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Conversion of Nitroxide Radicals by Phenolic and Thiol Antioxidants

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Nitrone/nitroso spin traps are often used for detection of unstable hydroxyl radical giving stable nitroxide radicals with characteristic electron spin resonance (ESR) signals. This technique may be useful only when the nitroxide radicals are kept stable in the reaction system. The aim of the present study is to clarify whether the nitroxide radicals are kept stable in the presence of the hydroxyl radical scavengers. Effect of hydroxyl radical scavengers on the ESR signals of nitroxide radicals, 2,2,6,6-tetramethyl-piperidine-*N*-oxyl (TEMPO) and the spin adduct (DMPO-OH) of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and hydroxyl radical, was examined. Although the ESR signals of TEMPO and the DMPO-OH spin adduct were unchanged on treatment with ethanol and dimethyl sulfoxide, their intensities were effectively decreased on treatment with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), cysteine, glutathione, 2-mercaptoethanol and metallothionein. Hence, the results of the detection of hydroxyl radical in the presence of phenolic and thiol antioxidants by the ESR technique using nitrone/nitroso spin traps may be unreliable.

Keywords: Nitroxide radical, hydroxyl radical, phenolic antioxidant, thiol antioxidant, spin trapping, electron spin resonance

INTRODUCTION

Electron spin resonance (ESR) spin trapping technique using nitrone/nitroso spin traps has been widely used for detection of unstable hydroxyl radical in the research fields of biology, biochemistry and medicine. Nitroxide radicals with the characteristic ESR signals are formed by the reaction of the nitrone/nitroso spin traps with hydroxyl radical. It is known, however, that NADPH,[1] ascorbic acid[2] and cells[3] can reduce the nitroxide radicals into ESR-silent species. Our previous studies have shown that some natural polyphenolic antioxidants convert a nitroxide spin adduct into ESR-silent species.[4] The ESR technique using nitrone/nitroso spin traps may be useful for detection of hydroxyl radical only when the nitroxide radicals are kept stable in the reaction system. The aim of the present study is to clarify whether the nitroxide radicals are kept stable in the presence of the hydroxyl radical scavengers.

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In the present study, effect of hydroxyl radical scavengers on the disappearance of the ESR signals of nitroxide radicals, 2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPO) and the spin adduct (DMPO-OH) of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and hydroxyl radical, was examined. Hydroxyl radical scavengers used were ethanol, dimethyl sulfoxide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), cysteine, glutathione and 2-mercaptoethanol. It was found that the phenolic and the thiol antioxidants decreased the intensities of the ESR signals of the nitroxide radicals. Hence, the results of the detection of hydroxyl radical in the presence of phenolic and thiol antioxidants by the ESR technique using nitrone/nitroso spin traps may be unreliable.

MATERIALS AND METHODS

Materials

DMPO (purity: more than 99.8% by gas chromatography) and TEMPO (about 98% by gas chromatography) were obtained from Labotec Company (Tokyo, Japan) and Sigma Chemical Company (St. Louis, MO, USA), respectively. Hydrogen peroxide (30%), iron (II) sulfate heptahydrate, ethanol, dimethyl sulfoxide and 2-mercaptoethanol were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cysteine was from Nacalai Tesque Company (Kyoto, Japan). Glutathione and metallothionein were from Sigma. Trolox was from Tokyo Chemical Industry (Tokyo, Japan).

ESR Spectroscopy

ESR spectra were obtained on an X-band JES-RE1X spectrometer (JEOL, Tokyo, Japan) equipped with a Mn^{2+} marker at room temperature using a capillary tube. The instrumental conditions were: field setting at 336.0 mT, scan range of 10 mT, modulation frequency of 100 kHz, microwave power of 10 mW and a modulation amplitude of 0.1 mT. The extremely right and left signals shown in each ESR spectrum are those of Mn^{2+} marker.

Treatment of TEMPO with Hydroxyl Radical Scavengers

TEMPO (20 μM) was dissolved in 0.1 M phosphate buffer (pH 7.4), and an aliquot of the solution was mixed to an equal volume of the buffer alone (control) and another aliquot was mixed to an equal volume of the buffer containing each hydroxyl radical scavenger at the indicated final concentration. The mixture was allowed to stand at room temperature for 1 h. The ESR spectrum of the mixture was recorded immediately and after 1 h. The ESR spectrum and the intensities of the signals of the solution of TEMPO alone were unchanged during standing for 1 h.

Spin Trapping of Hydroxyl Radical with DMPO in the Presence of Hydroxyl Radical Scavengers

A solution of each hydroxyl radical scavenger at the indicated final concentration, DMPO (0.1 M), hydrogen peroxide (0.1 mM) and iron (II) (0.1 mM) were mixed in this order in 0.1 M phosphate buffer (pH 7.4), and the mixture was kept at room temperature for 5 min for ESR spectroscopy.

Treatment of the DMPO-OH Spin Adduct with Hydroxyl Radical Scavengers

The DMPO-OH spin adduct was prepared by mixing DMPO (0.1 M), hydrogen peroxide (0.1 mM) and iron (II) (0.1 mM) in 0.1 M phosphate buffer (pH 7.4), and the mixture was kept at room temperature exactly for 5 min.

Water (5 μl) was added to 45 μl of the DMPO-OH spin adduct mixture, and ESR spectra of the mixture were recorded immediately (control 0 min) and exactly after 10 min (control 10 min). During the 10-min interval, the intensities of the ESR signals of DMPO-OH decreased in only a little extent.

A 5-μl aliquot of ethanol, dimethyl sulfoxide or a solution of other hydroxyl radical scavenger in water at the indicated final concentration was added to 45 μl of the DMPO-OH spin adduct mixture, and ESR spectra of the mixture were recorded exactly after 5 min (+sample 5 min) and after 15 min (+sample 15 min).

RESULTS

Firstly, effect of hydroxyl radical scavengers on the disappearance of nitroxide radical TEMPO was examined (Fig. 1). The 3-line ESR signals of TEMPO (10 μM) in phosphate buffer (pH 7.4), with hyperfine splitting constant (hfsc) of a_N =

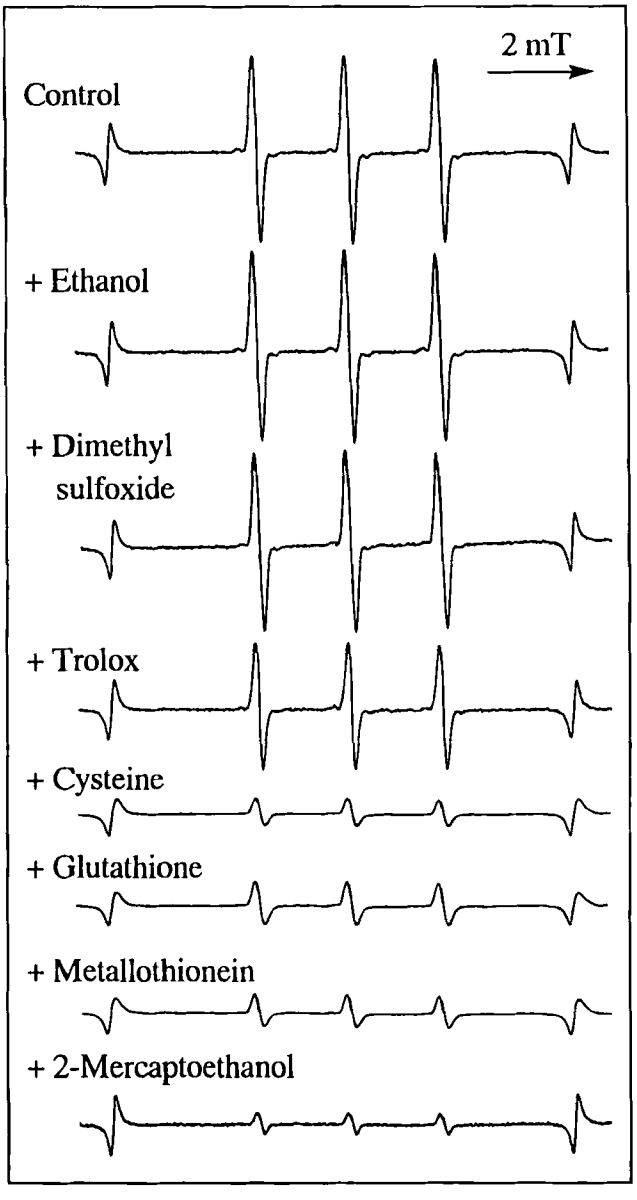

FIGURE 1 ESR spectra of TEMPO treated with hydroxyl radical scavengers. A solution of TEMPO (10 μM) in 0.1 M phosphate buffer (pH 7.4) was treated with none (control), ethanol (10 %), dimethyl sulfoxide (10 %), Trolox (10 mM), cysteine (0.1 M), glutathione (50 mM), metallothionein (1.7 mg/ml) or 2-mercaptoethanol (0.1 M) at room temperature for 1 h. The receiver gain was set at 500.

1.72 mT, were kept stable at room temperature during 1 h. When TEMPO was treated with well-known hydroxyl radical scavengers,[5–7] ethanol (10%) and dimethyl sulfoxide (10%), for 1 h, the ESR signals and their intensities were unchanged. When TEMPO was treated with a water-soluble phenolic antioxidant derivative of tocopherol, Trolox (10 mM), for 1 h, the intensities of the signals were significantly decreased. When TEMPO was treated with thiol antioxidants, cysteine (0.1 M), glutathione (50 mM), metallothionein (1.7 mg/ml), or 2-mercaptoethanol (0.1 M), for 1 h, dramatic decrease in the intensities of the signals was caused. The concentrations of the compounds effective to decrease 50% of the signal intensities were 20 mM for Trolox, 50 mM for cysteine, 60 mM for glutathione, 50 mM for 2-mercaptoethanol. Hence, it was concluded that although ethanol and dimethyl sulfoxide were not reactive toward the nitroxide radical, phenolic and thiol antioxidants converted the radical into ESR-silent species.

Secondly, the effect of hydroxyl radical scavengers on the appearance of the DMPO-OH adduct was examined by initial addition of the scavengers to the reaction mixtures. The DMPO-OH spin adduct was prepared by the reaction of DMPO (0.1 M), hydrogen peroxide (0.1 mM) and iron (II) (0.1 mM) in phosphate buffer (pH 7.4) in the absence and the presence of the scavengers at room temperature for 5 min. The characteristic 4-line ESR signals (ratio of the intensities, 1:2:2:1) with hfsc of $a_N = a_H = 1.49$ mT[5,8] were observed in the absence of the scavengers (Fig. 2, control). When ethanol (10%) or dimethyl sulfoxide (10%) was added before the reaction, the intensities of the ESR signals due to the DMPO-OH spin adduct extensively decreased. Instead, the 6-line ESR signals due to the DMPO-hydroxyethyl spin adduct[5,7,9,10] (Fig. 2, with ethanol) with hfsc of $a_N = 1.59$ mT and $a_H = 2.31$ mT appeared in the reaction with ethanol, and the 6-line ESR signals due to the DMPO-methyl spin adduct[9] (Fig. 2, with dimethyl sulfoxide) with hfsc of $a_N = 1.61$ mT and $a_H = 2.33$ mT appeared in the reaction with dimethyl sulfoxide. As well as other previous results,[5–10] these results reflected that ethanol and dimethyl sulfoxide had scavenged hydroxyl radical and concomitantly generated hydroxyethyl and methyl radicals.

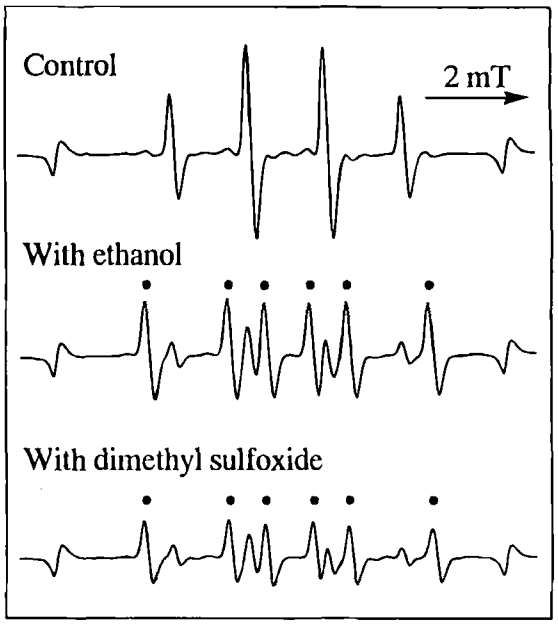

FIGURE 2 ESR spectra of a solution of water (control)/ethanol (10 %)/or DMSO (10 %), DMPO (0.1 M), hydrogen peroxide (0.1 mM), and iron (II) (0.1 mM) in 0.1 M phosphate buffer (pH 7.4) mixed in this order and kept at room temperature for 5 min. Receiver gain was set at 2000. Symbol (●) indicates the signals due to the DMPO-hydroxyethyl adduct and the DMPO-methyl adduct.

Thiol antioxidants, cysteine (10 mM), glutathione (50 mM) or 2-mercaptoethanol (0.1 M) that may scavenge hydroxyl radical,[11] was added before the reaction. The ESR signals due to the DMPO-OH spin adduct did not appear in the presence of cysteine (Fig. 3, with cysteine), the results being consistent with those of the earlier studies.[12] In the presence of glutathione, the intensities of the DMPO-OH signals were extensively decreased and new 4-line signals assignable as the DMPO-glutathione thiyl spin adduct[13,14] with hfsc of $a_N = 1.54$ mT and $a_H = 1.58$ mT appeared (Fig. 3, with glutathione). In the presence of 2-mercaptoethanol, the intensities of the DMPO-OH signals were similarly

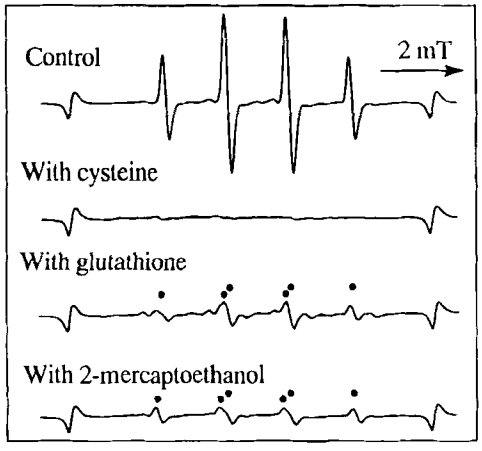

FIGURE 3 ESR spectra of a solution of water (control)/cysteine (10 mM)/glutathione (50 mM) or 2-mercaptoethanol (0.1 M), DMPO (0.1 M), hydrogen peroxide (0.1 mM) and iron (II) (0.1 mM) in 0.1 M phosphate buffer (pH 7.4) mixed in this order and kept at room temperature for 5 min. Receiver gain was set at 2000. Symbol (●) indicates the signals due to the DMPO-thiyl radical spin adducts.

decreased and new 4-line signals assignable as the DMPO-hydroxyethylthiyl spin adduct[13] with hfsc of a_N = 1.52 mT and a_H = 1.68 mT appeared (Fig. 3, with 2-mercaptoethanol). Thiol antioxidants looked as if they scavenged hydroxyl radical to form the corresponding thiyl radicals. In these reaction systems, however, there may be two problems: one is the possibility that the formation of hydroxyl radical was inhibited by chelation of iron (II) ion with the thiol antioxidants, another is the possibility that the DMPO-OH spin adduct formed reacted with the antioxidants.

Thirdly, the following experiments were conducted to estimate the effect of hydroxyl radical scavengers on the disappearance of the ESR signals of the DMPO-OH spin adduct after the adduct was produced. When the mixture of DMPO, hydrogen peroxide and iron (II) ion was allowed to stand at room temperature for 5 min (Fig. 4, control 0 min) and for additional 10 min (Fig. 4, control 10 min), the intensities of the DMPO-OH signals were little changed during the interval, indicating that the DMPO-OH spin adduct was kept stable during the 10-min interval. Effect of ethanol (10%) and dimethyl sulfoxide (10%) on the stability of the DMPO-OH spin adduct was examined by addition of each of these agents to the spin adduct solution at control 0 min and then standing for 5 min (Fig. 4, + sample 5 min). The intensities of the DMPO-OH signals were unchanged on treatment with these agents. The results indicate that ethanol and dimethyl sulfoxide did not react with the DMPO-OH spin adduct.

When Trolox (0.1 M) was added to the DMPO-OH spin adduct at control 0 min and the mixture was kept for 5 min, the intensities of the DMPO-OH signals were significantly decreased (Fig. 5). When cysteine (0.1 M) was added to the DMPO-OH spin adduct at control 0 min and the mixture was kept for 5 min, the DMPO-OH signals disappeared and the weak ESR signals with hfsc of a_N = 1.52 mT and a_H = 1.70 mT appeared (Fig. 6A). The signals were assignable as the DMPO-cysteine thiyl spin adduct.[12] The intensities of the signals decreased after 15 min. The DMPO-OH signals were significantly decreased on treatment with glutathione (0.5 M) for 5 min, and the signals completely disappeared after 15 min to be converted into the signals assignable as the DMPO-glutathione thiyl spin adduct with hfsc of a_N = 1.54 mT and a_H = 1.58 mT[13,14] (Fig. 6B). The DMPO-OH signals completely disappeared on treatment with 2-mercaptoethanol (1 M) for 5 min to be converted into the signals assignable as the DMPO-hydroxyethylthiyl spin adduct with a_N = 1.52 mT and a_H = 1.68 mT,[13] which were kept stable for additional 10 min (Fig. 6C). The concentrations of these phenolic and thiol antioxidants causing the decrease of 50% of the signal intensities of the DMPO-OH spin adduct were 10 μM for Trolox, 30 mM for cysteine, 50 mM for glutathione and 100 mM for 2-mercaptoethanol. These results indicate that the phenolic antioxidant effectively converted the DMPO-OH spin adduct into ESR-silent species, and the thiol antioxidants converted a part of the DMPO-OH spin adduct to give the corresponding thiyl radicals. The decrease in the

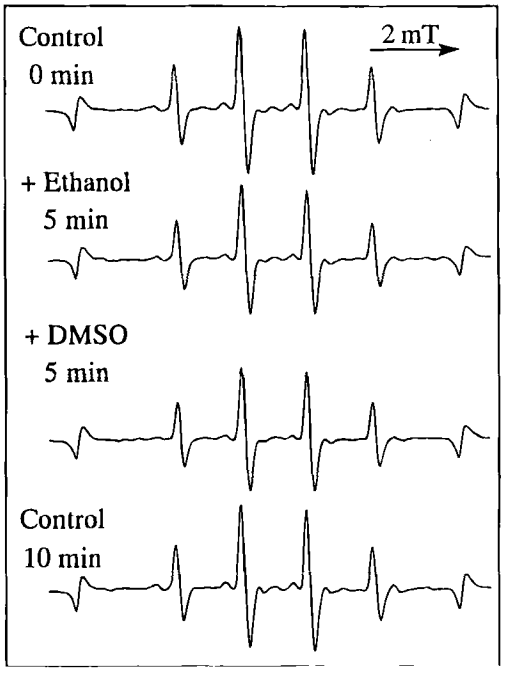

FIGURE 4 Effect of ethanol and dimethyl sulfoxide on the disappearance of the ESR signals of the DMPO-OH spin adduct, after the adduct was prepared. A mixture of DMPO (0.09 M), hydrogen peroxide (0.09 mM) and iron (II) (0.09 mM) in 0.1 M phosphate buffer (pH 7.4) was kept at room temperature for 5 min (control 0 min) and additional 10 min (control 10 min). The DMPO-OH preparation at control 0 min was treated with ethanol (10 %) or dimethyl sulfoxide (10 %) at room temperature for 5 min (+sample 5 min). Receiver gain was set at 2000. Symbol (●) indicates the signals due to the DMPO-thiyl radical spin adducts.

intensities of the DMPO-OH signals and the appearance of the DMPO-thiyl signals in the estimation of hydroxyl radical scavenging activity of thiol antioxidants observed above (Fig. 3) may reflect both the scavenging activity of the thiol antioxidants for hydroxyl radical and the reactivity of the anti-oxidants for the DMPO-OH spin adduct into ESR-silent species.

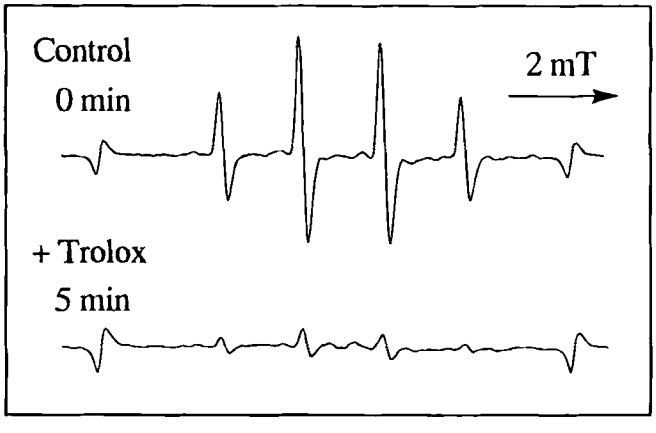

FIGURE 5 Effect of Trolox on the disappearance of the ESR signals of the DMPO-OH spin adduct, after the adduct was prepared. A mixture of DMPO (0.09 M), hydrogen peroxide (0.09 mM) and iron (II) (0.09 mM) in 0.1 M phosphate buffer (pH 7.4) was kept at room temperature for 5 min (control 0 min). The DMPO-OH preparation was treated with Trolox (0.1 M) at room temperature for 5 min (+ Trolox 5 min). Receiver gain was set at 2000.

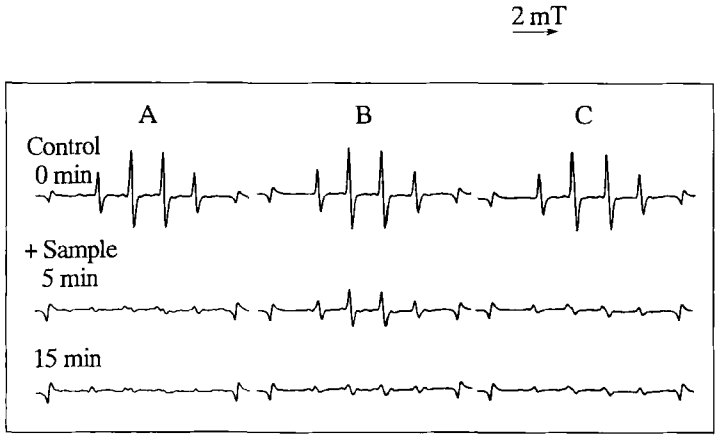

FIGURE 6 Effect of thiol antioxidants on the disappearance of the ESR signals of the DMPO-OH spin adduct, after the adduct was prepared. A mixture of DMPO (0.09 M), hydrogen peroxide (0.09 mM) and iron (II) (0.09 mM) in 0.1 M phosphate buffer (pH 7.4) was kept at room temperature for 5 min (control 0 min). The DMPO-OH preparation was treated with cysteine (0.1 M), glutathione (0.5 M) or 2-mercaptoethanol (1 M) at room temperature for 5 min (+sample 5 min) and 15 min (+sample 15 min). Receiver gain was set at 2000.

DISCUSSION

In the present study, it was demonstrated that the ESR signals of nitroxide radicals, TEMPO and the DMPO-OH spin adduct, were decreased on treatment with phenolic antioxidant Trolox and with thiol antioxidants, cysteine, glutathione and 2-mercaptoethanol. The DMPO-OH adduct was prepared by Fenton reagent in the present experiments. Active oxygen species generated from Fenton reaction of hydrogen peroxide and iron (II) ion are considered to be either hydroxyl radical or $Fe^{4+} = O$,[15] but it is not known which species produces the DMPO-OH spin adduct. The present results showed that phenolic and thiol antioxidants converted TEMPO and the DMPO-OH spin adduct into ESR-silent species. The observations obtained here supported our previous result[4] showing that plant polyphenolics, esculetin, epigallocatechin and epigallocatechin gallate effectively reduce the DMPO-OH spin adduct, and were consistent with the earlier results showing that NADPH,[1] ascorbic acid[2] and cells[3] can reduce the nitroxide radicals. While ethanol and dimethyl sulfoxide may donate hydrogen atom to hydroxyl radical to generate hydroxyethyl and methyl radicals, respectively, they could not donate hydrogen to the nitroxide radicals.

The present results may have important significance in the estimation of hydroxyl radical by ESR spin trapping technique using DMPO. Addition of DMPO to the systems in which hydroxyl radical is generating in the presence of phenolic or thiol antioxidants may not give the ESR signals of the DMPO-OH spin adduct. Silence in the ESR signals of the DMPO-OH spin adduct may reflect not only scavenging hydroxyl radical by phenolic and thiol antioxidants, but also converting the DMPO-OH spin adduct into ESR-silent species. It is difficult to distinguish two reactivities of these antioxidants for hydroxyl radical and for the DMPO-OH spin adduct.

Possible mechanisms of the decrease in the ESR signals of the DMPO-OH spin adduct in the reaction mixture of DMPO and hydroxyl radical in the presence of phenolic and thiol antioxidants might be offered as in Fig. 7. The reaction process of the spin trapping of hydroxyl radical by DMPO in the presence of phenolic antioxidants may be as follows. In one way, the phenolic antioxidants scav-

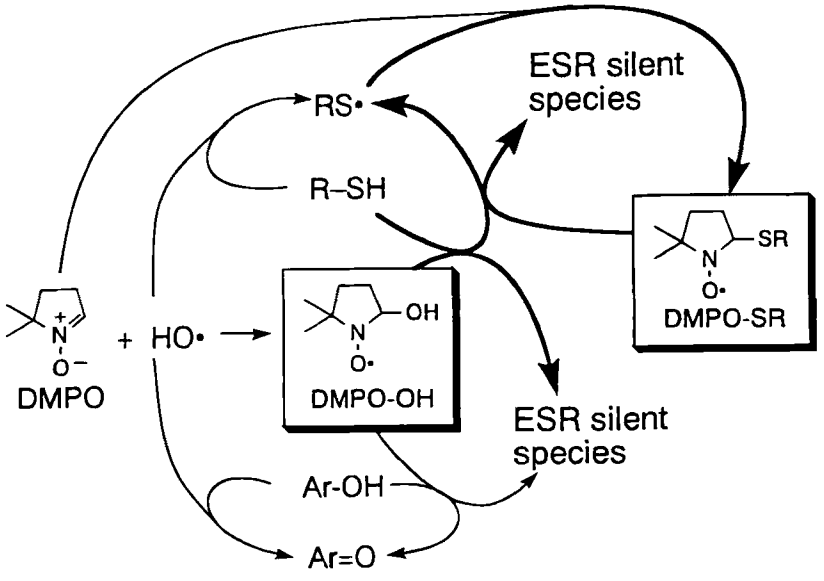

FIGURE 7 A possible mechanism for the disappearance of the ESR signals of the DMPO-OH spin adduct in the reaction mixture of DMPO and hydroxyl radical in the presence of phenolic and thiol antioxidants.

enged hydroxyl radical to cause the decrease in the DMPO-OH spin adduct. In another way, DMPO trapped hydroxyl radical to give the DMPO-OH spin adduct which was in turn converted by the phenolics into ESR-silent species. The reaction process of the spin trapping of hydroxyl radical by DMPO in the presence of thiol antioxidants may be more complex. In one way, the thiol antioxidants scavenged hydroxyl radical giving the corresponding thiyl radicals, which caused the decrease in the DMPO-OH spin adduct and the formation of the DMPO-thiyl spin adducts. In another way, DMPO trapped hydroxyl radical to form the DMPO-OH spin adduct, which was in turn converted into ESR-silent species by the thiol antioxidants generating the corresponding thiyl radicals. The thiyl radicals were trapped by DMPO to give the DMPO-thiyl spin adducts. The thiyl spin adducts may be converted by the thiol antioxidants into ESR-silent species.

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