Original Article

Scavenging of reactive oxygen species induced by hyperthermia in biological fluid

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The scavenging activity of rat plasma against hyperthermia-induced reactive oxygen species was tested. The glutathione-dependent reduction of a nitroxyl radical, 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl, which was restricted by adding superoxide dismutase or by deoxygenating the reaction mixture, was applied to an index of superoxide ($O_2^{\bullet-}$) generation. A reaction mixture containing 0.1 mM 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl and 1 mM glutathione was prepared using 100 mM phosphate buffer containing 0.05 mM diethylenetriaminepentaacetic acid. The reaction mixture was kept in a screw-top vial and incubated in a water bath at 37 or 44°C. The time course of the electron paramagnetic resonance signal of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl in the reaction mixture was measured by an X-band EPR spectrometer (JEOL, Tokyo, Japan). When the same experiment was performed using rat plasma instead of 100 mM PB, the glutathione-dependent reduction of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl, i.e., generation of $O_2^{\bullet-}$, was not obtained. Only the first-order decay reduction of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl, which indicates direct reduction of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl, was obtained in rat plasma. Adding 0.5% albumin to the phosphate buffer reaction mixture could almost completely inhibit $O_2^{\bullet-}$ generation at 37°C. However, addition of 0.5% albumin could not inhibit $O_2^{\bullet-}$ generation at 44°C, i.e., hyperthermic temperature. Ascorbic acid also showed inhibition of $O_2^{\bullet-}$ generation by 0.01 mM at 37°C, but 0.02 mM or more could inhibit $O_2^{\bullet-}$ generation at 44°C. A higher concentration of ascorbic acid showed first-order reduction, i.e., direct one-electron reduction, of 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl. Hyperthermia-induced $O_2^{\bullet-}$ generation in rat plasma can be mostly inhibited by albumin and ascorbic acid in the plasma.

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

The relationship of reactive oxygen species (ROS) with the effect of hyperthermia has been widely suggested. Several papers have reported evidence of ROS generation under hyperthermic conditions. Mitchell and Russo studied the effects of hyperthermic temperatures on the oxidative-reductive state of the cell by measuring glutathione (GSH) concentration and reported that continuous heating at 42.5°C or acute exposure at 43°C or 45.5°C resulted in rapid elevation of cellular GSH to 120–200% of control values.(1) Freeman *et al.*(2) suggested that heat shock promotes intracellular oxidative damage and that intracellular glutathione is necessary for protection. Yoshikawa *et al.*(3) reported that lipid peroxidation mediated by ROS plays an important role in the antitumor effect of hyperthermia. Superoxide ($O_2^{\bullet-}$) production in the skeletal muscle mitochondria of chickens in response to acute heat stress has been reported.(4) Various conditions of hyperthermic treatments of Chinese hamster ovary cells or ovarian carcinoma cells increased superoxide dismutase (SOD) activity of the cells.(5) The combination of hyperthermia and other treatments, such as radiation therapy, chemotherapy, etc., was investigated to sensitize the treatment.(6,7) The detailed mechanism of hyperthermic cell killing or sensitization to other stresses/treatments is still under investigation; however, interest in hyperthermia for clinical cancer treatment has been increasing.

Recently, Matsumoto *et al.*(8) reported that heating an aqueous solution containing oxygen can generate ROS, mainly as $O_2^{\bullet-}$, using an electron paramagnetic resonance (EPR) spin probing method. Nitroxyl radical is a stable free radical species when it exists alone in a water solution. Nitroxyl radical, however, can be oxidized by $O_2^{\bullet-}$, hydroxyl radical ($^{\bullet}OH$), and/or Fe^{3+} to be oxoammonium cation. Oxoammonium cation can react with GSH to make a stable diamagnetic compound, although the structure has not yet been elucidated. When a reaction mixture containing 4-hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL), a piperidine type nitroxyl radical, and a reduced form of glutathione (GSH) was heated, the EPR signal of the TEMPOL decreased, showing a characteristic decay curve shape with a delay before a steep reduction. This heating-induced decay of the EPR signal of TEMPOL could be suppressed by SOD or deoxygenation and could be an index of $O_2^{\bullet-}$ generation in the reaction mixture.

In this paper, temperature- and GSH-dependent reduction of TEMPOL was proposed as a method to develop an index of $O_2^{\bullet-}$ generation in rat plasma at hyperthermic temperature. The free radical scavenging ability of a plasma protein and ascorbic acid was analyzed using this method. ROS generation ability at hyperthermic temperature and the antioxidative ability of rat plasma was discussed.

Materials and Methods

Chemicals. 4-Hydroxyl-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL), SOD from human erythrocytes, hypoxanthine (HPX), xanthine oxidase (XOD), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Reduced glutathione (GSH) was purchased from Wako Chemical (Tokyo, Japan). 5,5-Dimethtyl-1-pyrroline-*N*-oxide (DMPO) was pur-

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chased from LABOTEC Co. (Tokyo, Japan). 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 (Milli-Q system, Merck Millipore, Billerica, MA) was used for preparing 100 mM PB.

Animals. Male 12-week-old Wister rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Rats were habituated for 1–2 weeks, then used for the experiment. Rat blood was collected from the abdominal artery under isoflurane anesthetization (2% in 2.5 L/min air flow). The blood was centrifuged at 1000 × g for 10 min to obtain the plasma fraction. The rat plasma was kept in ice until preparation of the reaction mixture.

Measurement of temperature-dependent reduction of TEMPOL. A reaction mixture containing 0.1 mM TEMPOL and 1 mM GSH was prepared using 100 mM PB. The reaction mixture was kept in a screw-top vial and incubated in a water bath at various temperatures (37, 40, 44, 50, or 70°C). The time course of the EPR signal of nitroxyl radical in the reaction mixture was measured by an X-band EPR spectrometer (JES-RE1X; JEOL, Tokyo, Japan) as described below. The experiments were repeated by adding 1.6 U/ml SOD or bubbling N_2 gas. The experiments at 37 and 44°C were repeated by bubbling 1% or 5% O_2 gas (balance gas was N_2). Experiments adding various concentrations (0.25–4%) of bovine serum albumin (BSA) to the reaction mixture were performed similarly at 37 and 44°C. The same experiments, adding various concentrations (0.005–0.04 mM) of ascorbic acid to the reaction mixture, were performed at 44°C with or without GSH. Another experiment was carried out using a reaction mixture containing 0.1 mM TEMPOL and 1 mM GSH prepared using rat plasma instead of 100 mM PB.

Measurement of $O_2^{\bullet-}$ scavenging ability of albumin. A reaction mixture containing xanthine oxidase (XOD), DMPO, and several concentrations of BSA was prepared. Aqueous solution of hypoxanthine (HPX) was added to the reaction mixture to start $O_2^{\bullet-}$ generation. The final concentrations of HPX, XOD, and DMPO were 0.5 mM, 0.1 U/ml, and 100 mM, respectively. The $O_2^{\bullet-}$ spin trapped by DMPO, i.e., DMPO-OOH, was measured by X-band EPR 1 min after starting the reaction. The EPR conditions were the same as below. The DMPO-OOH in the control reaction mixture containing 0% BSA was obtained, then the percentage inhibition of DMPO-OOH generated in the reaction mixtures containing various concentrations of BSA (0–4%) was estimated. The half-maximal inhibitory concentration (IC_{50}) value was estimated.

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 measured as soon as possible. The sample solution in the flat cell was placed back into the vial immediately after the measurement. The EPR conditions were as follows: microwave frequency 9.4 GHz, microwave power 4 mW, center field 334 mT, sweep width 10 mT, sweep speed 5 mT/min, modulation frequency 100 kHz, modulation amplitude 0.0079 mT, and time constant 0.03 s.

Results and Discussion

It is known that nitroxyl radicals are reduced in the presence of GSH.[8–10] Recently, it has been reported that the GSH-dependent reduction of nitroxyl radicals is a temperature-dependent reaction, and experimentally suggested that $O_2^{\bullet-}$ generation is probably related to this GSH-dependent nitroxyl reduction at relatively high temperature, i.e., 70°C.[11] In this paper, the relationship of $O_2^{\bullet-}$ generation to GSH-dependent TEMPOL reduction was again confirmed at hyperthermic temperature.

The GSH-dependent reduction of TEMPOL, a common nitroxyl radical, showed a characteristic decay curve shape, which has de-

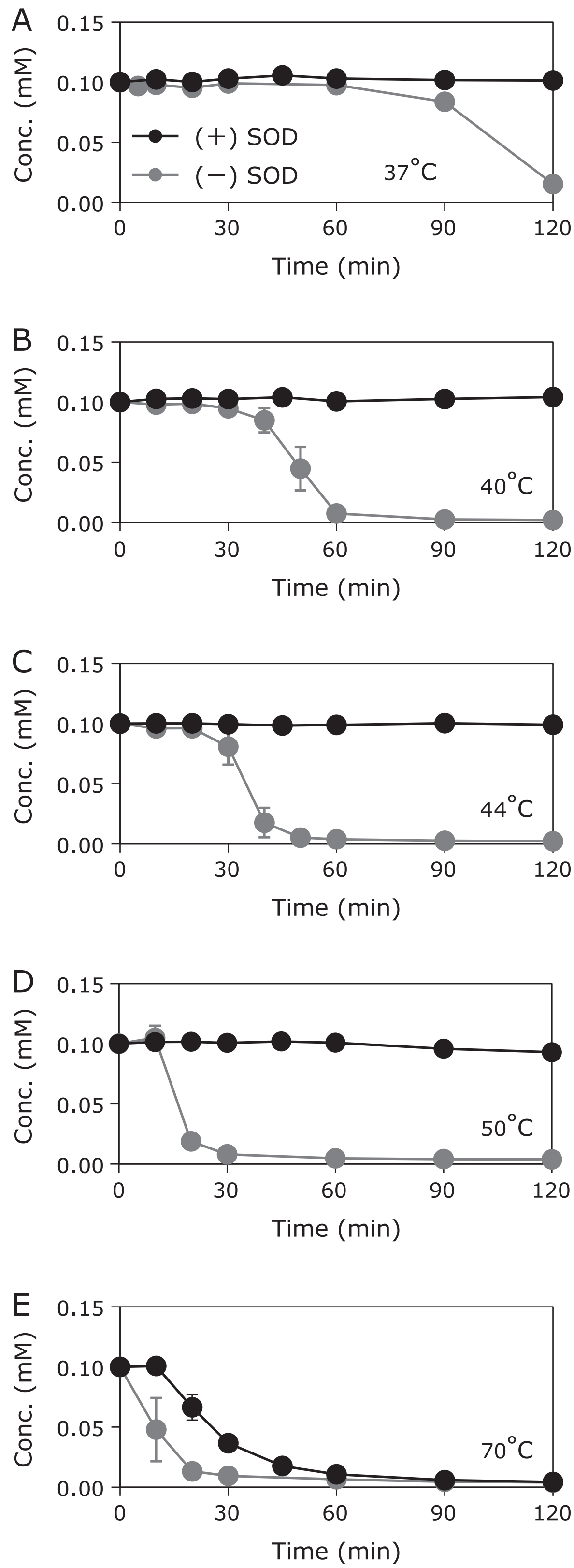

Fig. 1. Suppression of temperature- and GSH-dependent reduction of TEMPOL by SOD. A reaction mixture containing 0.1 mM TEMPOL and 1 mM GSH was prepared with 100 mM phosphate buffer (pH 7). The reaction mixture was incubated at (A) 37°C, (B) 40°C, (C) 44°C, (D) 50°C, and (E) 70°C. TEMPOL in the reaction mixture fell temperature dependently (gray marks). Addition of 1.6 U/ml SOD could stop (≤44°C) or suppress (>50°C) the reduction of TEMPOL (black marks). SOD may be deactivated at higher temperature than 50°C.

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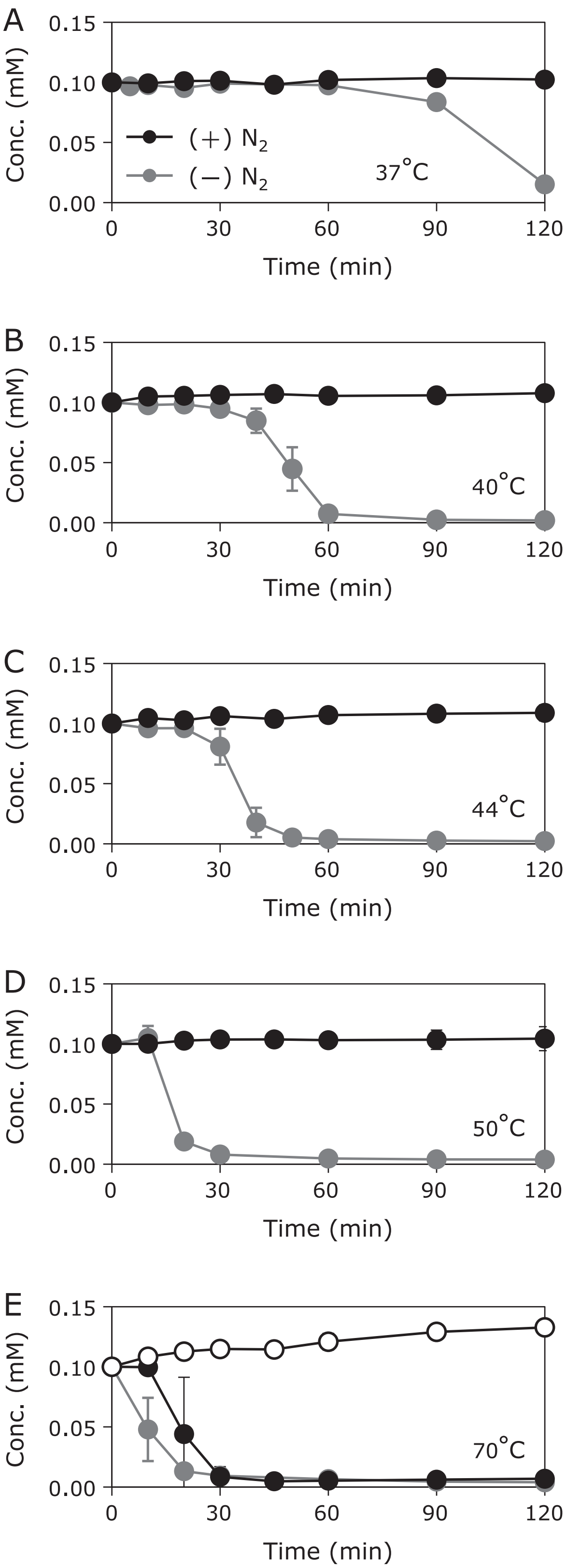

Fig. 2. Suppression of temperature- and GSH-dependent reduction of TEMPOL by bubbling N_2 gas (0.5 L/min). A reaction mixture containing 0.1 mM TEMPOL and 1 mM GSH was prepared with 100 mM phosphate buffer (pH 7). The reaction mixture was incubated at (A) 37°C, (B) 40°C, (C) 44°C, (D) 50°C, and (E) 70°C. TEMPOL in the reaction mixture fell temperature dependently (gray marks). Bubbling N_2 gas could stop (≤50°C) or weakly suppress (70°C) the reduction of TEMPOL (black marks). When N_2 flow rate was increased to 3 L/min, bubbling N_2 gas could stop the reduction of TEMPOL at 70°C (open circles in (E)).

layed time and sequential steep decay (gray marks in Fig. 1 and 2). The delay time became shorter and decay became steeper as the temperature increased. When 1.6 U/mL SOD was added to the reaction mixture, the reduction of TEMPOL was almost completely prevented in the experiment below 50°C (Fig. 1A–D). The reduction of TEMPOL was not fully prevented at 70°C by SOD, but the reduction was delayed slightly (Fig. 1E). SOD is a relatively stable enzyme. A solution of SOD in 0.1 M potassium phosphate, pH 7.5, showed no loss of activity after one hour at 60°C (product information; Sigma-Aldrich). Insufficient prevention of TEMPOL reduction at 70°C by SOD may not only be the result of slight inactivation of SOD but also due to burst $O_2^{\bullet-}$ generation at 70°C. No inhibition was observed even at 37°C, when the previously autoclaved SOD was used (data not shown).

Fig. 2 shows the effect of deoxygenating the reaction mixture on GSH-dependent TEMPOL reduction at various temperatures. Deoxygenating the reaction mixture by bubbling N_2 gas with a flow rate of 0.5 L/min could effectively prevent GSH-dependent TEMPOL reduction below 50°C. Bubbling N_2 gas at 3 L/min could inhibit GSH-dependent reduction of TEMPOL at 70°C (Fig. 2E). This result also suggests burst $O_2^{\bullet-}$ generation at 70°C.

The results in Fig. 1 and 2 indicate that GSH-dependent reduction at hyperthermic temperature was linked to the generation of $O_2^{\bullet-}$, which could be induced from dissolved oxygen in the reaction mixture. The chemical reaction mechanism of this hyperthermic $O_2^{\bullet-}$ generation is probably not so simple, since the reaction started after some delay. GSH itself may also be linked to $O_2^{\bullet-}$ generation at the hyperthermic temperature. Heating an aqueous solution of nitroblue tetrazolium (NBT), which can be reduced by $O_2^{\bullet-}$ to make blue formazan dye having absorption maximum at 560 nm, with the coexisting GSH could increase absorbance at 560 nm; however, no increase of absorbance at 560 nm was observed without GSH (data not shown). Unfortunately, direct evidence of $O_2^{\bullet-}$ generation by the EPR spin trapping method could not be obtained in this case as spin adducts were unstable at higher temperature. Regardless, these findings suggest that $O_2^{\bullet-}$ could be produced *in vivo* under similar hyperthermic conditions with fully dissolved oxygen. It has been reported that extracellular pO_2 is 20 mmHg in fat and 40 mmHg in muscle,[12] which are 1/8–1/4 of an air-equilibrated aqueous solution. Due to such low oxygen concentration in *in vivo* tissue, the thermal generation of $O_2^{\bullet-}$ *in vivo* may be negligible at 37°C, i.e., normal body temperature.

Fig. 3 shows GSH-dependent TEMPOL reduction profiles at 37°C and 44°C with the bubbling reaction mixture using 1% or 5% O_2 gas. At 37°C, GSH-dependent TEMPOL reduction, i.e., $O_2^{\bullet-}$ generation, was almost completely inhibited by 5% O_2 bubbling. At 44°C, however, $O_2^{\bullet-}$ generation could be expected even with 5% O_2 bubbling. The bubbling reaction mixture with 1% O_2 could stop the reduction of TEMPOL at 37°C and could prolong the delay time of TEMPOL reduction to almost 120 min at 44°C (gray squares in Fig. 3A and B). Fig. 3C shows the results of separate experiments with 1% O_2 bubbling at 37°C and 44°C during an 180 min time window. The reduction of TEMPOL was observed during 180 min incubation with 1% O_2 bubbling at 44°C, while 1% O_2 bubbling could inhibit the reduction of TEMPOL at 37°C. Therefore, hyperthermic $O_2^{\bullet-}$ generation could be expected under such low oxygen conditions.

The delay time of the TEMPOL reduction may change sensitively under uncontrollable, minor experimental conditions, especially in such a low oxygen atmosphere. In this experimental condition, i.e., 1% O_2 bubbling, the reduction of TEMPOL started with a delay time of around 90–120 min. The SD bars for the last 2 data points of gray squares in Fig. 3B increased, as one of three measurements in this data set showed 15% reduction at 120 min. This indicates the start of TEMPOL decay at this time point or later. The delay time of TEMPOL reduction with 1% O_2 bubbling at 44°C in Fig. 3C was, however, slightly shorter than 120 min.

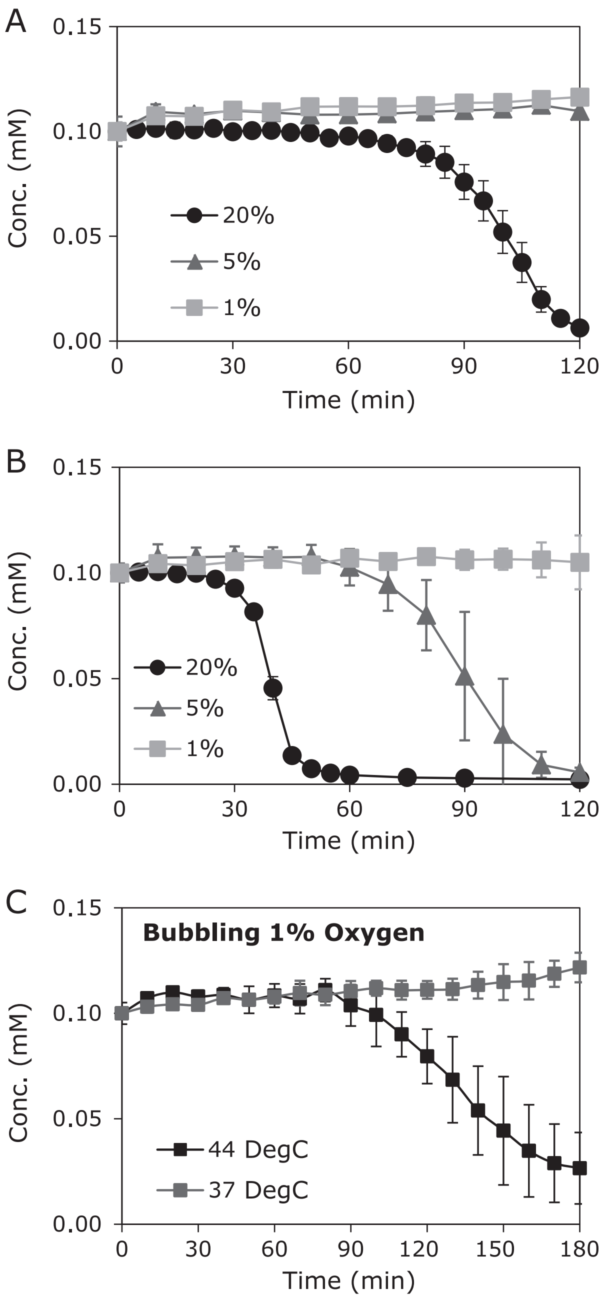

Fig. 3. Temperature- and GSH-dependent reduction of TEMPOL in a low O_2 atmosphere. The reaction mixture was incubated at (A) 37°C or (B) 44°C, bubbling gas containing 1% or 5% O_2. (C) Comparison of reduction profiles of TEMPOL when bubbling 1% O_2 at 37°C and 44°C. The bubbling rate was 0.5 L/min.

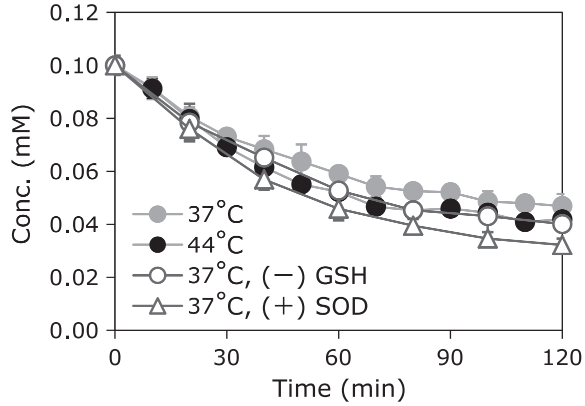

Fig. 4. Decay of TEMPOL in rat plasma. A reaction mixture containing 0.1 mM TEMPOL and 1 mM GSH was prepared using rat plasma instead of 100 mM phosphate buffer. When the reaction mixture was incubated at 37°C or 44°C, TEMPOL in the reaction mixture fell slowly but with no delay (black or gray solid circle, respectively). This TEMPOL reduction was GSH independent (open circle). SOD could not suppress this reduction of TEMPOL (open triangle). Male Wistar rats plasma were used for this experiment.

The relatively large error bars in Fig. 3C also suggest relatively large variations in the delay time.

Bubbling water with 1% O_2 can make around 0.01 mM oxygen concentration in water at 37°C, even at 44°C. Gas solubility in water generally decreases with a temperature increase. The solubility of oxygen in water at 37°C is roughly 1/2 of that at 0°C. However, the difference in oxygen solubility becomes lower at higher temperature; for example, the Bunsen solubility coefficient of oxygen in water at 0°C is 0.049 cm^3/cm^3 water, 0.023 cm^3/cm^3 water at 40°C, 0.019 cm^3/cm^3 water at 60°C, and 0.018 cm^3/cm^3 water at 80°C.[13] In addition, oxygen can dissolve in body fluid better than in water. For example, oxygen dissolves in plasma at 0.6 mmol/L, which is almost 3 times more than in water. Extracellular pO_2 levels in fat tissue, 20 mmHg, and in muscle, 40 mmHg, correspond to 0.027 and 0.054 mM O_2 concentration, respectively. Therefore, hyperthermic *in vivo* $O_2^{\bullet-}$ induction can be expected in such tissues.

A reaction mixture containing 0.1 mM TEMPOL and 1 mM GSH was prepared using rat plasma instead of 100 mM phosphate buffer. When the rat plasma-based reaction mixture was incubated at 37°C or 44°C, the decay profiles of TEMPOL (Fig. 4) showed different shapes from the characteristic decay profile shown in 100 mM PB. A reduction profile of TEMPOL similar to first-order decay and no delay could be observed in rat plasma. No marked difference was observed between the experiments at 37°C (black solid circle) and at 44°C (gray solid circle) in rat plasma. Addition of SOD also showed no effect on this reduction of TEMPOL in rat plasma. Since this reduction profile similar to first-order decay in rat plasma was also observed without the addition of GSH, this reaction can be estimated as a direct reduction of TEMPOL by various reductants, such as ascorbic acid, in rat plasma. These findings suggest that this first order-like TEMPOL decay observed in rat plasma has no relation with $O_2^{\bullet-}$. Therefore, it can be considered whether $O_2^{\bullet-}$ generated in rat plasma was scavenged or $O_2^{\bullet-}$ production in rat plasma was inhibited.

Antioxidative effects of serum albumin, such as hydroxyl radical scavenging ability, have been reported.[14] A possible reductant in rat plasma is ascorbic acid, because rats synthesize ascorbic acid. The concentration of albumin in ≥10-week Wistar rat plasma is around 4 g/dl (2007 data collection; Japan SLC, Inc., Hamamatsu, Japan). The concentration of ascorbic acid in 13-week-old male Wistar rats plasma was reported as around 0.04 mM.[15] Therefore, the effects of BSA and ascorbic acid on the GSH-dependent reduction of TEMPOL were tested again using 100 mM PB as the solvent.

Fig. 5 shows the effect of BSA on GSH-dependent reduction of TEMPOL at 37°C and 44°C. BSA inhibited the reduction concentration dependently. At 37°C, i.e., normal body temperature, 0.5% albumin could completely inhibit the reduction of TEMPOL (gray square in Fig. 5A); however, 0.5% albumin could not sufficiently stop the decay of TEMPOL at hyperthermic temperature, 44°C (gray square in Fig. 5B). Albumin concentration in rat plasma is around 4 g/dl (= 4%), a concentration sufficient to stop the GSH-dependent reduction of TEMPOL at hyper-

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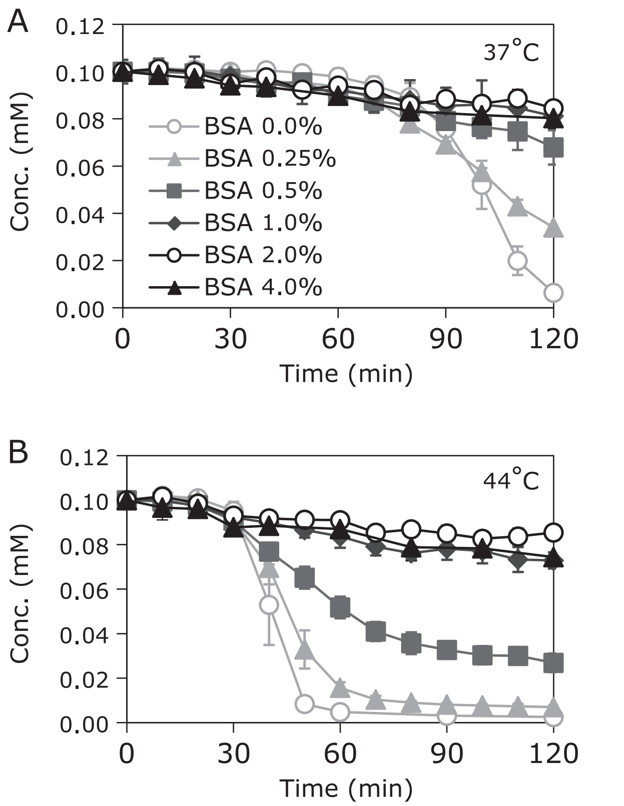

Fig. 5. Suppression of temperature- and GSH-dependent reduction of TEMPOL by bovine serum albumin (BSA). A reaction mixture containing 0.1 mM TEMPOL, 1 mM GSH, and various concentrations of BSA was prepared with 100 mM phosphate buffer (pH 7). The reaction mixture was incubated at (A) 37°C or (B) 44°C. Reduction of TEMPOL in the reaction mixture containing BSA was suppressed concentration dependently. 0.5% BSA was sufficient to suppress the reduction of TEMPOL at 37°C but not at 44°C.

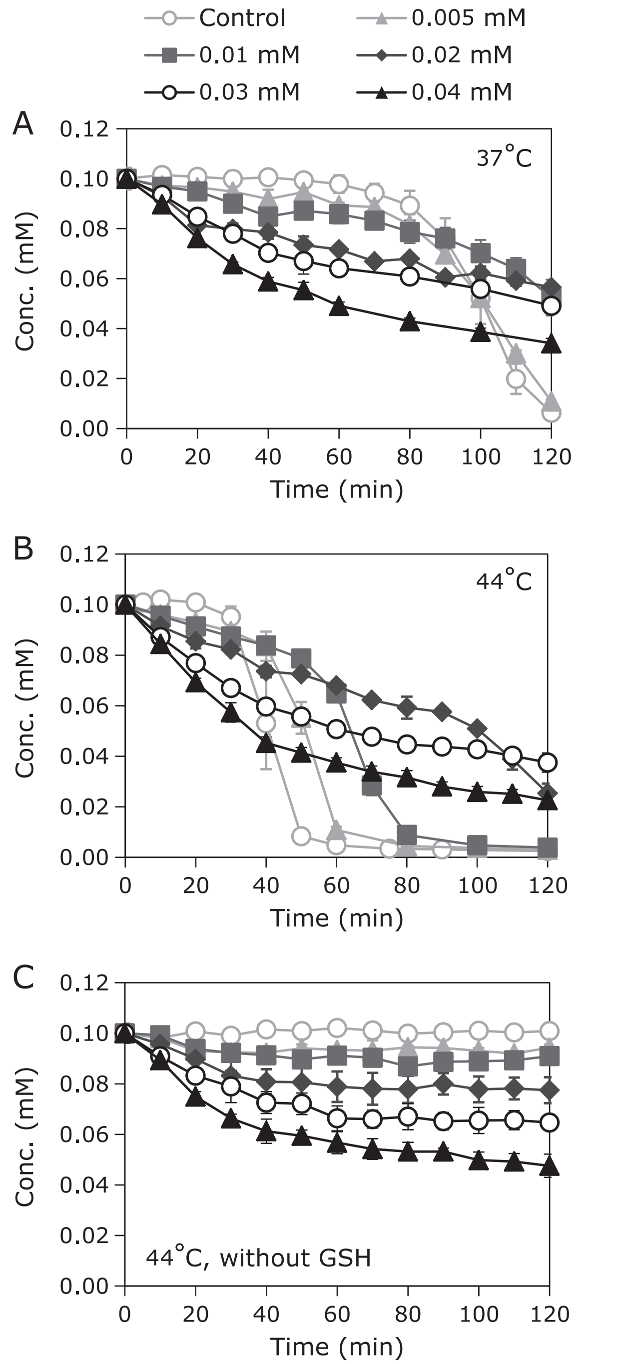

Fig. 6. Suppression of temperature- and GSH-dependent reduction of TEMPOL by ascorbic acid. A reaction mixture containing 0.1 mM TEMPOL, 1 mM GSH, and various concentrations of ascorbic acid was prepared with 100 mM phosphate buffer (pH 7). The reaction mixture was incubated at (A) 37°C or (B) 44°C. The experiment at 44°C was repeated (C) without GSH.

thermic temperature, 44°C. IC_{50} value of $O_2^{\bullet-}$ scavenging by BSA measured by the EPR spin trapping method using the HPX-XOD system as the $O_2^{\bullet-}$ source and DMPO as the spin trapping agent was 2.90%. For comparison, the IC_{50} value of ·OH scavenging by BSA reported in a previous paper was 3.14%.[16]

Fig. 6A and B show the effects of ascorbic acid on the GSH-dependent reduction of TEMPOL at 37°C and 44°C, respectively. Fig. 6C shows (GSH-independent) direct reduction of TEMPOL by ascorbic acid at 44°C. The GSH-dependent reduction profile of TEMPOL was switched to a profile similar to the first-order reaction depending on the concentration of ascorbic acid. The timing of the steep decay became later and, simultaneously, the first order-like decay became faster when the concentration of ascorbic acid became higher. The shapes of the decay profiles were finally exchanged (Fig. 6A and B). At 37°C, 0.005 mM ascorbic acid did not show marked inhibition of the reduction of TEMPOL (Fig. 6A), but 0.005 mM ascorbic acid delayed the reduction slightly (Fig. 6B). This result shows that ascorbic acid could inhibit the GSH-dependent reduction of TEMPOL, i.e., generation of $O_2^{\bullet-}$. Simultaneously, ascorbic acid could reduce TEMPOL directly (Fig. 6C). The rate of the first order-like reduction appears faster in the experiment with GSH (Fig. 6B, see 0.03 and 0.04 mM) than that without GSH (Fig. 6C).

Fig. 7 shows decay profiles of TEMPOL in 100 mM PB containing 1 mM GSH, 0.03 or 0.04 mM ascorbic acid, and 4% BSA at 44°C. The decay profiles were very similar to that observed in rat plasma (seen in Fig. 4). The characteristic steep decay following a slight delay, i.e., GSH-dependent reduction of TEMPOL, was not obtained, but a first order-like decay profile was observed. This is the result of the combination of $O_2^{\bullet-}$ scavenging by BSA and direct reduction of TEMPOL by ascorbic acid. As shown in Fig. 6B, 0.03–0.04 mM ascorbic acid itself is sufficient to inhibit GSH-dependent TEMPOL reduction. A very small amount of ascorbic acid may not scavenge $O_2^{\bullet-}$, but may inhibit the generation of $O_2^{\bullet-}$. These results suggest that hyperthermia-induced ROS in rat plasma can be mostly abolished by a relatively high concentration of albumin and an adequate amount of ascorbic

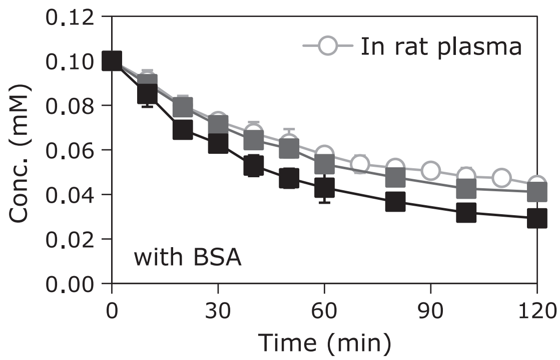

Fig. 7. Reduction profile of TEMPOL in reaction mixture containing biological concentration of ascorbic acid and BSA. A reaction mixture containing 0.1 mM TEMPOL, 1 mM GSH, 4% BSA, and various concentrations of ascorbic acid (gray squares: 0.03 mM, black squares: 0.04 mM) was prepared with 100 mM phosphate buffer (pH 7). Experiments were performed at 44°C.

acid in the plasma. In addition, the relatively strong reducing ability of ascorbic acid may be able to turn oxidative products back to their original state. Nevertheless, the stability of the oxidized product in rat plasma and/or sequential free radical reactions may be important to consider the practical effects of hyperthermia. If looked at from another point of view, the intracellular course of ROS could be more important to understand the biological effect of hyperthermia.

Conclusion

GSH-dependent reduction of TEMPOL at hyperthermic temperature was inhibited by adding SOD and/or deoxygenating (by bubbling N_2) the reaction mixture. This result evidently demonstrated the generation of superoxide in the aqueous reaction mixture at hyperthermic temperature. The generation of $O_2^{\bullet-}$, i.e., GSH-dependent reduction of TEMPOL, could not be observed when rat blood plasma instead of 100 mM PB was used to prepare the reaction mixture, although the first order decay-like reduction of TEMPOL could be observed. Adding 0.5% albumin to the reaction mixture could stop the reduction of TEMPOL at 37°C; however, 0.5% albumin could not completely stop the decay of TEMPOL at hyperthermic temperature, 44°C. Ascorbic acid also inhibited the generation of $O_2^{\bullet-}$, i.e., GSH-dependent reduction of TEMPOL, and in addition, ascorbic acid could directly reduce a stable radical, i.e., TEMPOL. Rat plasma has relatively strong antioxidative ability by scavenging free radicals and by its reducing ability.

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Mitchell JB, Russo A. Thiols, thiol depletion, and thermosensitivity. *Radiat Res* 1983; **95**: 471–485.
2 Freeman ML, Spitz DR, Meredith MJ. Does heat shock enhance oxidative stress? Studies with ferrous and ferric iron. *Radiat Res* 1990; **124**: 288–293.
3 Yoshikawa T, Kokura S, Tainaka K, *et al*. The role of active oxygen species and lipid peroxidation in the antitumor effect of hyperthermia. *Cancer Res* 1993; **53**: 2326–2329.
4 Mujahid A, Yoshiki Y, Akiba Y, Toyomizu M. Superoxide radical production in chiken skeletal muscle induced by acute heat stress. *Poult Sci* 2005; **84**: 307–314.
5 Loven DP, Leeper DB, Oberley LW. Superoxide dismutase levels in Chinese hamster ovary cells and ovarian carcinoma cells after hyperthermia or exposure to cycloheximide. *Cancer Res* 1985; **45**: 3029–3033.
6 Wang CC, Chen F, Kim E, Harrison LE. Thermal sensitization through ROS modulation: a strategy to improve the efficacy of hyperthermic intraperitoneal chemotherapy. *Surgery* 2007; **142**: 384–392.
7 Cui ZG, Kondo T, Feril Jr LB, Waki K, Inanami O, Kuwabara M. Effects of anitoxidants on X-ray- or hyperthermia-induced apoptosis in human lymphoma U937 cells. *Apoptosis* 2004; **9**: 757–763.
8 Matsumoto K, Okajo A, Nagata K, *et al*. Detection of free radical reactions in an aqueous sample induced by low linear-energy-transfer irradiation. *Biol Pharm Bull* 2009; **32**: 542–547.
9 Tada M, Yokoyama H, Ito O, Ohya H, Ogata T. Evaluation of the hepatic reduction of a nitroxide radical in rats receiving ascorbic acid, glutathione or ascorbic acid oxidase by *in vivo* electron spin resonance study. *J Gastronenterol Hepatol* 2004; **19**: 99–105.
10 Kuppusamy P, Li H, Ilangovan G, *et al*. Noninvasive imaging of tumor redox status and its modification by tissue glutathione levels. *Cancer Res* 2002; **62**: 307–312.
11 Matsumoto K, Nyui M, Kamibayashi M, Ozawa T, Nakanishi I, Anzai K. Temperature-dependent free radical reaction in water. *J Clin Biochem Nutr* 2012; **50**: 40–46.
12 Matsumoto A, Matsumoto S, Sowers AL, *et al*. Absolute oxygen tension (pO_2) in murine fatty and muscle tissue as determined by EPR. *Magn Reson Med* 2005; **54**: 1530–1535.
13 Winkler LW. Die Löslichkeit der Gase in Wasser. *Ber Deut Chem Ges* 1891; **24**: 89–101.
14 Liu SY, Chen CL, Yang TT, *et al*. Albumin prevents reactive oxygen species-induced mitochondrial damage, autophagy, and apoptosis during serum starvation. *Apoptosis* 2012; **17**: 1156–1169.
15 Hijova E, Nistiar F, Sipulova A. Changes in ascorbic acid and malondialdehyde in rats after exposure to mercury. *Bratisl Lek Listy* 2005; **106**: 248–251.
16 Ueno M, Nakanishi I, Matsumoto K. Method for assessing X-ray-induced hydroxyl radical-scavenging activity of biological compounds/materials. *J Clin Biochem Nutr* 2013; **52**: 95–100.

doi: 10.3164/jcbn.13-61