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Short Communication

Endogenous and New Synthetic Antioxidants for Peroxynitrite: Selective Inhibitory Effect of 5-Methoxytryptamine and Lipoic Acid on Tyrosine Nitration by Peroxynitrite

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ABSTRACT

The inhibitory effects of endogenous and synthetic compounds on the nitration and oxidation of L-tyrosine by peroxynitrite were examined. Nitration and oxidation activities of L-tyrosine by peroxynitrite were estimated by monitoring the formation of 3-nitrotyrosine and dityrosine with a high-performance liquid chromatography-ultraviolet (HPLC-UV)-fluorescence detector system. Glutathione and synthetic compounds ((2*S*,3*R*,4*S*)-*N*-ethylmercapto-3,4-dihydroxy-2-hydroxymethylpyrrolidine, L-*N*-dithiocarboxyproline) inhibited both the nitration and the oxidation reactions of L-tyrosine effectively. On the other hand, 5-methoxytryptamine and lipoic acid inhibited only the nitration reaction of L-tyrosine, and instead increased the oxidation reaction. It was assumed that 5-methoxytryptamine and lipoic acid reacted only with the nitrating species of peroxynitrite. This is the first report of a selective inhibitor for the nitrating reaction of peroxynitrite. Antiox. Redox Signal. 1, 239–244, 1999.

INTRODUCTION

Peroxynitrite (ONOO-) is formed from nitric oxide (NO) and superoxide *in vivo*. It has been reported to have a high activity for the oxidation of various biological components and for the nitration of free tyrosine and protein tyrosine residues. Because the nitration of tyrosine residues is one of the characteristic reactions of peroxynitrite, the presence of nitrotyrosine in tissues or cell cultures is often used as the marker of the production of peroxynitrite. Peroxynitrite causes various types of oxidative damage, for example, low-density lipoprotein (LDL) oxidation, lipid peroxidation, DNA strand breakage, and so on (White *et al.*, 1994; Darley-Usmar *et al.*, 1992, 1995). Additionally, the nitration of tyrosine is assumed to prevent the phosphorylation of tyrosine residues in the substrate proteins of tyrosine kinase (Gow *et al.*, 1996; Kong *et al.*, 1996). The oxidation and nitration activities of peroxynitrite play the pathological roles in oxidative stress. Various typical antioxidants were reported to have the inhibitory effects on the nitration of the tyrosine (Whiteman *et al.*,

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1996a,b). In a few reports, the fluorescent product, which was assumed to be dityrosine as an oxidation product, was formed simultaneously with the nitrotyrosine formation in the reaction of L-tyrosine and peroxynitrite (Van der Vliet *et al.*, 1994). In this study, the formation of nitrotyrosine and dityrosine in the reaction of L-tyrosine and peroxynitrite was confirmed, and then the inhibitory effects of the endogenous compounds and synthesized antioxidants on each of the oxidation and nitration reaction by peroxynitrite were examined.

MATERIALS AND METHODS

Chemicals

L-Tyrosine, 2,4-dihydroxy-DL-phenylalanine, *p*-fluoro-L-phenylalanine, glutathione (reduced form), and *dl*-lipoic acid were purchased from Wako Pure Chemical Ind. (Osaka, Japan). 3-Nitro-L-tyrosine, and 5-methoxytryptamine were from Aldrich (Milwaukee, WI). All reagents used were analytical grade. NEMP ((2*S*,3*R*,4*S*)-*N*-ethylmercapto-3,4-dihydroxy-2-hydroxymethylpyrrolidine) was obtained by hydrolysis of the precursor compound synthesized as described previously (Ikota and Hama-Inaba, 1996). DTCP (L-*N*-dithiocarboxyproline) was synthesized according to Shinobu *et al.* (1984). The structures and purity of synthesized NEMP and DTCP were confirmed by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy.

Peroxynitrite preparation

Peroxynitrite was synthesized as alkaline solution according to Whiteman *et al.* (1996b). Briefly, 1 ml of 1 *M* hydrogen peroxide (H_2O_2) was mixed with 1 ml of 1 *M* $NaNO_2$ in the glass tube under the acidic condition on an ice bath, and then rapidly quenched with 2 ml of 1.5 *M* NaOH. The quenched solution was treated with MnO_2 immediately, and then it was slowly frozen with dry-ice/acetone and the top layer of the frozen solution was collected. The concentrated peroxynitrite solution (25–126 m*M*) was obtained. The concentration of the peroxynitrite solution was determined spectrophotometrically from the absorbance at 302 nm (molar absorption coefficient = 1,670 M^{-1} cm^{-1}), and the prepared peroxynitrite solution was stored at −20°C until use. The stock solution was diluted to the desired concentration with 0.01 *N* NaOH after determining the concentration of the stock solution spectrophotometrically. The peroxynitrite solution without MnO_2 treatment was also prepared and used, but the formation of 3-nitro-L-tyrosine, 2,2′-dityrosine, and L-dopa were not significantly affected. H_2O_2 and sodium nitrite did not give any oxidized and nitrated products from L-tyrosine in this system.

Assignment of the dityrosine in the analytical experiment

The authentic sample of 2,2′-dityrosine was prepared from L-tyrosine and hydrogen peroxide in the presence of horseradish peroxidase at pH 9.5 at 37°C according to Gross and Sizer. (1959) and Nomura *et al.* (1990) with a slight modification. The reaction product was analyzed using HPLC with fluorescence detector, and the main product was confirmed as 2,2′-dityrosine by comparing its excitation and emission spectra (λmax; ex 295 nm and em 410 nm) with the reported ones in the literature (Lehrer and Fasman, 1967).

Reaction of peroxynitrite with L-tyrosine and analytical conditions

To the L-tyrosine (final concentration, 0–2 mM) solution in 0.1 *M* sodium phosphate buffer, pH 7.4, peroxynitrite (final concentration, 0–8 m*M*) was added at 37°C. After 10 min of incubation, *p*-fluorotyrosine (final concentration, 0.91 m*M*) was added to the reaction mixture as an internal standard, and the aliquot of the mixture was analyzed by the high-performance liquid chromatography ultraviolet (HPLC-UV)-fluorescence detector system. The HPLC conditions were as follows: column, TSK-GEL ODS 80-Ts 4.6 × 150 mm (TOSOH Corp., Tokyo, Japan); eluent, 0.1 *M* potassium phosphate, pH 3.5; flow rate, 1.0 ml/min; detection, absorbance at 274 nm and fluorescence at 410 nm (ex 295 nm). The 3-nitro-L-tyrosine produced was quantified with the standard curve. The reaction of peroxynitrite and L-tyrosine was also carried out at pH 9.5 and pH 11.5 as same manner.

Effects of various compounds on nitrotyrosine and dityrosine formation

In the presence of various concentrations of testing compounds, L-tyrosine (1 m*M*) was incubated with peroxynitrite (0.2 m*M*) at 37°C for 10 min in neutral buffer solution, pH 7.4. After addition of fluorotyrosine as an internal standard, the formation of 3-nitro-L-tyrosine and 2,2′-dityrosine were estimated, and the IC_{50} value of each testing compound was determined both for 3-nitro-L-tyrosine and for 2,2′-dityrosine formation. The amount of 3-nitro-L-tyrosine formed was determined with the standard curve. The fluorescent product formation was represented as the percent for the fluorescence intensity in the control experiment.

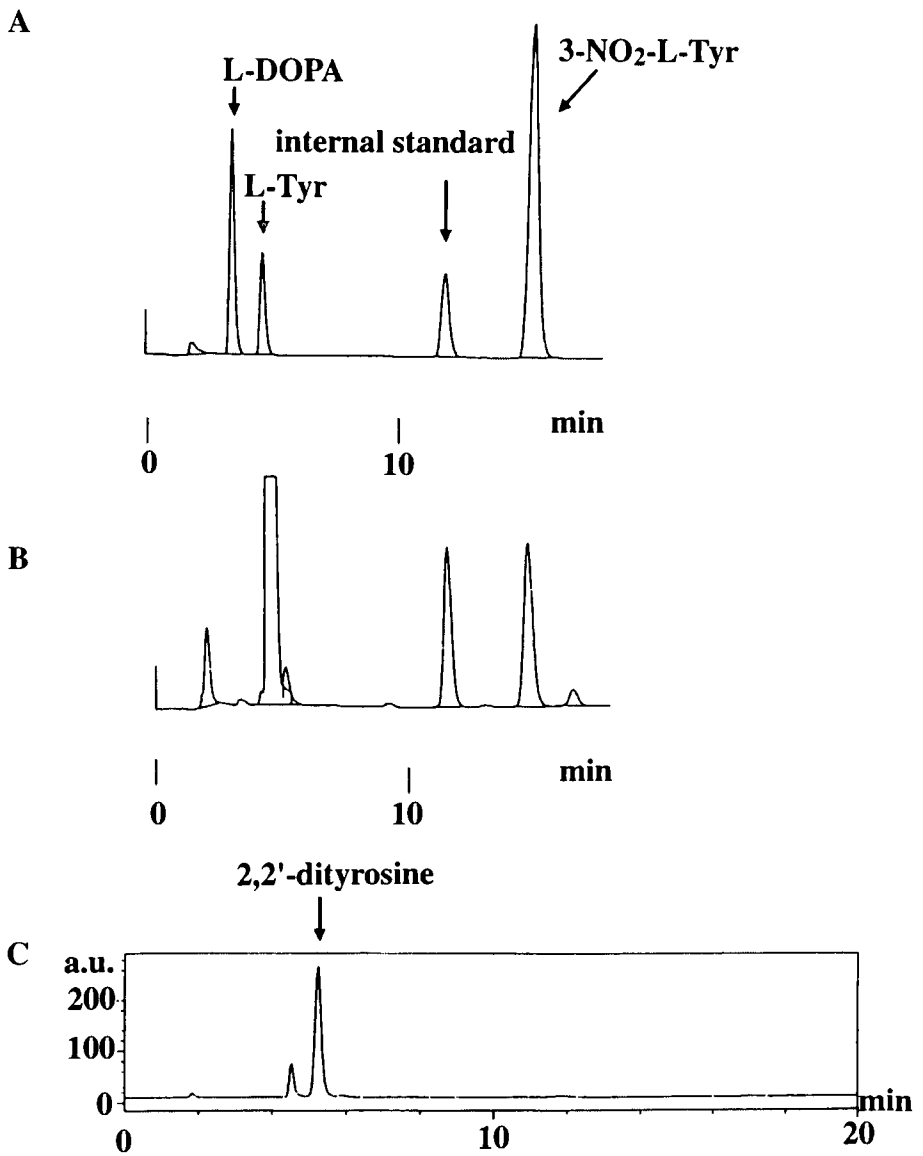

FIG. 1. HPLC charts of the authentic and reaction products. A. The authentic mixture of L-dopa, L-tyrosine (L-Tyr), *p*-fluoro-L-tyrosine (internal standard), and 3-nitro-L-tyrosine (3-NO_2-L-Tyr); output from UV (274 nm) detector. **B.** The reaction mixture of 1 m*M* L-tyrosine and 0.2 m*M* peroxynitrite; output from UV (274 nm). **C.** The reaction mixture monitored by fluorescence detector (ex 295 nm, em 410 nm). The HPLC condition was as described in Materials and Methods.

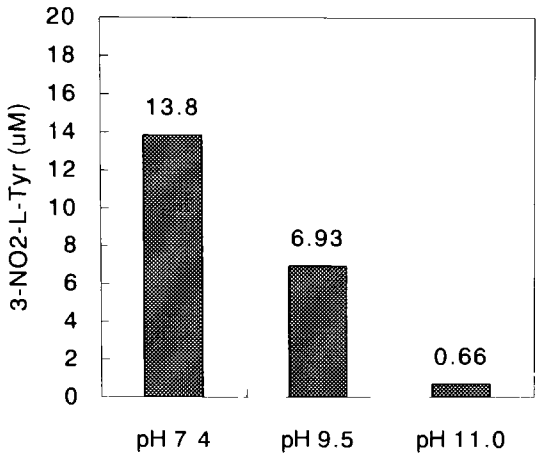

FIG. 2. 3-Nitro-L-tyrosine formation by peroxynitrite with L-tyrosine at different pH. In the phosphate buffer (pH 7.4–11.0), L-tyrosine (1 m*M*) was treated with peroxynitrite (0.2 m*M*) as described in Materials and Methods. The amount of 3-nitro-L-tyrosine formed for the 10 min reaction was determined by HPLC with the standard curve.

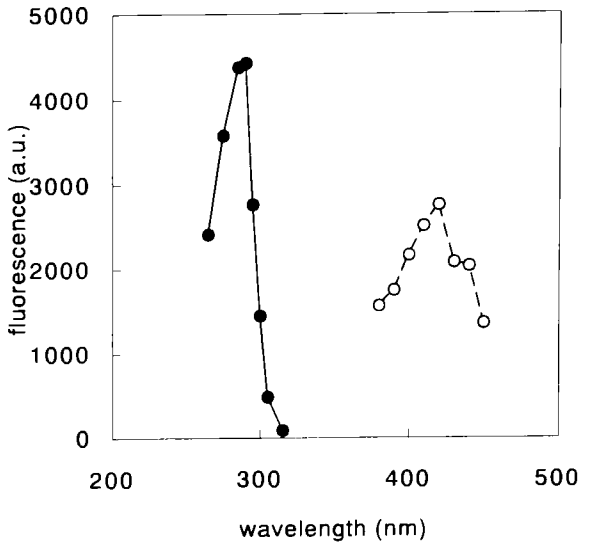

FIG. 3. Excitation and fluorescence spectra of the fluorescent product from the reaction of L-tyrosine and peroxynitrite. (●) excitation spectra monitored at 410 nm fluorescence; (○) fluorescence spectra excited by 295 nm light.

RESULTS

Reaction of peroxynitrite with L-tyrosine

The reaction products of peroxynitrite with L-tyrosine were analyzed by HPLC and a tandemly jointed UV-fluorescence detector system. In the reaction of peroxynitrite and L-tyrosine, it was confirmed that 3-nitrotyrosine was detected as a major product, as reported (Van der Vliet *et al.*, 1994) (Fig. 1). The formation of 3-nitro-L-tyrosine was dependent on the concentration of both of L-tyrosine and peroxynitrite, and was suppressed under higher pH conditions (Fig. 2). These results suggest that 3-nitrotyrosine was a reaction product of L-tyrosine and a protonated form of peroxynitrite (peroxynitrous acid) rather than an anion form. It was also confirmed that a fluorescent product was formed (Van der Vliet *et al.*, 1994), which was assigned as 2,2′-dityrosine by comparing its retention time and fluorescence spectra (Fig. 3) with the authentic sample prepared as described in Materials and Methods.

Effects of synthetic and endogenous compounds on the nitration and oxidation reaction by peroxynitrite

Inhibitory effects of synthetic compounds and endogenous compounds on the reaction of peroxynitrite with L-tyrosine were examined. In the presence of 5-methoxytryptamine (1 in Fig. 4) and *dl*-lipoic acid (2), it was shown that the formation of 2,2′-dityrosine was not inhibited, but rather increased, and 3-nitrotyrosine was significantly inhibited. Glutathione, a typical antioxidant, inhibited the formation of both 3-nitrotyrosine and 2,2′-dityrosine. The synthetic compounds NEMP (3 in Fig. 4) and DTCP (4 in Fig. 4) also effectively inhibited the formation of 3-nitrotyrosine and 2,2′-dityro-

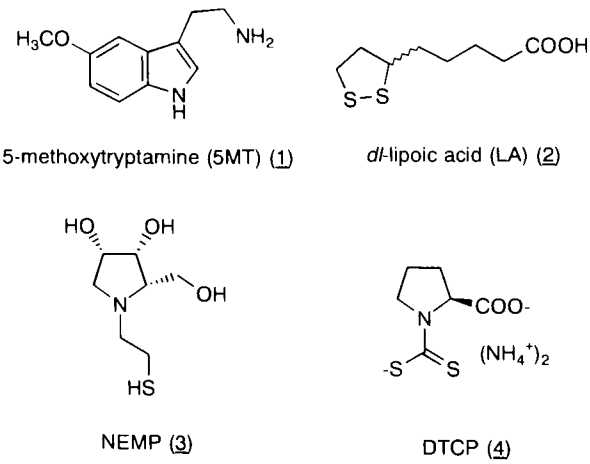

FIG. 4. Structures of endogenous and synthetic compounds used in the inhibitory experiment.

sine. The IC_{50} values of these compounds were almost the same as that of glutathione in this reaction. L-cystine, a typical oxidized form of thiol, showed weak inhibition of the 3-nitrotyrosine and 2,2′-dityrosine formation (Fig. 5).

DISCUSSION

From the results of the reaction of peroxynitrite and L-tyrosine, 3-nitro-L-tyrosine and 2,2′-dityrosine were considered to be typical products of the nitration and oxidation reaction of peroxynitrite with L-tyrosine, respectively. The formation of 3-nitrotyrosine was dependent on the concentration of peroxynitrite and L-tyrosine. In neither case of the treatment of decomposed peroxynitrite nor hydrogen peroxide with L-tyrosine was 3-nitrotyrosine formed.

The formation of 3-nitrotyrosine by peroxynitrite was effectively inhibited by glutathione, NEMP, DTCP, 5-methoxytryptamine, and lipoic acid (Fig. 5A). The formation of 2,2′-dityrosine was also inhibited by low doses of glutathione, NEMP, and DTCP (Fig. 5B). In the case of 5-methoxytryptamine and lipoic acid, however, the 2,2′-dityrosine formation was not inhibited and instead increased at the low dose (Fig. 5B). From this result, 5-methoxytryptamine and lipoic acid were suggested to be selective inhibitors for the tyrosine nitration by peroxynitrite.

L-Dopa was also detected in this reaction mixture, but its amount was decreased at a higher concentration of peroxynitrite due to the further oxidation of dopa by peroxynitrite. Actually, the further oxidation product peak of L-dopa by the reaction with peroxynitrite was detected at a retention time of 16–17 min under this condition using the HPLC-electrochemical detector system. The formation of L-dopa was also not affected in the presence of 5-

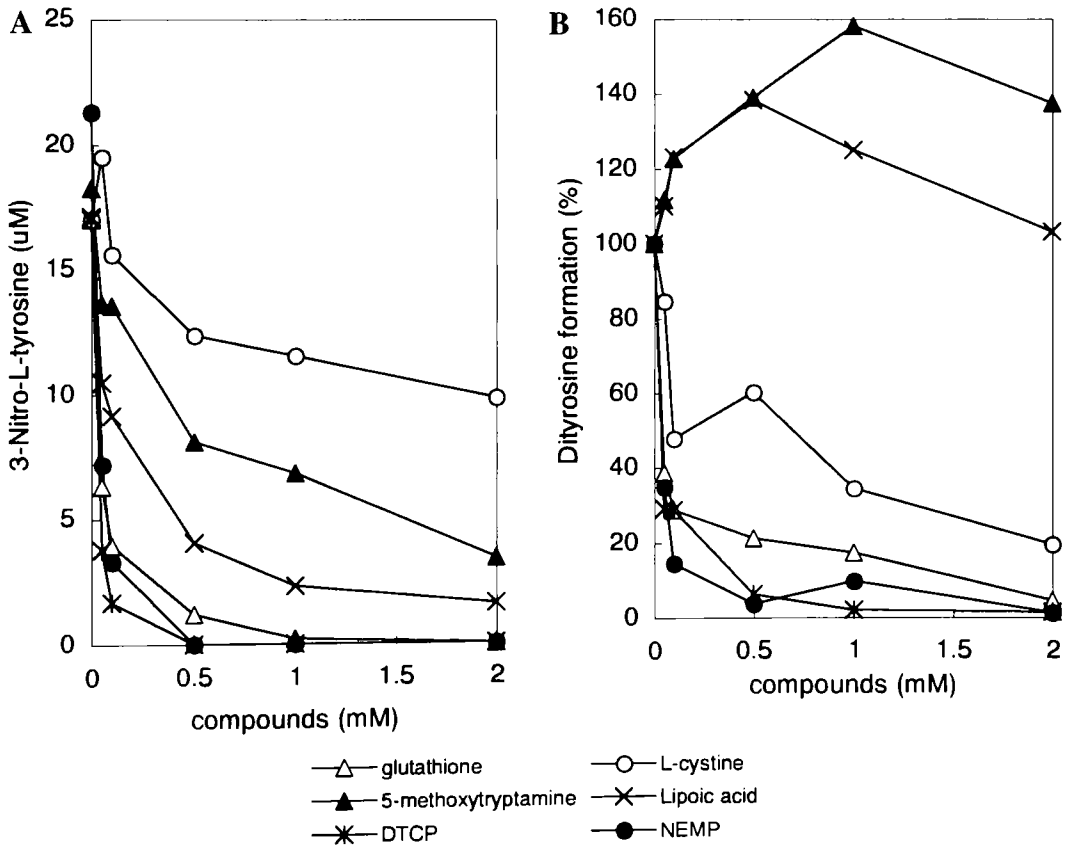

FIG. 5. Formation of 3-nitro-L-tyrosine and 2,2′-dityrosine by L-tyrosine and peroxynitrite reaction in the presence of endogenous and synthetic compounds. L-Tyrosine (1 m*M*) was treated with 0.2 m*M* peroxynitrite, and then the formation of 3-nitro-L-tyrosine and 2,2′-dityrosine was measured as described in Materials and Methods. **A.** 3-Nitro-L-tyrosine formation (μM). **B.** 2,2′-Dityrosine formation as percent for the formation without test compounds.

methoxytryptamine or *dl*-lipoic acid. On the other hand, in the presence of glutathione, NEMP, and DTCP, the formation of L-dopa was first increased and then decreased in a dose-dependent manner, accompanied by the decrease of the further oxidation product of L-dopa. This also suggested that glutathione, NEMP, and DTCP inhibited the oxidation reaction of peroxynitrite and L-tyrosine, but 5-methoxytryptamine or *dl*-lipoic acid did not affect it.

By monitoring the formation of 3-nitro-L-tyrosine and 2,2′-dityrosine simultaneously, it was revealed that 5-methoxytryptamine and *dl*-lipoic acid have a selective inhibitory activity for the nitration reaction of peroxynitrite. This result suggests that the nitration and the oxidation by peroxynitrite may proceed at least partly in the separate way. These reagents are assumed to play potentially important roles against reactive oxygen/nitrogen species (ROS/RNS), and are suggested to be useful tools for investigating ROS/RNS reactions. Further investigation regarding the reaction mechanisms of tyrosine with peroxynitrite and the functional damages and modifications of proteins by peroxynitrite, as well as the protective activity of these compounds found in this study, are now in progress.

ABBREVIATIONS

DTCP, L-*N*-dithiocarboxyproline; H_2O_2, hydrogen peroxide; HPLC-UV, high-performance liquid chromatography-ultraviolet; LDL, low-density lipoprotein; NEMP, (2*S*, 3*R*, 4*S*)-*N*-ethylmercapto-3,4-dihydroxy-2-hydroxymethylpyrrolidine; NMR, nuclear magnetic resonance; NO, nitric oxide; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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