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Formation of TEMPOL-hydroxylamine during reaction between TEMPOL and hydroxyl radical: HPLC/ECD study

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Abstract
Nitroxyl radicals are important antioxidants that have been used to protect animal tissues from oxidative damage. Their reaction with hydroxyl radical (•OH) is generally accepted to be the mechanism of antioxidant function. However, the direct interaction of nitroxyl radicals with •OH does not always provide a satisfactory explanation in various pH, because the concentration of hydrogen ion may affect the generation of secondary •OH-derived radicals. In the present study, it was confirmed that the reaction between 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) and •OH generated TEMPOL-hydroxylamine, 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPON) and TEMPON-hydroxylamine using HPLC coupled with electrochemical detection. In the absence of NADH, TEMPOL-H may be generated by the reaction with secondary •OH-derived radicals in acidic condition. In the presence of NADH, a large proportion of the non-paramagnetic products was TEMPOL-H. Finally, it was clarified that TEMPOL-H was generated during dopamine metabolism, which is believed to be one of the •OH sources in pathological processes such as Parkinson's disease.

Keywords: *Hydroxyl radical, nitroxyl radical, nitroxide, tempol, ESR, HPLC*

Abbreviations: *ESR, electron spin resonance; ECD, electrochemical detection; HPLC, high performance liquid chromatography; MAO, monoamine oxidase; •OH, hydroxyl radical; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; TEMPOL-H, 4-hydroxy-2,2,6,6-tetramethyl-N-hydroxypiperidine; TEMPON, 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl; TEMPON-H, 4-oxo-2,2,6,6-tetramethyl-N-hydroxypiperidine*

Introduction

Accumulating evidences suggest that oxidative damage contributes to various disease processes. Nitroxyl radicals have been used as antioxidants to protect animal tissues from oxidative damage following indomethacin-induced gastric ulcer [1], brain ischemia reperfusion [2,3] and Parkinson's disease model [4]. Reactive oxygen species (ROS) are generated in these disease states through neutrophil infiltration [5] or dopamine metabolism [6]. Therefore, the effective reactions of nitroxyl radicals with ROS such as superoxide anion radical and hydroxyl radical (•OH) are generally proved to be a key mechanisms for antioxidant function. However, the direct interaction of nitroxyl radical with ROS does not always provide a satisfactory explanation for antioxidant activity in various conditions such as pH, because the concentration of hydrogen ion may

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affect the generation of secondary •OH-derived radicals [7,8]. Therefore, it remains unknown how nitroxyl radicals react with •OH at various pH.

The hydroxyl radical is experimentally produced by exposing H_2O_2 to ultraviolet (UV) rays. Using this •OH generation system, Saito et al. [9] determined two reaction sites of 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL, Figure 1A) with •OH: the hydroxyl group at 4-position of the piperidine ring and the nitroxyl group. When •OH reacts with the hydroxyl group of TEMPOL, 4-oxo-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPON, Figure 1B) is generated. This finding is based on the result that the hyperfine coupling constant for the nitrogen atom (a^N) of this signal is the same as that of TEMPON [9,10]. Furthermore, Willson [11] suggested that the final product might be the oxoammonium cation, when •OH reacts with the nitroxyl group at almost the diffusion-controlled rate [12]. The oxoammonium cation was reduced to the hydroxylamine by NADH [13].

One limitation of the ESR technique, however, is that TEMPOL may lose their paramagnetism in the reaction solution. Until now, no methodology was presented for the direct determination of the non-paramagnetic products formed between TEMPOL and •OH. The generation of TEMPON is also based on differences which exist between the hyperfine coupling constant of TEMPOL and that of TEMPON. In order to identify the reaction products, insufficient to determine the reaction products, further studies are needed to determine the reaction products by using more specific methods.

In this report, we detected the reaction products of TEMPOL generated during the UV photolysis of H_2O_2, using high performance liquid chromatography (HPLC) coupled with electrochemical detection (ECD), to clarify how TEMPOL reacted with •OH. This technique can readily detect nitroxyl radicals and non-paramagnetic products such as hydroxylamine derivatives in the same sample, as these agents are well resolved on a C-18 reverse phase column. We also studied the effect of pH and NADH as electron donors, to examine the reaction mechanism that might occur *in vivo*. Finally, we clarified that TEMPOL was converted to the corresponding hydroxylamine during dopamine metabolism, which is believed to be one of the •OH sources in pathological processes such as Parkinson's disease.

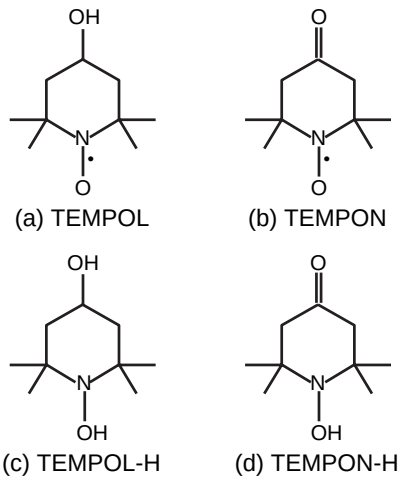

Figure 1. Chemical structures of TEMPOL (A), TEMPON (B), TEMPOL-H (C) and TEMPON-H (D).

Materials and methods

Chemicals

TEMPOL and TEMPON were obtained from Sigma-Aldrich Co. (MO, USA). The reduced form of TEMPOL, 4-hydroxy-2,2,6,6,-tetramethyl-N-hydroxypiperidine (TEMPOL-H, Figure 1C), was prepared using H_2 bubbled over Pt powder [14]. 4-Oxo-2,2,6,6,-tetramethyl-N-hydroxypiperidine (TEMPON-H, Figure 1D) was prepared by the reduction of TEMPON using ascorbate [15]. Potassium ferricyanide, hydrogen peroxide, iron sulphate heptahydrate, dopamine hydrochloride, citric acid, sodium dihydrogen phosphate dihydrate, ascorbic acid sodium salt and lithium perchlorate were purchased form Wako Pure Chemical Industry (Osaka, Japan). Methanol (HPLC grade) was purchased from Nacalai Tesque, Inc (Kyoto, Japan). NADH was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Monoamine oxidase (MAO) B human was obtained from Sigma-Aldrich Co. (MO, USA). All the reagents were dissolved in Mcilvaine buffer solution (pH 2.2–8.0) [16]. Ultrapure water was used in all experiments (Millipore Co.; MA, USA).

Chemical reaction of TEMPOL in H_2O_2 solution caused by UV irradiation

The hydroxyl radical was generated by UV photolysis of H_2O_2 as described previously [9]. Briefly, TEMPOL (25 μM) dissolved in buffer solution was mixed with H_2O_2 (7.5 mM) and NADH (25 μM) in a brown vial. The mixture then was transferred to a disposable micro-pipette (Drummond Scientific Co., PA, USA) and the X-band ESR spectra were recorded. After the ESR measurements, the mixture was irradiated with UV light for 1, 3, 5, 7, 10 and 15 min in the ESR cavity to generate •OH and the ESR spectra were recorded again. The UV irradiation was performed with an SX-UI 251HQ irradiation unit equipped with a 250 W extra-high pressure UV lamp (USH-250SC) (Ushio Co., Ltd., Tokyo, Japan). A UV filter with cut-off wavelength of 300 nm was used to prevent UV-induced destruction of the nitroxyl group, which has a maximum absorbance of 230 nm.

ESR measurements

X-band ESR spectra were recorded using a JEOL JES-1X ESR spectrometer (microwave power: 5 mW; amplitude of 100 kHz field modulation: 0.06 mT; time constant: 0.03 s; sweep rate: 1 min/10 mT). The ESR spectra were analysed with an ESR Data Analyser (JEOL Co. Ltd., Akishima, Japan).

Measurement of reaction products using the HPLC/ECD system

TEMPOL and its reaction products were assayed by HPLC coupled to ECD. Separation was achieved with a C18 reverse-phase column (MCM column 150*4.6 mm, MC Medical Inc., Tokyo Japan). The mobile phase contained 10 mM lithium perchlorate with 30% (v/v) methanol. The HPLC flow rate was 0.5 mL/min. Electrochemical detection was carried out with a Coulochem III detector (ESA Laboratories, Inc., MA, USA) equipped with a guard cell (M5020) and analytical cell set (M5011). The guard cell was set at + 850 mV, electrode 1 at 200 mV and electrode 2 at 800 mV.

TEMPOL-derived reaction products during dopamine metabolism

TEMPOL (25 μM) dissolved in buffer solution was mixed with dopamine (1 mM), MAO (1 mg protein/mL), $FeSO_4$ (10 μM) and NADH (25 μM) in a brown vial. The reaction mixture was used for the X-band ESR and HPLC experiments.

Results

Loss of ESR TEMPOL signal caused by UV photolysis of H_2O_2

The ESR spectrum of TEMPOL consisted of triplet with the same peak height and a hyperfine coupling constant of 1.69 mT (data not shown). A slight decrease in the ESR signal was observed during UV irradiation in the absence of H_2O_2 (Figure 2A–D, indicated by open and closed circles). The ESR signal loss was appreciable at pH 2.2 and 5.0 following the UV photolysis of H_2O_2 (Figure 2A and B, respectively, indicated by open squares) in the absence of NADH. With NADH in the reaction system, we observed notable ESR signal loss at pH 2.2, 5.0 and

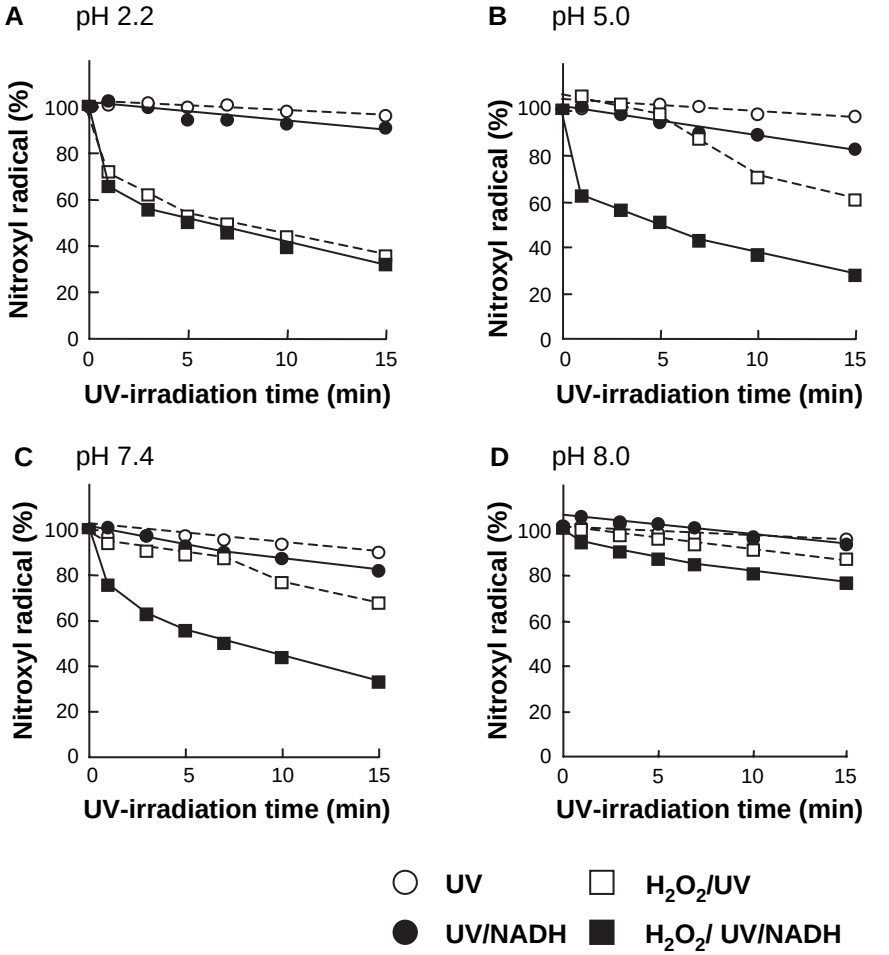

Figure 2. ESR signal decay of nitroxyl radicals by the UV photolysis of H_2O_2. ESR spectra were recorded for a mixture of TEMPOL and with or without NADH at pH 2.2 (A), 5.0 (B), 7.4 (C) or 8.0 (D) during the UV photolysis of H_2O_2.

7.4 (Figure 2A–C, indicated by closed squares). At pH 8.0, TEMPOL was present at ~80% of its original level 15 min after the reaction (Figure 2D).

HPLC/ECD analysis of TEMPOL, TEMPON and their hydroxylamines

Figure 3A shows a typical HPLC chromatogram of a mixture of TEMPOL, TEMPON and their hydroxylamines. The peaks of TEMPOL-H, TEMPOL, TEMPON and TEMPON-H appeared at 14.3, 16.2, 18.3 and 24.5 min, respectively. A peak at 10.5 min caused by measurement noise was always observed.

The fraction obtained at peak 1 did not yield an ESR spectrum (Figure 3B, ESR spectrum 1). However, the addition of potassium ferricyanide (1 mM) to this fraction resulted in an ESR spectrum for TEMPOL. The fractions defined by peaks 2 and 3 gave ESR spectra that were typical to TEMPOL and TEMPON, respectively (Figure 3B, ESR spectra 2 and 3). Although the fraction defined by peak 4 did not yield an ESR spectrum (Figure 3B, ESR spectrum 4), the ESR spectrum for TEMPON appeared upon the addition of potassium ferricyanide. The hyperfine couplings of TEMPOL and TEMPON were $a^N = 1.69$ and $a^N = 1.60$ mT, respectively.

The concentration dependencies of the HPLC/ECD signals for TEMPOL-H, TEMPOL, TEMPON and TEMPON-H are shown in Figure 3C.

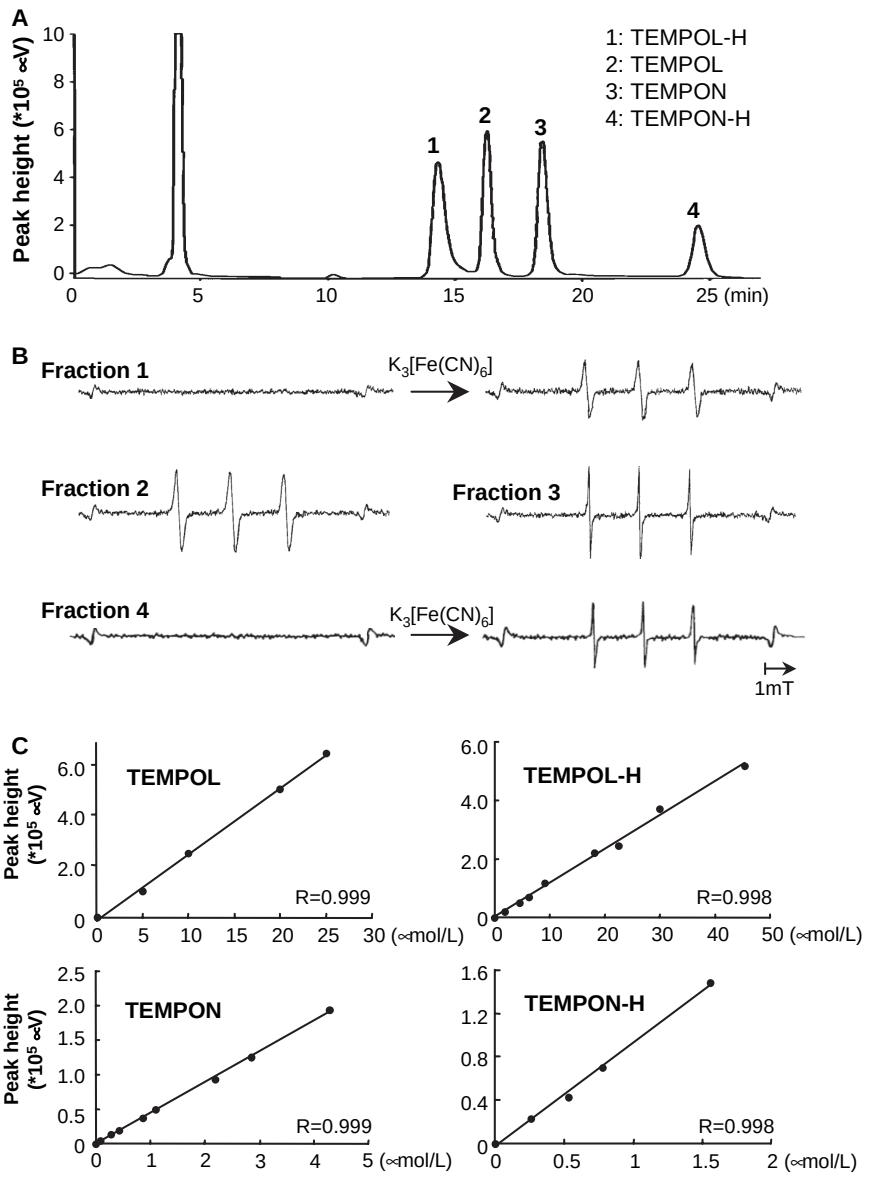

Figure 3. HPLC/ECD chromatogram of a standard solution (A), ESR spectrum of each HPLC peak (B) and concentration dependence of the HPLC/ECD signals of the standard solution (C). The conditions for HPLC/ECD or ESR are described in Materials and methods. The fractions defined by the HPLC peaks were collected and subjected to ESR measurement. The numbers of the ESR spectra correspond to the numbers of the HPLC peaks.

Identification of the reaction products resulting from the UV photolysis of H_2O_2

Figure 4 shows a typical HPLC chromatogram of the reaction mixture containing TEMPOL and H_2O_2 at pH 7.4. Small peaks of TEMPOL-H, TEMPON and TEMPON-H were observed 15 min after UV irradiation in the absence of NADH (Figure 4A). Addition of NADH to the reaction mixture caused a significant increase in TEMPOL-H signal (Figure 4B). After 15 min of reaction, the percentage of each product is shown in Figure 5. The generation of TEMPON was confirmed only at pH 7.4 and 8.0. At pH 2.2 and 5.0, TEMPON-H only was observed and not TEMPON. In the absence of NADH, the generation of TEMPOL-H was confirmed by HPLC/ECD and its percentage decreased as pH increase. In the presence of NADH, TEMPOL-H was generated significantly. The amount of undetermined products was calculated.

TEMPOL-derived reaction products during dopamine metabolism

Schematic representation of $^{\bullet}OH$ generation and typical ESR spectrum of the TEMPOL during dopamine metabolism are shown in Figure 6A. The ESR signal loss was appreciable and then the generation of corresponding hydroxylamine was also observed by HPLC/ECD (Figure 6B). A control reaction in which MAO was omitted showed no loss of TEMPOL (Figure 6B, indicated by dotted line). In this control reaction, the peaks of NADH, dopamine and TEMPOL appeared at 3.2, 10.2 and 16.2 min, respectively. After the reaction was initiated by MAO, an appreciable increase in the peak of DOPAC was observed at 8.1 min (Figure 6B, indicated by straight line). A peak caused by enzyme solution was observed at 4.9 min. The level of TEMPOL-H generation was close agreement with the decrease of TEMPOL.

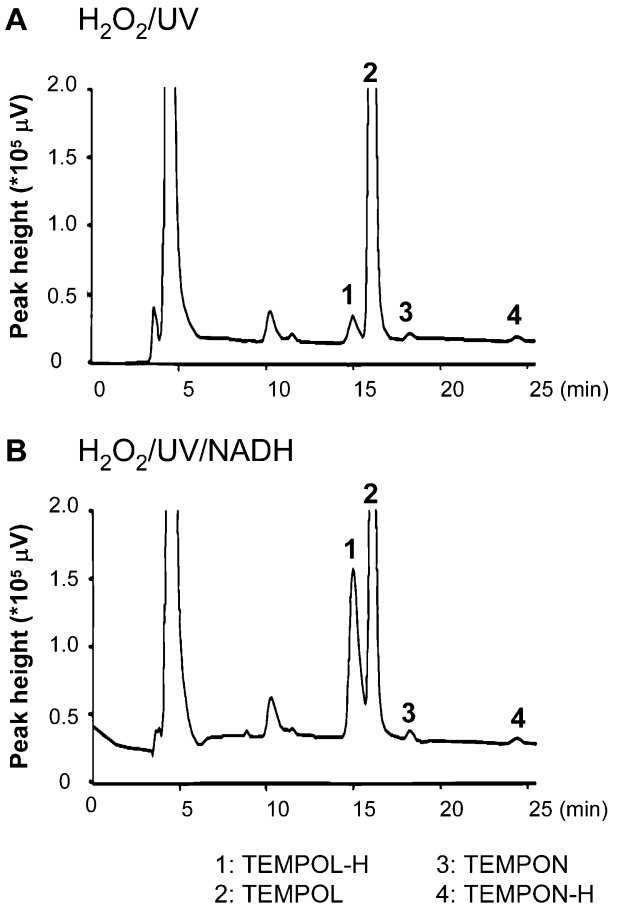

Figure 4. HPLC/ECD chromatogram of TEMPOL and its reaction products generated by the UV photolysis of H_2O_2 in the absence (A) and presence of NADH (B) at pH 7.4. The sample was subjected to HPLC analysis after UV irradiation (15 min).

Discussion

Nitroxyl radicals are considered as effective antioxidants and they have been extensively used to protect various tissues from oxidative stress. Elucidation of scavenging mechanisms is crucial for their therapeutic use. There is little information regarding interactions of nitroxyl radical with $^{\bullet}OH$ at various physiological pH. In this study, using an HPLC/ECD technique, we have identified the generation of TEMPOL-derived products such as TEMPOL-H, TEMPON and TEMPON-H in the $^{\bullet}OH$ generation system at pH 2.2–8.0. Reaction mechanisms are discussed in the following paragraphs.

The hydroxyl radical is produced by the UV photolysis of H_2O_2. According to previous reports, the corresponding hydroxylamine was not directly generated when TEMPOL reacted with $^{\bullet}OH$ [11]. However, we observed the generation of TEMPOL-H in the absence of a reducing agent (Figure 4A and 5). We realize that other reactive species may exist under our experimental conditions. For example, $^{\bullet}H$ may be generated by the reaction of H^+ with $^{\bullet}OH$ [8]. A carbonate radical anion may be also generated by the reaction of $^{\bullet}OH$ with trace dissolved carbonate ion. Therefore, $^{\bullet}H$ or a carbonate radical anion may be generated upon the UV photolysis of H_2O_2. These species are reported to cause the loss of paramagnetism by nitroxyl radicals [7,11,17,18].

TEMPON was observed at pH 7.4 and 8.0 and some was converted to the corresponding hydroxylamine (Figure 5). As one explanation for the formation of TEMPON, Saito et al. [9] suggested that TEMPON formation is triggered by the abstraction of a hydrogen atom from the 4-position of the TEMPOL molecule by $^{\bullet}OH$, similar to the abstraction of the α-hydrogen from alcohols.

In the absence of NADH, TEMPOL-H and TEMPON-H may be generated by the reaction of the nitroxyl group with $^{\bullet}H$ or carbonate radical anion. The rate constants for the reaction of

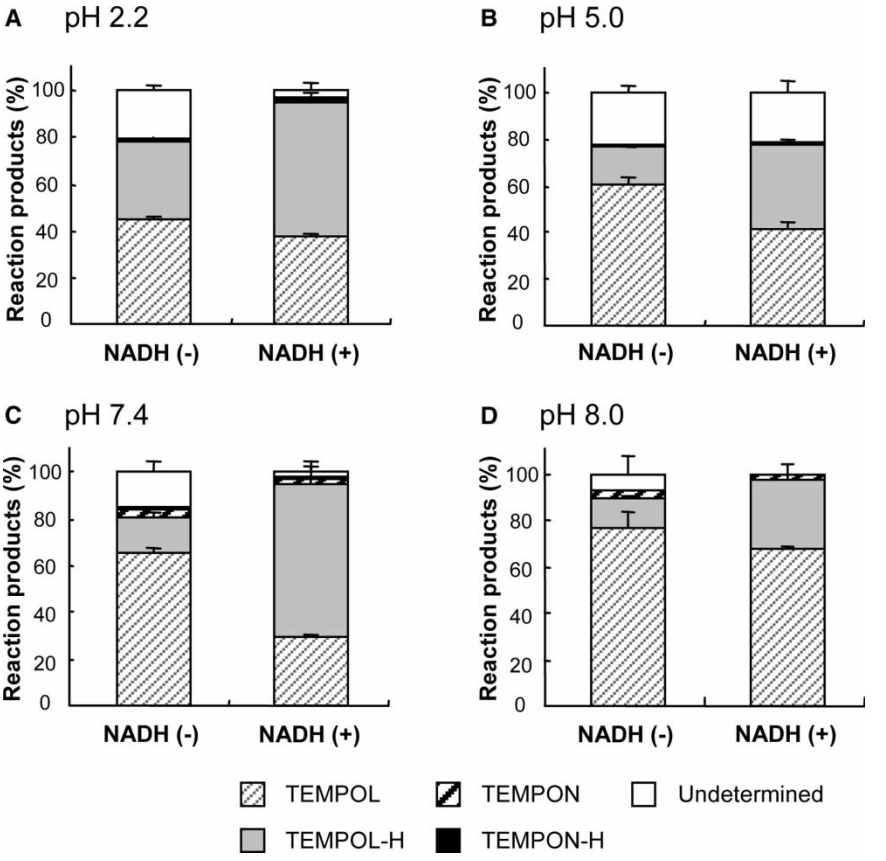

Figure 5. TEMPOL and its reaction products estimated by HPLC/ECD at pH 2.2 (A), 5.0 (B), 7.4 (C) and 8.0 (D). The samples were subjected to HPLC analysis after UV irradiation (15 min). Values are means ± SEM.

TEMPOL and TEMPON with $^{\bullet}H$ are reported to be $4.9^{\star}10^{9}$ and $8.0^{\star}10^{9}$ $m^{-1}s^{-1}$, respectively [17].

$$>N\text{-}O^{\bullet} + {}^{\bullet}H \rightarrow> N\text{-}OH \quad (1)$$

Carbonate radical anion may be involved in the generation of hydroxylamine. Hydroxyl radical is trapped by the dissolved carbonate ion, resulting in the generation of carbonate radical anion. This species reacts with the nitroxyl group and reduces it to the hydroxylamine [7].

$$>N\text{-}O^{\bullet} + {}^{\bullet}CO_2^{-} - + H^{+} \rightarrow> N\text{-}OH + CO_2 \quad (2)$$

Reactions (1) and (2) are likely to progress in acid solution, because the concentration of H^{+} is important for the chemical reaction [7,8]. In fact, more TEMPOL-H was generated at low pH (Figure 5). TEMPON was also converted to TEMPON-H in acid solution.

In the presence of NADH, in addition to the above reaction, TEMPOL may be subjected to combined oxidation and reduction. Willson [11] suggested that the oxoammonium cation might be generated by the reaction between nitroxyl group and $^{\bullet}OH$. The presence of oxoammonium cation could be confirmed by its two-electron reduction to the hydroxylamine by NADH [13]. TEMPOL may be oxidized to the oxoammonium cation by $^{\bullet}OH$ and the oxoammonium cation could be in turn reduced to TEMPOL-H by NADH, as shown in reactions (3)–(5).

$$>N\text{-}O^{\bullet} + {}^{\bullet}OH \rightarrow> N\text{-}O\text{-}OH \quad (3)$$

$$>N\text{-}O\text{-}OH \rightarrow> N^{+} = O + OH^{-} \quad (4)$$

$$>N^{+} = O + NADH \rightarrow> N\text{-}OH + NAD^{+} \quad (5)$$

We have also confirmed the formation of TEMPOL-H during dopamine metabolism (Figure 6B). As mechanisms of dopamine-induced oxidative stress, dopamine catalysed by MAO is accompanied by formation of hydrogen peroxide, which may be converted to $^{\bullet}OH$ in the presence of iron [6]. Therefore, it was proposed that TEMPOL-H was generated when TEMPOL reacted with $^{\bullet}OH$ during dopamine metabolism according to reactions (3)–(5).

In the present study, we could not determine all the reaction products, especially those generated in the absence of NADH. Candidate substances as undetermined products might be oxoammonium cations. NADH contributes to the reduction of oxoammonium cation to hydroxylamine. In fact, TEMPOL-H generation in the presence of NADH may be related to a marked decrease in the undetermined products. As another substance, Nigam et al. [18] suggested that >N-O-OH, generated through reaction (3), could be protonated to >N-O-OH $(H)^{+}$. On the

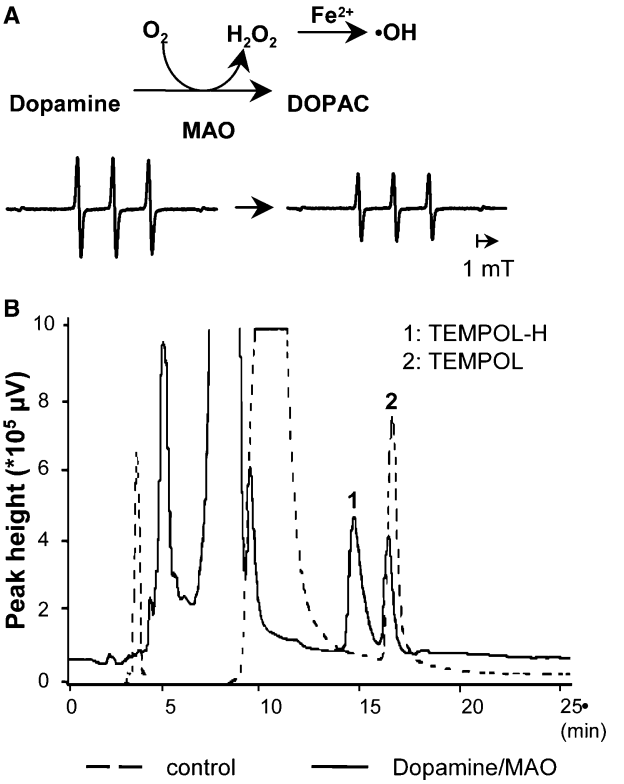

Figure 6. Schematic representation of •OH generation during dopamine metabolisms and ESR spectrum of TEMPOL (A) and HPLC/ECD chromatogram of reaction products (B). ESR spectra of nitroxyl radicals were recorded in dopamine/MAO system for 15 min and then the samples were subjected to HPLC analysis. The experimental conditions are described in Materials and methods.

other hand, we cannot explain why there was ~20% undetermined product at pH 5.0, even in the presence of NADH. The highly reactive molecule •OH might also react with a site on TEMPOL besides the hydroxyl group or the nitroxyl group. The undetermined products may be identified by future studies.

In this study, we contribute novel information about TEMPOL-derived reaction products obtained using HPLC/ECD. In the absence of NADH, TEMPOL-H may be generated by the reaction between TEMPOL and secondary •OH-derived radicals in acidic condition. In the presence of NADH, a large proportion of the non-paramagnetic products was TEMPOL-H at pH 2.2, 5.0 and 7.4. These results suggest that TEMPOL-H generation occurs at various physiological pH, including in the stomach or acidic organelles. As a potent oxidant, •OH can attack critical bio-molecules and cause cell damage. TEMPOL may function as an antioxidant *in vivo* partly through the pH-dependent scavenging mechanisms.

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References

[1] Utsumi H, Yasukawa K, Soeda T, Yamada K, Shigemi R, Yao T, Tsuneyoshi M. Noninvasive mapping of reactive oxygen species by in vivo electron spin resonance spectroscopy in indomethacin-induced gastric ulcers in rats. J Pharmacol Exp Ther 2006;317:228–235.

[2] Kato N, Yanaka K, Hyodo K, Homma K, Nagase S, Nose T. Stable nitroxide Tempol ameliorates brain injury by inhibiting lipid peroxidation in a rat model of transient focal cerebral ischemia. Brain Res 2003;979:188–193.

[3] Yamato M, Egashira T, Utsumi H. Application of *in vivo* ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. Free Radic Biol Med 2003;35:1619–1631.

[4] Liang Q, Smith AD, Pan S, Tyurin VA, Kagan VE, Hastings TG, Schor NF. Neuroprotective effects of TEMPOL in central and peripheral nervous system models of Parkinson's disease. Biochem Pharmacol 2005;70:1371–1381.

[5] Kasazaki K, Yasukawa K, Sano H, Utsumi H. Non-invasive analysis of reactive oxygen species generated in NH4OH-induced gastric lesions of rats using a 300 MHz *in vivo* ESR technique. Free Radic Res 2003;37:757–766.

[6] Hermida-Ameijeiras A, Mendez-Alvarez E, Sanchez-Iglesias S, Sanmartin-Suarez C, Soto-Otero R. Autoxidation and MAO-mediated metabolism of dopamine as a potential cause of oxidative stress: role of ferrous and ferric ions. Neurochem Int 2004;45:103–116.

[7] Madden KP, Taniguchi H. *In situ* radiolysis time-resolved ESR studies of spin trapping by DMPO: reevaluation of hydroxyl radical and hydrated electron trapping rates and spin adduct yields. J Phys Chem 1996;100:7511–7516.

[8] Shimizu M. Strong interaction between the ring system and the ionosphere of Saturn. Moon and the Planets 1980; 22:521–522.

[9] Saito K, Takeshita K, Ueda J, Ozawa T. Two reaction sites of a spin label, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), with hydroxyl radical. J Pharm Sci 2003; 92:275–280.

[10] Deffner U, Schimmack W. Letter: Radiation effects on aqueous solutions of the nitroxyl free radical TMPN (2,2,6,6-tetramethyl-4-piperidinol-N-oxyl). Int J Radiat Biol Relat Stud Phys Chem Med 1976;29:71–75.

[11] Willson RL. Reaction of triacetoneamine-N-oxyl with hydroxyl radicals. Int J Radiat Biol Relat Stud Phys Chem Med 1972;21:401–403.

[12] Takeshita K, Saito K, Ueda J, Anzai K, Ozawa T. Kinetic study on ESR signal decay of nitroxyl radicals, potent redox probes for in vivo ESR spectroscopy, caused by reactive oxygen species. Biochim Biophys Acta 2002;1573:156–164.

[13] Krishna MC, Grahame DA, Samuni A, Mitchell JB, Russo A. Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. Proc Natl Acad Sci USA 1992; 89:5537–5541.

[14] Zhdanov RI, Komarov PG. Sterically-hindered hydroxylamines as bioactive spin labels. Free Radic Res Commun 1990; 9:367–377.
[15] Bobko AA, Kirilyuk IA, Grigor'ev IA, Zweier JL, Khramtsov VV. Reversible reduction of nitroxides to hydroxylamines: roles for ascorbate and glutathione. Free Radic Biol Med 2007;42:404–412.
[16] Mcilvaine TC. A buffer solution for colorimetric comparison. J Biol Chem 1921;49:183–186.
[17] Asmus KD, Nigam S. Kinetics of nitroxyl radical reactions. A pulse-radiolysis conductivity study. Int J Radiat Biol Relat Stud Phys Chem Med 1976;29:211–219.
[18] Nigam S, Asmus KD, Willson RL. Electron transfer and addition reactions of free nitroxyl radicals with radiation induced radicals. J Chem Soc Faraday Trans 1976;72:2324–2340.