Temperature-dependent free radical reactions were investigated using nitroxyl radicals as redox probes. Reactions of two types of nitroxyl radicals, TEMPOL (4-hydroxyl-2,2,6,6-tetramethylpiperidine-N-oxyl) and carbamoyl-PROXYL (3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl), were tested in this paper. Heating a solution containing a nitroxyl radical and a reduced form of glutathione (GSH) caused temperature-dependent decay of electron paramagnetic resonance (EPR) signal of the nitroxyl radical. Heating a solution of the corresponding hydroxylamine form of the nitroxyl radical showed EPR signal recovery. The GSH-dependent reduction of nitroxyl radicals at 70°C was suppressed by antioxidants, spin trapping agents, and/or bubbling N₂ gas, although heating carbamoyl-PROXYL with GSH showed temporarily enhanced signal decay by bubbling N₂ gas. Since SOD could restrict the GSH-dependent EPR signal decay of TEMPOL, O₂⁻ is related with this reaction. O₂⁻ was probably generated from dissolved oxygen in the reaction mixture. Oxidation of the hydroxylamines at 70°C was also suppressed by bubbling N₂ gas. Heating a solution of spin trapping agent, DMPO (5,5-dimethyl-1-pyrroline-N-oxide) showed a temperature-dependent increase of the EPR signal of the hydroxyl radical adduct of DMPO. Synthesis of hydroxyl radical adduct of DMPO at 70°C was suppressed by antioxidants and/or bubbling N₂ gas. The results suggested that heating an aqueous solution containing oxygen can generate O₂⁻.

**Key Words:** reactive oxygen species, electron paramagnetic resonance, redox probe, nitroxyl radical, hyperthermia

Interest in hyperthermia for clinical cancer treatment has been increasing recently, especially in combination use with existing treatments, i.e. chemotherapy, radiotherapy and immunotherapy, to increase the efficiency of those treatments; however, the mechanism of hyperthermical cell killing or sensitization to other stresses/treatments has been unclear. The relation of reactive oxygen species (ROS) with the effect of hyperthermia has also been increasing recently, especially in combination use with existing treatments, i.e. chemotherapy, radiotherapy and immunotherapy, to increase the efficiency of those treatments; however, the mechanism of hyperthermical cell killing or sensitization to other stresses/treatments has been unclear. The relation of reactive oxygen species (ROS) with the effect of hyperthermia has been widely alleged and/or reported.¹⁻⁴

Nitroxyl radicals have been conventionally used as chemical redox probes in *in vitro* and *in vivo* experiments in electron paramagnetic resonance (EPR) spectroscopy.⁵⁻⁷ Nitroxyl radicals have been highlighted recently as redox-sensitive MR contrast agents.¹⁸⁻¹⁹ Nitroxyl paramagnetic radical can be directly detected using EPR or indirectly detected through a proton T₁ shortening effect using MRI; however, diamagnetic hydroxylamines, which are a one-electron reduced form of nitroxyl radicals, cannot be detected by either method. Changing spectroscopic behavior through redox transformation of nitroxyl radicals has been utilized to detect free radical reactions in a sample. A nitroxyl radical can be oxidized to the corresponding oxoammonium cation by several oxidants, such as 'OH, O₂⁻, and/or Fe³⁺ ion (Eq. 1).

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The oxoammonium cation was reduced to the corresponding hydroxylamine by receiving hydrogen from hydrogen donors, such as dihydroroticamidine adenine dinucleotide (NADH), dihydroroticamidine adenine dinucleotide phosphate (NADPH), and/or a reduced form of glutathione (GSH) (Eq. 2).

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A recent study¹¹ also found that oxoammonium may irreversibly react with GSH to make a redox-stable complex (Eq. 3), while the structure of this redox-stable complex is still unclear.

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In this paper, temperature-dependent free radical reactions were investigated using nitroxyl radicals as redox probes to examine whether ROS generation can be anticipated for the effect of hyperthermia. The results suggested the temperature-dependent induction of ROS formation in an aqueous solution. The possible mechanisms of temperature-dependent free radical reactions in water are discussed.

**Materials and Methods**

**Chemicals.** 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl (CmP, which is also known as 3CP or carbamoyl-PROXYL) and 4-hydroxyl-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) are among the most widely used nitroxyl radicals in electron paramagnetic resonance (EPR) spectroscopy.¹⁻¹⁰ Nitroxyl radicals have been conventionally used as chemical redox probes in *in vitro* and *in vivo* experiments in electron paramagnetic resonance (EPR) spectroscopy.⁵⁻⁷ Nitroxyl radicals have been highlighted recently as redox-sensitive MR contrast agents.¹⁸⁻¹⁹ Nitroxyl paramagnetic radical can be directly detected using EPR or indirectly detected through a proton T₁ shortening effect using MRI; however, diamagnetic hydroxylamines, which are a one-electron reduced form of nitroxyl radicals, cannot be detected by either method. Changing spectroscopic behavior through redox transformation of nitroxyl radicals has been utilized to detect free radical reactions in a sample. A nitroxyl radical can be oxidized to the corresponding oxoammonium cation by several oxidants, such as 'OH, O₂⁻, and/or Fe³⁺ ion (Eq. 1).

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were purchased from Sigma-Aldrich (St. Louis, MO). 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from LABOTEC Co. (Tokyo, Japan). 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline-N-oxide (CYPMPO) was synthesized as reported elsewhere.\(^{(12)}\)

The hydroxylamine forms of TEMPOL and CmP, i.e. TEMPOL-H and CmP-H, were a gift from Dr. Murali C. Krishna of the National Cancer Institute (NIH, Bethesda, MD). Other chemicals used in this study were of analytical grade. As the basic solvent of reaction mixtures, 100 mM phosphate buffer (pH 7.0) containing 0.05 mM diethylenetriaminepentaacetic acid (DTPA) (100 mM PB) was prepared and used for all experiments. Deionized water (deionized by the Milli-Q system) was used to prepare 100 mM PB.

**EPR signal decay of nitroxyl radicals by heating.** A reaction mixture containing 0.1 mM nitroxyl radical (TEMPOL or CmP) and 1 mM GSH was prepared using 100 mM PB. The reaction mixture was kept in a screw-top vial and was incubated in a heat block at various temperatures (0, 24, 37, 50, 70, or 90°C). Time course of EPR signal in an aliquot of the reaction mixture was measured with X-band EPR spectrometer. Experiments were carried out at various temperatures (0, 24, 37, 50, 70, or 90°C). (A) Time course of TEMPOL in the reaction mixture. (B) Time course of CmP in the reaction mixture. Marks and bars indicate average and SD of at least 3 experiments.

![Fig. 1. Temperature-dependent decay of nitroxyl radicals under coexisting GSH. The reaction mixture containing 0.1 mM nitroxyl radical (TEMPOL or CmP) and 1 mM GSH was incubated in a water bath at various temperatures. The reaction mixture was prepared with 100 mM PB, containing 0.05 mM DTPA. Time course of EPR signal in an aliquot of the reaction mixture was measured with X-band EPR spectrometer. Experiments were carried out at various temperatures (0, 24, 37, 50, 70, or 90°C). (A) Time course of TEMPOL in the reaction mixture. (B) Time course of CmP in the reaction mixture. Marks and bars indicate average and SD of at least 3 experiments.](image1)

![Fig. 2. Inhibition of heat-induced decay of nitroxyl radicals by antioxidants. The reaction mixture containing 0.1 mM nitroxyl radical (TEMPOL or CmP), 1 mM GSH, and 600 mM of an anti-oxidant (α-mannitol, DMSO, or ethanol) was incubated in a heat block at 70°C. (A) Time course of TEMPOL in the reaction mixture. (B) Time course of CmP in the reaction mixture. Marks and bars indicate average and SD of at least 3 experiments.](image2)

**EPR signal decay of nitroxyl radicals by ROS.** To generate 'OH in the reaction mixture, a reaction mixture containing 0.1 mM nitroxyl radical (TEMPOL or CmP), 1 mM GSH, and 1 mM H\(_2\)O\(_2\) was prepared using 100 mM PB, and then the reaction mixture was irradiated by ultraviolet B (290 nm) at room temperature. To generate O\(_2^-\) in the reaction mixture, a reaction mixture containing 0.1 mM nitroxy radical (TEMPOL or CmP), 1 mM GSH, 0.05 mM hypoxanthine (HPX), and 0.01 U/mL xanthine oxidase (XOD) was prepared using 100 mM PB, and then the reaction mixture was incubated at room temperature. The time course of the EPR signal of nitroxyl radical in the reaction mixture was measured by an X-band EPR spectrometer (JEOL, Tokyo, Japan) as described below.

**Oxidation of hydroxylamines with ROS.** To see the reaction of hydroxylamines with 'OH, a reaction mixture containing 0.1 mM TEMPOL-H or CmP-H and 1 mM H\(_2\)O\(_2\) was prepared using 100 mM PB. The reaction mixture was irradiated by ultraviolet B (290 nm). The time course of EPR signal intensity in the reaction mixture was measured with an X-band EPR spectrometer (JEOL, Tokyo, Japan) as described below.
course of EPR signal intensity in the reaction mixture after starting the reaction (adding HPX) was recorded.

Oxidation of hydroxylamines by heating. A reaction mixture containing 0.1 mM TEMPOL-H or CmP-H was prepared using 100 mM PB. The reaction mixture was heated at 70°C. The experiments were repeated under N₂ bubbling. The experiments were also repeated adding 1.6 U/ml SOD. The time course of EPR signal intensity in the reaction mixture after heating was recorded.

Induction of OH-spin adduct of spin trapping agents by heating. DMPO was added to 100 mM PB containing 0.05 mM DTPA (pH 7.0) to make the final concentration of 225 mM. The reaction mixture was kept in a screw-top vial and was incubated in a water bath at various temperatures (37, 50, 70, or 90°C). The time course of DMPO-OH formed in the reaction mixture was measured by an X-band EPR spectrometer (JEOL) as follows. Using CYPMPO instead of DMPO, the same procedures were tested again. The experiment was repeated with the addition of several concentrations of a OH scavenger, such as DMSO or mannitol, at 70°C.

X-band EPR Measurement. An aliquot (120–130 μl) of the reaction mixture was sampled in a quartz flat cell, set in a TE-mode cavity using a special cell holder, and was measured as soon as possible. The sample solution in the flat cell was put back into the vial immediately after the measurement. The EPR conditions were as follows: microwave frequency was 9.4 GHz, microwave power was 4 mW, center field was 334 mT, sweep width was 10 mT, sweep speed was 5 mT/min, modulation frequency was 100 kHz, modulation amplitude was 0.0079 mT, and time constant was 0.03 s.

Results and Discussion

When the aqueous solution of nitroxyl radicals was heated with coexisting GSH, the EPR signal of nitroxyl radicals decreased time- and temperature-dependently (Fig. 1). This EPR signal decay was not observed without GSH. The EPR signal decay of TEMPOL showed the delay time of starting the reaction. The delay time was shortened with increasing temperature, and the reaction rate also became faster temperature-dependently (Fig. 1A). The delay time to start the reaction at room temperature was around 3–4 h. The EPR signal was stable for more than 24 h when the reaction mixture was kept on ice, as shown in a previous report. The reaction of CmP, however, did not show a delay to the initiation of EPR signal loss. The reaction rate of CmP became faster temperature-dependently (Fig. 1B).

Fig. 3 shows the effect of various antioxidants on the EPR signal loss of nitroxyl radicals at 70°C in the reaction mixture containing GSH. A relatively high concentration (600 mM) of antioxidants did not have any notable effects on the reaction of TEMPOL, except that DMSO showed very slight suppression of the reaction. The reaction of CmP, however, could be suppressed by a high concentration of antioxidants.

Fig. 4 shows the effect of a spin trapping agent, DMPO, on the EPR signal loss of nitroxyl radicals in the reaction mixture at 70°C. Adding DMPO to the reaction mixture of TEMPOL suppressed the EPR signal loss of TEMPOL depending on the concentration of DMPO; however, DMPO did not have an effect on the delay time to the initiation of EPR signal loss (Fig. 3A).
DMPO also suppressed the reaction of CmP. Another spin trapping agent, CYPMPO, showed stronger suppression of the reactions of both TEMPOL and CmP (Fig. 4).

EPR signal loss of TEMPOL when heating the reaction mixture was not obtained when GSSG was used instead of GSH (Fig. 5A). The EPR signal, however, temporarily decreased and again recovered when NAD(P)H was used instead of GSH (Fig. 5B). This temporary decrease of the EPR signal may due to the re-oxidation of hydroxylamine to the corresponding nitroxyl radical. This behavior was observed for the reaction of CmP (Fig. 6).

The EPR signal loss of TEMPOL by heating in the reaction mixture containing GSH was suppressed by bubbling the reaction mixture with N₂ gas (Fig. 7A). The start of the decrease of the EPR signal was markedly delayed by N₂ gas bubbling, although the reaction could not be stopped completely. On the other hand, the EPR signal loss of CmP by heating with coexisting GSH increased with N₂ gas bubbling compared with air, and the EPR signal of CmP gradually recovered (Fig. 7B). DMPO could restrict this EPR signal decay of CmP under N₂ bubbling conditions (data not shown). The results in Fig. 7 suggest that the oxygen dissolved in the reaction mixture is related with the temperature-dependent free radical formation in the reaction mixture, while the reaction mechanisms were different between TEMPOL and CmP.

Fig. 8 shows EPR signal losses of nitroxyl radicals in the reaction mixture containing GSH induced by chemically generated ‘OH or O₂⁻. ‘OH was generated by irradiating UVB to H₂O₂. O₂⁻ was generated by reacting with PHX and XOD. The GSH-dependent reduction of nitroxyl radicals can be caused by either ‘OH or O₂⁻. Both TEMPOL and CmP lost most of the EPR signal relatively quickly when the reaction was induced by ‘OH. O₂⁻ could also reduce the EPR signal of both nitroxyl radicals; however, the reaction of CmP under generating O₂⁻ appeared smaller than that with TEMPOL, which lost almost all EPR signal

Fig. 5. Reaction of TEMPOL in the heated reaction mixture containing GSSG, NADH, or NADPH instead of GSH. The reaction mixture containing 0.1 mM TEMPOL and 1 mM GSSG, NADH, or NADPH was incubated at 70°C. (A) Time courses of TEMPOL in the reaction mixture. (B) Detailed time course of TEMPOL in the reaction mixture containing NAD(P)H. (C) Effect of DMPO on the reaction of TEMPOL. Marks and bars indicate average and SD of at least 3 experiments.

Fig. 6. Reaction of CmP in the heated reaction mixture containing GSSG, NADH, or NADPH instead of GSH. The reaction mixture containing 0.1 mM CmP and 1 mM GSSG, NADH, or NADPH was incubated at 70°C. (A) Time courses of CmP in the reaction mixture. (B) Detailed time course of CmP in the reaction mixture containing NAD(P)H. (C) Effect of DMPO on the reaction of CmP. Marks and bars indicate average and SD of at least 3 experiments.
under generating $O_2\cdot^-$. TEMPOL has been reported as a SOD mimicking reagent.\(^{(13,14)}\) The results in Fig. 8 show that TEMPOL may sensitively react with $O_2\cdot^-$ compared with CmP.

Fig. 9 shows the effect of SOD and CAT on the heat-induced EPR signal decay of TEMPOL. SOD could restrict the EPR signal decay of TEMPOL at both 37°C and 70°C, while CAT could not, suggesting that the $O_2\cdot^-$ generated in the reaction mixture is related with the heat-induced EPR signal loss of TEMPOL, while the mechanism of $O_2\cdot^-$ generation is still in progress. SOD was deactivated at 70°C; therefore, SOD could not stop the reaction at 70°C but delayed the reaction (Fig. 9B); however, the reaction at 37°C was almost stopped during 120 min experimentation (Fig. 9A).

Fig. 10 shows the results of oxidizing hydroxylamine forms of TEMPOL or CmP (TEMPOL-H or CmP-H) to the corresponding nitroxyl radicals in several ways, such as exposing hydroxylamine to chemically generated $\cdot OH$, $O_2\cdot^-$, or simply heating the aqueous solution of a hydroxylamine. EPR signals increased quickly when the hydroxylamines were exposed to $\cdot OH$ (open circles). EPR signal intensities increased gradually when the hydroxylamines were exposed to $O_2\cdot^-$ (open diamonds). The oxidation of hydroxylamines can be caused also by either $\cdot OH$ or $O_2\cdot^-$. When the hydroxylamines were heated at 70°C, the EPR signal intensities gradually increased, similar to the reaction with $O_2\cdot^-$ (triangle). This heat-induced EPR signal growth was restricted by N₂ gas bubbling (square). Both TEMPOL-H and CmP-H showed similar time course patterns of reactions, while the reactions were greater for CmP-H than TEMPOL-H.

When the aqueous reaction mixture containing DMPO was heated, the EPR signal of DMPO-OH radical was observed (Fig. 11A). The EPR signal intensity of DMPO-OH increased time- and temperature-dependently (Fig. 11B). The temperature-dependent formation of DMPO-OH was inhibited by the addition of $\alpha$-mannitol and/or DMSO (Fig. 12 A and B). This observation did not coincide with a previous report by Shoji et al.\(^{(15)}\) who reported that the formation of DMPO-OH was suppressed under an argon atmosphere, using ultra-pure water, or the addition of EDTA; however, the addition of $\alpha$-mannitol or DMSO did not affect the formation of DMPO-OH. In contrast to the previous report, the formation of DMPO-OH was suppressed by the relatively high concentration of $\cdot OH$ scavengers in this paper, in which the experimental system contained 0.05 mM DTPA. Therefore, most DMPO-OH formation in water by heating was not via scavenging $\cdot OH$; however, part of it was via scavenging $\cdot OH$. The $\cdot OH$-independent formation of DMPO-OH may not suppressed by such metal ion chelators. The EPR signal of DMPO-OH was not observed in this experiment. DMPO-OH may develop and be transformed to DMPO-OH quickly, since N₂ gas bubbling could stop the appearance of the DMPO-OH signal (data not shown).

Heating a solution containing GSH and nitroxyl radical caused EPR signal decay of the nitroxyl radical temperature-dependently. This heat-induced EPR signal decay of nitroxyl radicals was suppressed by adding EPR spin trapping agents, such as DMPO.
and CYPMPO, or a relatively high concentration of free radical scavengers, such as DMSO, α-mannitol, and ethanol, although the reaction of TEMPO was only slightly weakened by DMSO. This suggests that the generation of ROS in the reaction mixture related to the EPR signal decay.

Heating a solution containing NAD(P)H and a nitroxyl radical caused temporary EPR signal decay of the nitroxyl radical, which recovered subsequently to the initial level. This temporary EPR signal decay of nitroxyl radical reacting with NAD(P)H was eliminated by adding a spin trapping agent, DMPO. Since NAD(P)H can cause temporary EPR signal decay, nitroxyl radicals were oxidized to oxoammonium by ROS.

The EPR signal loss of TEMPO by heating in a reaction mixture containing GSH was suppressed by bubbling N₂ gas, while the EPR signal loss of CmP by heating with coexisting GSH increased with N₂ gas bubbling. DMPO can restrict this EPR signal decay of CmP under N₂ bubbling conditions (data not shown). Both TEMPO and CmP lost most of the EPR signal relatively quickly when the reaction was induced by chemically generated 'OH. The chemically generated O²⁻ also reduced the EPR signal of both nitroxyl radicals with coexisting GSH; however, the reaction of CmP was less than that of TEMPO. SOD could restrict the EPR signal decay of TEMPO at both 37°C and 70°C, while CAT could not. Since SOD could restrict the GSH-dependent EPR signal decay of TEMPO, it was suggested that O²⁻ generated in the reaction mixture was related to this reaction. EPR signals increased quickly when the hydroxylamines were exposed to photo-chemically generated 'OH. EPR signal intensities increased gradually when the hydroxylamines were exposed to chemically generated O₂⁻. Increasing the nitroxyl EPR signal by heating a hydroxylamine was suppressed by N₂ bubbling. Heating a DMPO solution showed the temperature-dependent generation of DMPO-OH spin adducts. This heat-induced generation of DMPO-OH could be suppressed by adding a high concentration of anti-oxidants to the reaction mixture. N₂ gas bubbling could stop the appearance of the DMPO-OH signal by heating. This suggests that the generation of ROS in the reaction mixture.

Conclusion

The results in this paper suggest that heating an aqueous solution containing oxygen can generate ROS. Since SOD could restrict the GSH-dependent EPR signal decay of TEMPO, the main ROS generated in reaction mixtures was estimated as O₂⁻. Since bubbling N₂ gas restricted this reaction, O₂⁻ was probably generated from dissolved O₂ in the reaction mixture while the mechanism of O₂⁻ generation was still in progress. O₂⁻ was partly transformed to 'OH, since antioxidants can slightly restrict the GSH-dependent EPR signal decay of both TEMPO and CmP.
Abbreviations

- CAT: catalase
- CmP: 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-N-oxyl
- CYPMPO: 5-(2,2-dimethyl-1,3-propoxycyclophosphoryl)-5-methyl-1-pyrroline-N-oxide
- DMPO: 5,5-dimethyl-1-pyrroline-N-oxide
- DMSO: dimethylsulfoxide
- DTPA: diethylenetriaminepentaacetic acid
- GSH: reduced form of glutathione
- GSSG: oxidized form of glutathione
- HPX: hypoxanthine
- NADH: dihydronicotinamide adenine dinucleotide
- NADPH: dihydronicotinamide adenine dinucleotide phosphate
- ROS: reactive oxygen species
- SOD: superoxide dismutase
- TEMPOL: 4-hydroxyl-2,2,6,6-tetramethylpiperidine-N-oxyl
- XOD: xanthine oxidase

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