively prevented the breaking induced by $ONOO^-$/ONOOH: the increased number of type 4 comets was lowered and the decreased number of type 1 comets was raised with the increase of the doses of the antioxidants (Figure 4B). In this case the concentrations of these antioxidants required for effective inhibition were about 50-fold higher than that of $ONOO^-$/ONOOH.

For examination of the effect of β-carotene against DNA breaking by $ONOO^-$/ONOOH, a solvent that can dissolve both β-carotene and $ONOO^-$/ONOOH was necessary. Phosphate buffer containing 20% tetrahydrofuran was found to be suitable, because both β-carotene and $ONOO^-$/ONOOH were soluble in the solvent and DNA breaking induced by 20 μM $ONOO^-$/ONOOH was not affected by the solvent (data not shown). The effect of β-carotene between 0 and 10 μM in the buffer containing 20% tetrahydrofuran on DNA breaking induced by 20 μM $ONOO^-$/ONOOH is shown in Figure 5A. The increased number of type 4 comets was lowered and the decreased number of type 1 comets was raised with the increase of the doses of β-carotene. The concentration of β-carotene required for effective inhibition was lower than the concentration of $ONOO^-$/ONOOH.

Cellular DNA of HL-60 cells was cleaved by incubation with SIN-1, which is known to release nitric oxide and superoxide reacting with each

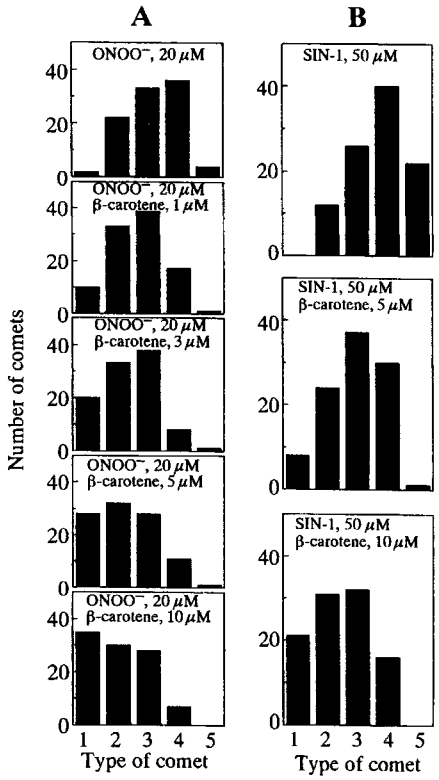

FIGURE 5 Effect of β-carotene on the DNA breaking of HL-60 cells caused by $ONOO^-$/ONOOH or SIN-1. The cells were treated with 20 μM of $ONOO^-$/ONOOH (A) or 50 μM SIN-1 (B) in the presence of β-carotene at the indicated final concentration in the phosphate buffer (pH 7.5) containing 20% tetrahydrofuran.

other to form $ONOO^-$/ONOOH in an aqueous solution;[11] in the phosphate buffer in a dose-dependent manner upto 200 μM: the number of type 1 and type 2 comets reflecting only slight DNA breaks were decreased and the number of type 4 comets was increased with the increasing doses of SIN-1 (Figure 3B). Mannitol at 1.0 mM was inactive to prevent DNA breaking induced by 50 μM SIN-1. Ascorbate and Trolox at 1.0 mM effectively prevented DNA breaking: the increased number of type 4 comets was lowered and the decreased numbers of type 1 and 2 comets were raised (Figure 4C). The effect of β-carotene between 0 and 10 μM in the buffer containing 20% tetrahydrofuran on DNA breaking induced by 50 μM SIN-1 is shown in Figure 5B. The increased number of type 4 comets was lowered and decreased numbers of type 1 and 2 comets were raised with the increasing dose of β-carotene. The concentration of β-carotene required for effective inhibition was as low as below the concentration of SIN-1. The results obtained using SIN-1 were similar to those obtained by $ONOO^-$/ONOOH. In conclusion, β-carotene prevented DNA breaking induced by $ONOO^-$/ONOOH much more effectively than ascorbate and Trolox.

DISCUSSION

It has been shown that treatment of DNA with peroxynitrous acid gives 8-hydroxydeoxyguanosine[12,13] which is in turn rapidly degraded by the same treatment.[14] In contrast, 8-nitroguanine[15] and 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine[16] are produced on treatment of guanine and 2′-deoxyguanosine with peroxynitrous acid, respectively, and the former has been detected in DNA treated with peroxynitrous acid.[17] Treatment of supercoiled DNA with peroxynitrous acid causes single strand breaks as assessed by agarose gel electrophoresis,[3,4] and treatment of rat thymocytes with peroxynitrous acid causes cellular DNA breaking as assessed by fluorescence analysis of DNA unwinding.[5] Monitoring of cellular DNA damage may be the best way for evaluating the effect of peroxynitrite on DNA damage. In the present study, another evidence for the cellular DNA breaking by peroxynitrous acid and SIN-1 by alkaline single gel electrophoresis (comet) assay was shown.

Peroxynitrous acid may release hydroxyl radical or hydroxyl radical-like species by homolysis,[8,18] or otherwise it may become activated to a reactive form symbolized as $ONOOH^*$.[2,8] Effect of hydroxyl radical scavengers on DNA breaking induced by peroxynitrous acid has been reported. Mannitol is not effective to prevent

the breaking of supercoiled DNA[3] and the cellular DNA of rat thymocytes[5] induced by peroxynitrous acid, indicating that hydroxyl radical is not involved in the breaking. Unexpectedly, dimethylsulfoxide enhances the damages of supercoiled and cellular DNA.[3,5] In the present investigation, similar effects of mannitol and dimethyl sulfoxide were observed. Reaction of dimethyl sulfoxide with peroxynitrous acid may create another active species to break DNA single strands. Breaking of supercoiled DNA is inhibited by seleno-compounds such as selenocystine, selenomethionine and ebselen[4] and the cellular DNA damage is inhibited by Trolox.[5]

The cellular DNA breaking of HL-60 cells induced by peroxynitrous acid was prevented by ascorbate, Trolox and β-carotene as assessed by alkaline single cell gel electrophoresis (comet) assay. The concentrations of ascorbate and Trolox required for effective inhibition were much higher than those of peroxynitrous acid. In the DNA damage induced by peroxynitrous acid, β-carotene was found to be the most effective inhibitor. The concentrations of β-carotene required for effective inhibition were lower than the concentration of peroxynitrous acid.

It has been shown that peroxynitrous acid effectively destroys β-carotene, and β-carotene has high reactivity to peroxynitrous acid even in the presence of α-tocopherol and ascorbyl palmitate.[6] Chemistry of the reaction of β-carotene with peroxynitrous acid may be complexed and has not yet been clarified, whereas β-carotene molecules may be destroyed into many non-ultraviolet-absorbing components. The reaction of α-tocopherol or Trolox with peroxynitrous acid has been shown to produce tocopherone cations which are in turn hydrolyzed into tocopherylquinones.[19,20] Reaction of ascorbate with peroxynitrous acid may involve the oxidation of ascorbate. These reactions may be involved in the protective effects of these antioxidants against DNA breaking induced by peroxynitrous acid.

Acknowledgements

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