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Artificial Radical Generating and Scavenging Systems: Synthesis and Utilization of Photo-Fenton Regent in Biological Systems

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A photo-labile compound which is bioinactive but, upon irradiation with light, yields bioactive species is called as "caged compound". Photolysis of caged compounds generating bioactive species, has become a general method to produce a desired amounts of bioactive species in the specific time interval at the desired place or area of the target biological systems. For this purpose, we designed and synthesized caged hydroxyl radical., "Photo-Fenton Reagent" NP-III. NP-III has a strong absorption maximum at 377 nm and yields hydroxyl radicals upon UV light irradiation. The antioxidant activity of the α -lipoic acid and other naturally occurring compounds has been examined by using NP-III as a molecular probe. For example, upon photoirradiation of NP-III with BSA or apolipoprotein of human low density (LDL), the significant oxidative modifications were observed in both cases. The oxidation was completely suppressed in the presence of α-lipoic acid, which clearly demonstrates the strong hydroxyl radical scavenging activity of α-lipoic acid. Other applications of NP-III will also be described

Keywords: Photo-Fenton Reagent, Hydroxyl Radical, DNA Oxidation, Protein Oxidation, Antioxidant

INTRODUCTION

Hydroxyl radical is so reactive that it abstracts hydrogen atom or electron from organic molecules and adds to unsaturated bonds of organic compounds with almost diffusion controlled rates in the vicinity of its generation. However, since hydroxyl radical is electrophilic, it reacts readily with electron rich compounds than electron deficient compounds although the selectivity is quite low. There are several hydroxyl radical generating methods; (a) radiolysis of water, (b) redox reactions of hydrogen peroxide, (c) photolysis of organic hydroperoxides or hydrogen peroxide. The second one (b) involves the Harber-Weiss reaction and the Fenton reaction. The third approach (c) is intriguing because the hydroxyl radical generation can easily be controlled by the externally optical devices. We designed and synthesized caged hydroxyl radical, *i.e.*, "Photo-Fenton Reagent" NP-III. NP-III has a strong absorption maximum at 377 nm (ε = 28.2 cm^{-1} mM^{-1}) and yields hydroxyl radicals upon UV light irradiation. The antioxidant activity of many antioxidatnts including α-lipoic acid and other naturally occurring compounds has also been examined by using NP-III as a molecular probe. By comparing the inhibitory rate induced by NP-III by the addition of antioxidants, we can easily evaluate the strength of the antioxidant acitivity of the compounds.

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MATERIALS & METHODS

Preparation of Photo-Fenton Reagent (NP-III). Photo-Fenton reagent was prepared according to the method previously reported [1]. We describe here briefly. To a solution of N, N'-(bis-2,2-dimethoxyethyl)-1,4,5,8- naphthalimide (1.11 g, 2.5 mmol) (prepared from the reaction of naphthalene-1,4,5,8-tetracarboxylic anhydride and 2,2-dimethoxyethylamine) in dry dichloromethane (50 ml), is added excess hydrogen peroxide in dry ether, prepared by extracting 30% aqueous hydrogen peroxide with ether. Triflic acid (0.24 ml, 2.5 mmol) is added to the resulting solution at 0 °C with a syringe. The solution is stirred for 1hr at 0 °C. After the reaction, the mixture was poured into ice water. The dichloromethane layer is separated, and washed twice with water. Evaporation of the dichloromethane layer at 0 °C yields a yellow solid which is washed with cold ethyl ether to give analytically pure NP-III as a yellow solid

Oxidation of Salicylic Acid by NP-III in the Presence of 1,2-Dithiane-3,4-diol or α-Lipoic Acid. Irradiated NP-III solutions were prepared in acetonitrile to a concentration ten-fold the final concentration. Then, to a solution of 250 μl of 2 mM of salicylic acid, 50 μl of 1mM NP-III and 150 μl of 27 mM citrate and 30 mM acetate buffer was added 50 μl of 1,2-dithiane-3,4-diol to final concentrations of 0, 100, 200, 500, 1000, and 5000 μM. Samples were then irradiated for 30 min. A duplicate set of samples covered with aluminum foil served as dark controls. The irradiation was provided by transilluminator (wavelength, >350 nm) at a distance of 10 cm. After the reaction, the reaction mixture was subjected to HPLC-ECD system to detect the formation of 2,3-dihydroxy-benzoic acid and 2,5-dihydroxy-benzoic acid. The yield for the formation of hydroxylated salicylate was determined by comparing the peak areas to those of the standards [2].

Oxidation of Bovine Serum Albumin by NP-III in the Presence of 1,2-Dithiane-3,4-diol or α-Lipoic Acid. The concentration of BSA was adjusted to 2 mg/ml in 0.1M sodium phosphate buffer (pH 7.2).

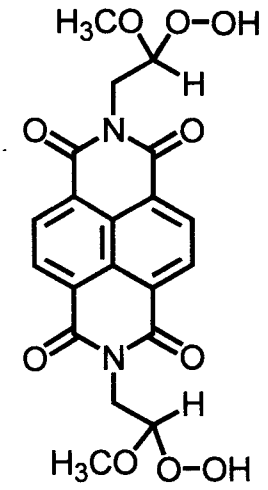

Fig. 1 Structure of NP-III

NP-III solution of 100 μM was prepared by diluting 1 mM NP-III solution in acetonitrile by re-distilled water. Solutions containing 800 μl of BSA, 100 μM of 100 μl of NP-III and 100 μl of 1,2-dithiane-3,4-diol or α-lipoic acid at final concentrations of α-lipoic acid of 5000, 2500, 1000, 500, 200, and 100 μM were directly irradiated for 15 min. A duplicate set of samples covered with foil served as dark controls. The carbonyl content of the BSA was measured as described [3]. Briefly, the reaction mixture was treated with 2,4-dinitrophenylhydrazine at room temperature for 2h. After the reaction the mixed solution was washed with ethyl alcohol : ethyl acetate (1 : 1, v / v) three times. The protein pellet was dissolved in 6 M guanidine-HCl solution (pH 2.3) and the absorbance at 360 nm was measured.

RESULTS & DISCUSSION

According to the laser flash photolysis study of "Photo-Fenton" reagent, NP-III, the lowest singlet state of NP-III has predominantly nπ * character whereas 1,8-naphthalimide derivatives have ππ* character. So, only NP-III can undergo the γ–hydrogen abstraction upon photoirradiation. In the case of NP-III, the

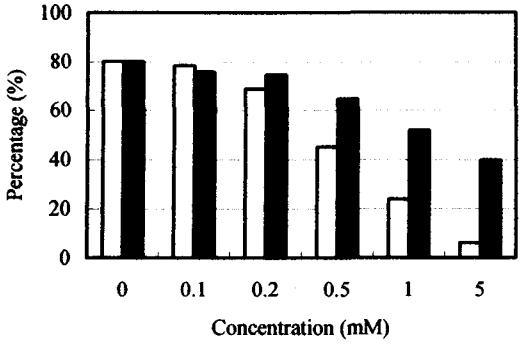

Fig.2. Oxidation of Salicylic Acid by NP-III in the Presence of α-Lipoic Acid. Salicylic acid (2 mM) dissolved in 27 mM citrate and 30 mM acetate buffer was incubated with 1 mM of NP-III in the presence of various concentrations of (■)1,2-dithiane-3,4-diol or (□)α-lipoic acid. After irradiation the sample solutions were subjected to HPLC-ECD and the amounts of the hydroxylated salicylic acid were determined by comparing the peak area with those of standards of 2,3- and 2,5-dihydroxy-benzoic acid.

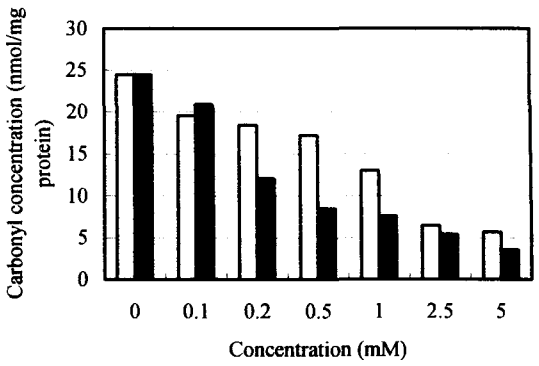

Fig. 3. Oxidation of Bovine Serum Alubumin by NP-III in the Presence of α-Lipoic Acid. BSA (2mg / ml) was incubated with 10 μM of NP-III in the presence of various concentrations of (■)1,2-dithiane-3,4-diol or (□) α-lipoic acid in 0.1 M phosphate buffer solution (pH 7.0). After the irradiation, the sample solution was reacted with dinitrophenylhydrazine for 1h at room temperature then 10% trichloroacetic acid (final concentration) was added to precipitate protein. The protein pellet was washed three times with ethyl alcohol-ethyl acetate (v / v, 1 : 1) and then it was dissolved in 6 M guanidine - HCl solution. The readings were taken at 360 nm using 22000 $M^{-1}cm^{-1}$ of DNPH to calculate protein carbonyls.

photoprocess (quantum yield, 0.03) leads to the formation of an oxygen-centered radical and the release of hydroxyl radical [4]. Based on these mechanistic findings, we carried out the biological damages induced by hydroxyl radical by using NP-III upon photoirradiation.

As previously described, NP-III produces the hydroxyl radical and induces the damages to many biological substrates including proteins [3], DNA, [5,6], and human LDL [3]. The protective effect of many antioxidants toward hydroxyl radical can be evaluated by using NP-III. In fact, we have evaluated the antioxidant activity of lipoic acid [7], dihydrolipoic acid [8], seleno-lipoic acid, [9] water-soluble chitosan derivatives, [10] amino acid derivatives [11] and fermented papaya extract [12].

In this study, we compared the strength of the antioxidant activity of 1,2-dithiane-3,4-diol and α-lipoic acid. Hydroxyl radical scavenging activity of 1,2-dithiane-3,4-diol and α-lipoic acid was confirmed by the inhibition of the formation of dihydroxy-benzoic acid from the reaction of salicylic acid with NP-III. We have already reported that reaction of NP-III with salicylic acid induced the formation of 2,3- and 2,5-dihydroxybenzoic acid [2]. The formation of dihydroxybenzoic acid was completely suppressed in the presence of 5mM of LA (Fig. 2). However, in the case of 1,2-dithiane-3,4-diol, the formation of hydroxylated salicylate was suppressed *ca.* 50% at this concentration (Fig.2).

We also examined the inhibitory activity of these two compounds by examining the inhibition of the formation of protein carbonyls of BSA induced by NP-III upon photoirradiation. When the BSA solution was irradiated in the presence of 10 μM of NP-III for 15 min., the protein carbonyl concentration of BSA increased to 22.5 nmol / mg protein. The protein carbonyl concentration of BSA was significantly decreased in the

presence of 1,2-dithiane-3,4-diol. For example, in the presence of 1 mM 1,2-ditiane-3,4-diol (100 times to NP-III), the protein carbonyl concentration decreased to 7.6 nmol / mg protein under the same reaction conditions. The inhibition effect of 1,2-ditiane-3,4-diol was observed at 200 μM of 1,2-ditiane-3,4-diol concentration (Fig. 3). A similar result was obtained in the case of α-lipoic acid, however, the inhibitory activities are not so high to those observed of 1,2-ditiane-3,4-diol (Fig.3). As far as examining the hydroxyl radical quenching activity, there is not so much difference between 1,2-dithiane-3,4-diol and α-lipoic acid. α-Lipoic acid has a strained 1,2-dithiolane ring chromophore and 1,2-dithiane-3,4-diol has a six-membered ring. The observed antioxidant activities of these compounds are mainly due to these strained S-S ring chromophore. Further studies concerning the antioxidant works are in progress in this laboratory.

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