Phenolic Antioxidants Prevent Peroxynitrite-Derived Collagen Modification in Vitro

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To investigate the prevention of protein modification by food components, effects of natural antioxidants on in vitro modification of collagen by peroxynitrite were examined using a polyclonal antibody specific to 3-nitrotyrosine. To perform the assay with many samples at a time, the inhibitory effects of antioxidants were evaluated using an enzyme-linked immunosorbent assay. Polyphenols such as caffeic acid, curcumin, and flavonoids showed strong inhibitory effects on the formation of 3-nitrotyrosine in peroxynitrite-modified collagen. The inhibitory effects of caffeic acid and its related compounds on nitration of Tyr were then further investigated. Both caffeic acid, ferulic acid, and *p*-coumaric acid inhibited the modification, whereas cinnamic acid did not. By the treatment of *p*-coumaric acid with peroxynitrite, nitrated *p*-coumaric acid was detected in the reaction mixture using a liquid chromatograph-mass spectrometer (LC-MS). These results suggest that some phenolic antioxidants may prevent tissue injury by oxidants including peroxynitrite in vivo.

Keywords: *Peroxynitrite; collagen; antioxidants; nitrotyrosine; antibody*

INTRODUCTION

Oxidative stress has been implicated in the processes of many diseases. Significant amounts of superoxide (O_2^-) and nitric oxide (NO) are considered to be produced in inflammation and ischemic reperfusion. NO reacts with O_2^- to yield peroxynitrite, which is considered to participate in some diseases. The treatment of protein with peroxynitrite causes the formation of 3-nitrotyrosine (Ischiropoulos et al., 1992), which is considered as a marker for the generation of peroxynitrite (Crow et al., 1995). The 3-nitrotyrosine residue has been detected in atherosclerotic lesions (Beckman et al., 1994), endotoxin shock (Szabó et al., 1995a; Wizemann et al., 1994), ischemic lung injury (Ischiropoulos et al., 1995a), and lung sections of patients with acute lung injury (Haddad et al., 1994) using specific monoclonal and polyclonal antibodies that recognize nitrotyrosine in proteins (Beckman et al., 1994). In addition, 3-nitrotyrosine has been detected in the serum and synovial fluid from rheumatoid patients (Kaur and Halliwell, 1994). Using HPLC, nitrotyrosine was detected in the proteins of plasma and polymorphonuclear leukocytes from human whole blood after activation with phorbol ester or calcium ionophore (Salman-Tabcheh et al., 1995). These results indicate the participation of peroxynitrite (3-nitrotyrosine) in many diseases.

In this way, it may be important to prevent these peroxynitrite-derived injuries. Whiteman et al. (1996) have showed that both oxidized and reduced lipoic acid could efficiently protect against the damage of α1-antiproteinase by peroxynitrite. Frears et al. (1996) report that desferrioxamine, thiourea, methionine, tryptophan, and tyrosine have protective effects on the inactivation of tissue inhibitor of metalloproteinase-1 (TIMP-1) by peroxynitrite. However, the effects of antioxidants, except for tocopherol, ascorbic acid, or glutathione, on the prevention of protein modification by peroxynitrite have not been investigated.

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Collagen is the major component of various tissue and organs. Collagen modification with aging or diseases has become of interest. We have examined the oxidative modifications of collagen (Uchida et al., 1990; Kato et al., 1992) and then found that oxidation of proline leads to the fragmentation of the peptide bond, accompanied by the formation of 2-pyrrolidone (Kato et al., 1992). Nitrotyrosine staining in *Helicobacter pylori* gastritis occurred in components of the extracellular matrix (Mannick et al., 1996). Collagen may be modified by peroxynitrite or other nitration species in vivo.

We investigated the effects of natural antioxidants on the modification of collagen and L-Tyr (as a model for Tyr residue) by synthetic peroxynitrite in vitro. In this paper, we have reported that some phenolic antioxidants show strong inhibitory effects on nitration of tyrosine residue by peroxynitrite. After food intake, phenolic antioxidants may protect against biological damage by peroxynitrite and also active oxygen species.

MATERIALS AND METHODS

Materials. Collagen IV (from human placenta), BSA, melittin, β-carotene, α-tocopherol, taxifolin, and 3-nitrotyrosine were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide was obtained from Mitsubishi Gas Co. (Tokyo, Japan). Collagen I (from bovine tendon), uric acid, butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), ferulic acid, mannitol, and cinnamic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Caffeic acid, gallic acid, chlorogenic acid, and quercetin were obtained from Nacarai Tesque, Inc. (Kyoto, Japan). Complete and incomplete Freund's adjuvant were obtained from Difco (Detroit, MI). An ECL kit and peroxidase-labeled anti-rabbit IgG goat antibody were purchased from Amersham (England). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). Epigallocatechingallate (EGCG) and epicatechingallate (ECG) were gifts from Mitsui Norin Co., Ltd. (Tokyo, Japan).

Curcumin and tetrahydrocurcumin (THC) were prepared from turmeric, which was a gift from Daiwa Kasei Co., Ltd. (Saitama, Japan), as described previously (Sugiyama et al., 1996).

Synthesis of Peroxynitrite. Peroxynitrite was made by the procedure described by the methods of Hughes and Nicklin

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(1970). Briefly, an ice-cold solution of 0.6 M HCl, 0.7 M H_2O_2 (10 mL) was simultaneously added to a well-stirred, cooled (4 °C) solution of 0.6 M $NaNO_2$ (10 mL), immediately followed by the addition of 1.5 M NaOH (20 mL). The synthetic solution in a 50-mL capped centrifuge tube was frozen at −20 °C. Peroxynitrite formed a yellow top layer due to freeze fractionation, which was retained for further studies. The top layer typically contained 150−200 mM peroxynitrite as determined by UV-absorbance spectroscopy at 302 nm in 1.2 M NaOH ($\epsilon_{302nm} = 1670\ M^{-1}\ cm^{-1}$) (Hughes and Nicklin, 1968).

Reaction Conditions. Collagen I (0.5 mg/mL) and L-Tyr (1 mM) were dissolved in 0.1 M phosphate buffer (pH 7.4) and then reacted with peroxynitrite at room temperature with vigorous mixing and kept for 15 min. The reaction mixture was stored at −20 °C prior to analysis. In order to exclude the effects of contaminated sodium chloride, sodium hydroxide, nitrite, nitrate, and hydrogen peroxide on the modification, as a control, decomposed peroxynitrite was prepared by prior addition of peroxynitrite to phosphate buffer and then mixed with the substrate (collagen/L-Tyr).

Antibody Preparation. Antiserum against nitrotyrosine was prepared using the methods of Beckman et al. (1994). Briefly, keyhole limpet hemocyanin (8 mg) in 5 mL of 100 mM phosphate buffer (pH 7.4) plus 1 mM Fe^{3+} EDTA was mixed rapidly with 2 mM peroxynitrite. After dialysis against phosphate-buffered saline (PBS) overnight at 4 °C, 500 μL of the protein (0.5 mg) was emulsified with an equal volume of complete Freund's adjuvant and was intramuscularly injected into a New Zealand White (NZW) rabbit. After one month, 0.5 mg of nitrated keyhole limpet hemocyanin was emulsified with an equal volume of incomplete adjuvant and was injected as a booster at 2-week intervals prior to adequate antibody generation.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (6% acrylamide) was prepared and run according to the method of Laemmli (1970) using a stacking gel of 3%. Twenty micrograms of protein were applied to each lane of gel. The gels were stained with Coomassie Brilliant Blue. For estimation of nitrotyrosine in protein, the migrated protein in the gel was transblotted to a PVDF membrane, incubated for 1 h at room temperature with 4% skim milk for blocking, washed with 0.1 M Tris-buffered saline (pH 7.4) containing 0.05% Tween 20, and treated with antiserum (1/500). This procedure was followed by the addition of horseradish peroxidase conjugated to goat anti-rabbit IgG immunogloblin (1/2000) and ECL reagent (Amersham) using the protocol recommended by the manufacturer. The bands were visualized by autoradiography.

Characterization of the Antibody by Enzyme-Linked Immunosorbent Assay. Cross-reactivity testing with various compounds and assay of 3-nitrotyrosine formation were done by competitive ELISA. The coating agent was nitrated BSA, the amount of 3-nitrotyrosine in BSA being about 0.80 mol/mol Tyr as estimated by HPLC and amino acid analysis of the hydrolysates. The nitrated BSA was prepared by the treatment of BSA (1.6 mg/mL) with a solution of peroxynitrite (2 mM)/Fe^{3+}EDTA (1 mM), followed by dialysis against PBS. Fifty microliters of nitrated BSA solution (0.03 mg/mL in PBS) were dispensed into each well of a microtiter plate and kept overnight at 4 °C. Competitors or proteins (50 μL) in PBS were mixed with 50 μL of antiserum (1/500) in PBS containing 1% BSA and allowed to stand overnight at 4 °C. The coating solution was discarded, and the wells were washed three times with PBS containing 0.05% Tween 20 (TPBS) and then with water. The wells were blocked with 200 μL of 4% skim milk for 1 h at 37 °C with gentle shaking. After washing, the samples reacted overnight (90 μL) were applied on the wells and incubated with gentle shaking at 37 °C for 1 h. After washing, the well was incubated with 100 μL of peroxidase-labeled anti-rabbit IgG goat antibody (1/5000) in TPBS for 1 h at 37 °C with shaking. After washing, 100 μL of *o*-phenylenediamine solution (*o*-phenylenediamine 5 mg/30% H_2O_2 10 μL/0.1 M citrate−phosphate buffer (pH 5.5) 10 mL) was added to each well. The plate was periodically gently shaken at room temperature until adequate color developed. The color development was stopped by the addition of 50 μL of 2 N H_2SO_4 to each well. The absorbance at 492 nm was measured with a multiplate reader (SPECTRA MAX 250, Molecular Devices Corporation, CA).

Determination of Dityrosine and 3-Nitrotyrosine by HPLC. Dityrosine and 3-nitrotyrosine were determined by reversed-phase HPLC on a Develosil ODS-HG-5 column (4.6 × 250 mm) (Nomura Chemical Co., Ltd., Aichi, Japan) as described (Kato et al., 1994). The hydrolysates were dissolved in 0.01 M HCl before injection into HPLC. Ten microliters of the sample were applied to the column and eluted with a solution of 0.5% acetic acid/methanol (29/1) at a flow rate of 0.8 mL/min. The detection of dityrosine was performed by a fluorescence detector TOYO SODA FS8000 (excitation wavelength, 300 nm; emission wavelength, 400 nm). Nitrotyrosine was determined by absorbance at 274 nm. These identifications were performed by coelution with authentic samples and their UV-absorbances determined by a photodiode array detector. The amounts of dityrosine and 3-nitrotyrosine were calculated by comparison with standard curves of authentic samples. Dityrosine was prepared as described previously (Kato et al., 1994). The purity of synthetic dityrosine (96%) was determined by reversed-phase HPLC (ODS-HG-5, 4.6 × 250 mm) by absorbance at 280 nm using 0.5% acetic acid/methanol (29/1) as the eluent. Standard 3-nitrotyrosine was commercially obtained from Sigma Chemical Co. (St. Louis, MO).

Assay for Determination of Nitrated Collagen by ELISA. To confirm the formation of 3-nitrotyrosine in peroxynitrite-modified collagen, the reduction of 3-nitrotyrosine to 3-aminotyrosine was performed by the treatment of peroxynitrite-modified collagen with sodium dithionite as described (Mozhaev et al., 1988). Peroxynitrite-modified collagen was coated on the well at various concentrations and allowed to stand at 4 °C overnight. After washing three times with TPBS then water, 100 μL of the reducing agent ($Na_2S_2O_4$, 6 mM) was added to the well, and the plate was soaked for 5 min. As a control, instead of reducing agent, 100 μL of water was added to the well. The plate was washed and was blocked by 4% skim milk for 1 h at 37 °C with gentle shaking. The antiserum (1/1000, in PBS containing 0.5% BSA) was added to the well after washing. After incubation for 2 h and then washing, the well was incubated with 100 μL of peroxidase-labeled anti-rabbit IgG goat antibody (1/5000) in TPBS for 1 h at 37 °C with shaking. The color development was performed as described above.

To investigate the inhibitory effects of antioxidants on collagen nitration, the reaction mixture was diluted with a 50-fold vol of PBS, and 50 μL of the diluted solution was dispensed in the well and kept overnight at 4 °C. After washing, 100 μL of antiserum (1/1000) in PBS containing 0.5% BSA was added to the well and soaked for 2 h at 37 °C. The treatments with the second antibody and color development were performed as described above.

Characterization of Peroxynitrite-Modified *p*-Coumaric Acid. Peroxynitrite (1 mM) was added to *p*-coumaric acid (1 mM) in 0.1 M phosphate buffer (pH 7.4) and then immediately mixed. An aliquot of the reaction mixture was applied to a liquid chromatograph connected with PLATFORM II (VG Biotech, Tudor Toad Altrincham, WA, United Kingdom), and the modified product was identified by mass spectrometry with a negative electrospray interface. The separation of *p*-coumaric acid and the products was performed by reversed-phase HPLC on a Develosil ODS-HG-5 column (4.6 × 250 mm) (Nomura Chemical Co., Ltd., Aichi, Japan). Ten microliters of the sample was applied to the column and eluted with a solution of 0.1% acetic acid/methanol (1/1) at a flow rate of 0.8 mL/min. The UV detection was performed by absorbance at 280 nm. The amounts of nitrated *p*-coumaric acid were evaluated on the basis of the molar coefficient of 3-nitrotyrosine (Zhu et al., 1996) because sufficient amounts of nitrated compound could not be obtained.

To confirm the formation of the nitrated compound, the reduction of the compound was performed as described below. *p*-Coumaric acid (1 mM) was dissolved in 0.1 M phosphate buffer (pH 7.4), and synthetic peroxynitrite (1 mM) was added

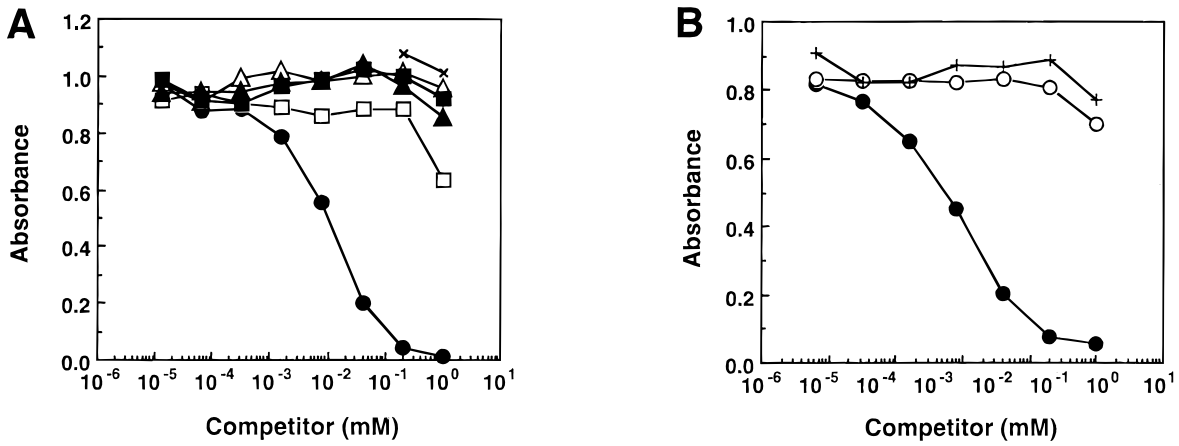

Figure 1. Cross-reactivities of various compounds with the polyclonal antibody. The cross-reactivities were examined by competitive ELISA as described in Materials and Methods. The coating agent was nitrated BSA (0.03 mg/mL). Symbols: (●), 3-nitrotyrosine; (□), 3-aminotyrosine; (△), tyrosine; (▲), dityrosine; (■), DOPA; (×), phenylalanine; (○), 3-chlorotyrosine; (+), *o*-phosphotyrosine.

to the solution. The solution was applied to a C18 cartridge (Sep-Pak, Waters, Milford, MA), and the adsorbed products were eluted with 5 mL of methanol. The eluate was evaporated and an aliquot was reduced for 5 min by the addition of 9 vol of 20 mM Tris-HCl buffer (pH 8.0) containing 3 mM $Na_2S_2O_4$. After the reduction, the solution was subjected to HPLC, and the elution was monitored by the absorbance at 280 nm. The conditions of the HPLC were the same as those of LC-MS (see above).

RESULTS

To evaluate the inhibitory effects of some antioxidants on modification of proteins by peroxynitrite, we prepared a polyclonal antibody specific to 3-nitrotyrosine. Nitrated keyhole limpet hemocyanin (KLH) was injected into an NZW rabbit according to the method of Beckman et al. (1994), and the antiserum obtained was characterized by competitive ELISA (Figure 1, parts A and B). The reaction between the antibody and nitrated BSA adsorbed on the well was inhibited in the presence of free 3-nitrotyrosine. The antibody to nitrated KLH did not recognize L-Tyr and L-Phe. Aminotyrosine at a high concentration slightly inhibited the binding of antibody to nitrated BSA (coating agent). Recently, it has been reported that 3-chlorotyrosine is formed by the reaction between Tyr and HOCl (Domigan et al., 1995). However 3-chlorotyrosine is not recognized by the antibody (Figure 1B). Synthetic peroxynitrite solution contains hydrogen peroxide and trace metal ions, which can catalyze the oxidation of biomolecules. Dityrosine and 3,4-dihydroxyphenylalanine (DOPA), which are known to be modified products of tyrosine residue by oxidative treatment (Kato et al., 1994, 1995), had no cross-reactivity (Figure 1A).

It is known that tryptophan residue is also decomposed by the treatment of protein with peroxynitrite (Ischiropoulos et al., 1995b). In order to examine the cross-reactivity of tryptophan-derived compounds, melittin, which contains no tyrosine residue but has one tryptophan residue, was modified by peroxynitrite, and the cross-reactivities of the native and modified melittin were then investigated using competitive ELISA. The results of about 52% loss of tryptophan fluorescence in melittin by peroxynitrite showed that considerable modification of the tryptophan residue occurred. However, no cross-reactivity of both native and peroxynitrite-treated melittin was observed (data not shown). In addition, the native and peroxynitrite-treated melittins

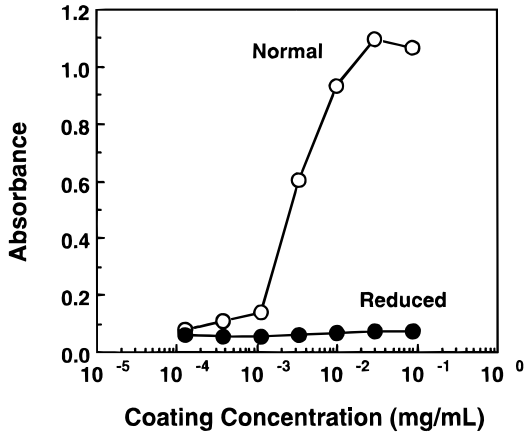

Figure 2. Effect of reduction with dithionite on nitrated collagen. Collagen (0.5 mg/mL) was reacted with 1 mM peroxynitrite. The peroxynitrite-modified collagen was coated on the well overnight at 4 °C. After washing, the modified collagen was reacted in the presence (●) or absence (○) of a reducing agent at room temperature for 5 min. The plate was washed and treated with antiserum and then a second antibody.

were subjected to SDS-PAGE (15%) and then Western blotting. None of the immunoreactive materials was observed by immunological detection (data not shown). Kynurenine, which is known to be one of products of oxidized Trp, has no antigenicity using competitive ELISA (data not shown). In this way, it is clear that the antibody is highly specific to 3-nitrotyrosine.

We have observed the formation of immunoreactive materials in peroxynitrite-modified collagen (Kato et al., unpublished results). In order to clarify the formation of 3-nitrotyrosine in collagen, peroxynitrite-modified collagen was reduced by sodium dithionite, and the reactivity of the modified collagen was then investigated because the nitrotyrosine residue is converted to aminotyrosine by reduction with sodium dithionite (Mozhaev et al., 1988). If nitrotyrosine is formed in the peroxynitrite-treated collagen, the cross-reactivity should disappear after treatment with sodium dithionite. As shown in Figure 2, the reactivity of nitrated collagen with the antibody completely disappeared after the treatment with reductant. The result suggests that 3-nitrotyrosine formed in peroxynitrite-modified collagen was changed to 3-aminotyrosine by reduction.

To investigate the prevention of collagen nitration by antioxidants, the changes in collagen were examined by

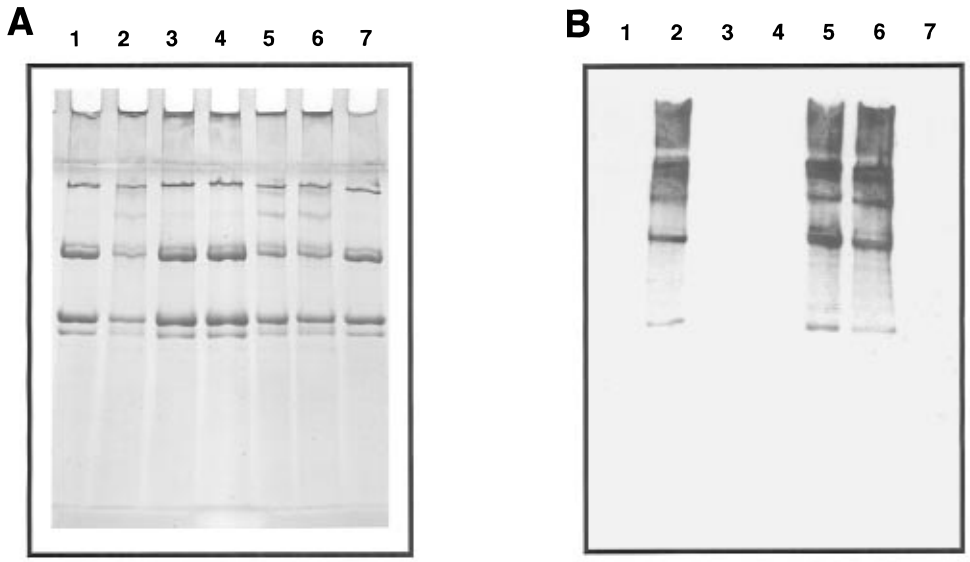

Figure 3. Effects of epigallocatechingallate (EGCG) and epicatechingallate (ECG) on the nitration of collagen determined by SDS-PAGE (A) and immunoblot analysis (B). Collagen (0.5 mg/mL) was reacted with 1 mM peroxynitrite in the presence or absence of antioxidants (0.2 mM). Native and peroxynitrite-modified collagens were applied to SDS-PAGE (6.5%). Two gels were prepared, and one of the migrated gels was stained with Coomassie Brilliant Blue (A). The other gel was electrotransblotted to a PVDF membrane as described in Materials and Methods. The blotted membrane was treated by antiserum to 3-nitrotyrosine, and the binding of the antibody to the membrane was visualized by ECL reagent using the peroxidase-labeled anti-rabbit IgG antibody (B). Lanes: (1) Native collagent, I, (2) + 1 mM peroxynitrite, (3) + 1 mM peroxynitrite + 0.2 mM ECG, (4) + 1 mM peroxynitrite + 0.2 mM EGCG, (5) + 1 mM peroxynitrite + 10% DMSO, (6) + 1 mM peroxynitrite + 1% DMSO, (7) + 1 mM decomposed peroxynitrite.

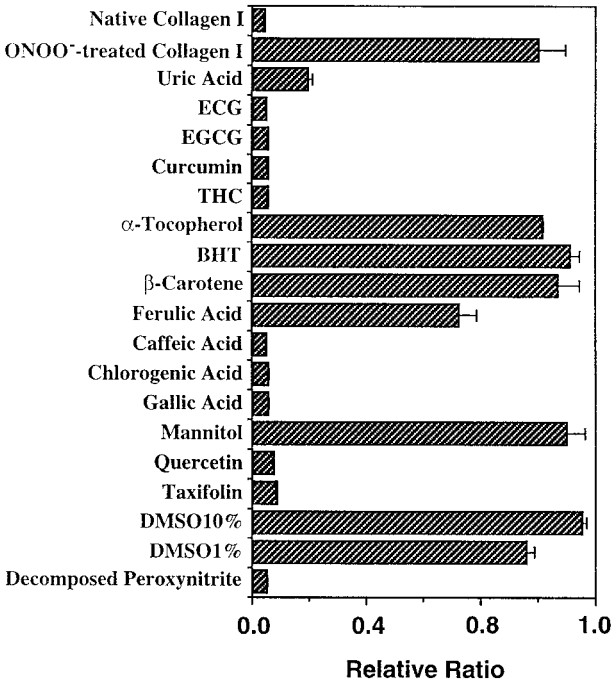

Figure 4. Inhibitory effects of antioxidants on nitration of collagen determined by ELISA. The reaction was performed as described in Figure 3 legend. The reaction mixture was diluted with PBS and was then dispensed on a microtiter plate. The results are represented as the relative ratio against peroxynitrite-treated collagen in the absence of antioxidants. The data represent mean ± standard deviation of three determinations.

SDS-PAGE and Western blotting. As shown in Figure 3, modification of collagen by peroxynitrite was prevented by epicatechingallate (ECG) and epigallocatechingallate (EGCG). Water-insoluble materials were dissolved in DMSO prior to the addition of the reaction mixture. The final concentration of DMSO in the reaction mixture was 10%. No effect of dimethyl sulfoxide (DMSO), used as a solvent of lipophilic antioxidants, on the modification was observed (Figure 3, lanes 5 and 6). However, this assay using Western blotting cannot accommodate many samples simultaneously. We then established the assessment of prevention using ELISA.

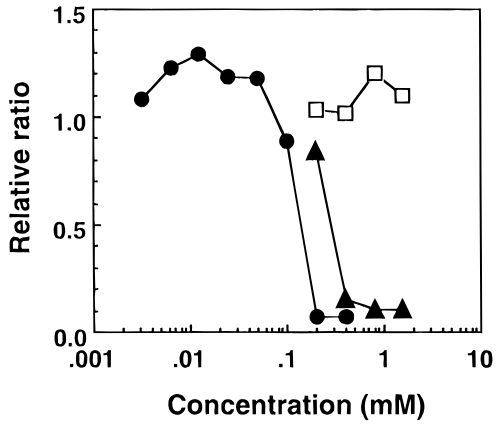

Figure 5. Effects of concentration of antioxidants on nitrate of collagen. Collagen was reacted with 1 mM peroxynitrite in the presence of cinnamic acid (□), *p*-coumaric acid (▲), or caffeic acid (●). The results are represented as the relative ratio against peroxynitrite-treated collagen in the absence of antioxidants.

The results of ELISA are summarized in Figure 4. Uric acid is reported as a scavenger of peroxynitrite (Skinner et al., 1995; Szabó et al., 1995b). The inhibitory effect was derived from the modification of uric acid itself by peroxynitrite, accompanied by the formation of allantoin or parabanic acid (Skinner et al., 1995). Some phenolic antioxidants (such as caffeic acid, curcuminoids, and flavonoids) exhibited strong inhibitory effects. Caffeic acid and related compounds were used as inhibitors, and the effect on nitration of collagen was further investigated. The nitrotyrosine formation in peroxynitrite-modified collagen decreased with increasing concentration of caffeic acid and *p*-coumaric acid (Figure 5). This ELISA assay is relative and based on

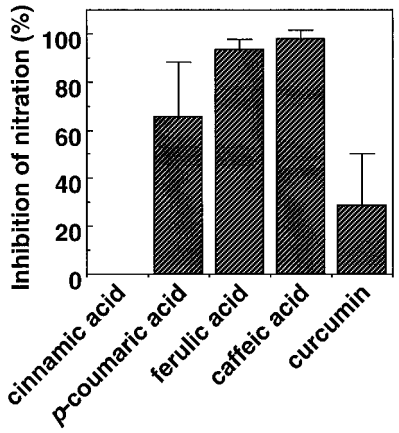

Figure 6. Inhibitory effects of caffeic acid (0.2 mM), ferulic acid (0.2 mM), curcumin (0.2 mM), cinnamic acid (0.2 mM), and *p*-coumaric acid (0.2 mM) on nitration of L-Tyr (1 mM) by 1 mM peroxynitrite. The result was expressed as inhibition % of nitration of L-Tyr. The data represent mean ± standard deviation of three determinations.

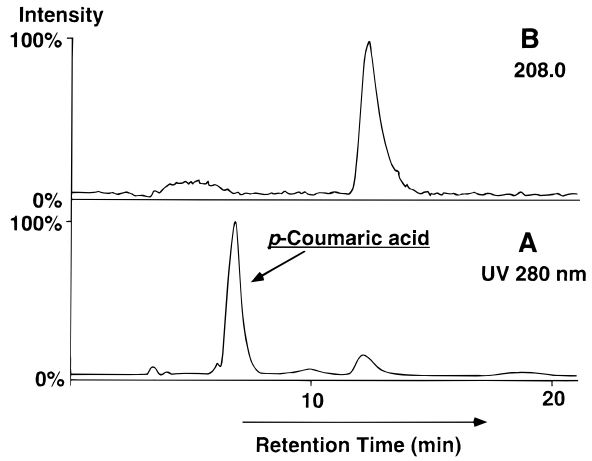

Figure 7. LC-MS for peroxynitrite-modified *p*-coumaric acid. *p*-Coumaric acid was treated with peroxynitrite, and the formed products were applied to reversed-phase HPLC connected with MS (PLATFORM II, VG Biotech, United Kingdom). The elution was monitored by absorbance at 280 nm (A). The detection of nitrated *p*-coumaric acid was performed by LC-MS with electrospray negative mode scanned by $(M - H)^-$ ion, 208 (B).

the time-dependent chromophore formation which reflects the amount of nitrated collagen. Therefore, it will not allow the determination of subtle differences in the various antioxidants, whereas the inhibitory effects of many samples can be simultaneously measured. Then, L-Tyr was also used as a model of the Tyr residue in protein, and the modification and its prevention by antioxidants were examined using HPLC. 3-Nitrotyrosine and dityrosine were formed by treatment of L-Tyr with peroxynitrite (data not shown). The inhibitory effects of L-Tyr modification were evaluated on the basis of the inhibition of 3-nitrotyrosine formation. The formation of 3-nitrotyrosine was suppressed by the addition of *p*-coumaric acid, curcumin, ferulic acid, or caffeic acid but not cinnamic acid (Figure 6). The methoxy compound (ferulic acid) as well as caffeic acid inhibited the modification of L-Tyr by peroxynitrite. To clarify the mechanism of inhibition, as a first step, *p*-coumaric acid was used as one of the model antioxidants. We considered that the coumaric acid has a simple structure and that the modified compound can probably be easily characterized. Peroxynitrite (1 mM) was added to *p*-coumaric acid (1 mM) in 0.1 M phosphate buffer (pH 7.4) and then mixed immediately. An aliquot of the reaction mixture was subjected to LC-MS, and the elution of products was then monitored at 280 nm (Figure 7A). The major product provided the $(M - H)^-$ ion, 208 (Figure 7B). The product had a yellowish color similar to that of nitrotyrosine. The peak disappeared after the treatment with sodium dithionite (data not shown). The result indicates the conversion of the nitro group to an amino group by the reduction. From these results, the product is supposed to be a nitrated *p*-coumaric acid. The loss of *p*-coumaric acid by peroxynitrite was 45%, and the yield of nitrated *p*-coumaric acid from the loss of *p*-coumaric acid was approximately 5.8%. The other modified *p*-coumaric acid derived from dimerization or oxidation must be formed.

DISCUSSION

The 3-nitrotyrosine residues formed *in vivo* may have some biological effects. Peroxynitrite-promoted nitration will result in permanent impairment of the cyclic cascades that control signal transduction processes and regulate cell cycles (King et al., 1996). In this way, the inhibition of peroxynitrite-derived nitration of protein may aid in prevention of biological disorders or diseases. Recently, the inhibitory effects of hydroxyl radical scavengers, amino acids, metal ion chelators, and lipoic acid on the nitration of proteins have been investigated (Whiteman et al., 1996; Frears et al., 1996). We focused on some food-derived antioxidants for screening the inhibitors of nitration. The specific antibody to 3-nitrotyrosine was prepared for the evaluation according to the methods of Beckman et al. (1994). In a preliminary study, using the antibody, the inhibitory effects of antioxidants on the nitration of collagen was examined by Western blotting. As shown in Figure 3, ECG and EGCG have strong inhibitory effects. However, the methods are not suitable for the simultaneous analysis of many samples. We then developed an assay system using enzyme-linked immunosorbent assay (ELISA).

Uric acid inhibited the nitration of collagen by peroxynitrite (Figure 4). It has been reported that pretreatment with uric acid reduced the cellular damage caused by either peroxynitrite or by induction of inducible nitric oxide synthetase with proinflammatory cytokines or lipopolysaccharide (Szabó et al., 1995b). The inhibitory mechanism is probably derived from the degradation of uric acid itself to allantoin or parabaric acid (Skinner et al., 1995). Phenolic antioxidants have considerable inhibitory effects on the nitration. As far as we know, the inhibitory effects of phenolic antioxidants on the peroxynitrite-induced nitration have not been reported. The inhibitory effects probably correlated with their antioxidative activities. However, the relationship between antioxidative activity and inhibitory effect of nitration is not clear. Curcumin has the strong inhibitory effect of nitration of collagen (Figure 4). Using L-Tyr as a substrate, the inhibitory effect of curcumin was not so strong (Figure 6). The reason for the difference is unknown. The affinity of the antioxidant with substrates (collagen or L-Tyr) may be related to the inhibitory mechanism.

To estimate the mechanism of inhibition, *p*-coumaric acid was chosen as a typical phenolic antioxidant. The formation of nitrated compounds was confirmed by LC-MS (Figure 7). We have identified one of the peroxynitrite-modified phenolic antioxidants. This suggests that one of the inhibition mechanisms by phenolic antioxi-

dants is the competitive reaction with nitration of the protein-bound tyrosine. The peroxynitrite-derived injuries in vivo may be prevented by food components (phenolic antioxidants) after food intake. However, further studies on the prevention of peroxynitrite-induced modification by food components, especially an in vivo study, are needed.

ABBREVIATIONS USED

EGCG, epigallocatechingallate; ECG, epicatechingallate; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PVDF, polyvinylidene difluoride; LC-MS, liquid chromatography−mass spectrometry; DOPA, 3,4-dihydroxyphenylalanine; DMSO, dimethyl sulfoxide; THC, tetrahydrocurcumin; BHT, butylated hydroxytoluene.

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