Free Rad. Res., Vol. 29, pp. 127–135
Reprints available directly from the publisher
Photocopying permitted by license only

Ozone Exposure Generates Free Radicals in the Blood Samples *In Vitro*. Detection by the ESR Spin-Trapping Technique

IKUKO UENO[a,*], MIKIO HOSHINO[a], TOSHIAKI MIURA[b] and NARIKO SHINRIKI[c]

[a] *The Institute of Physical and Chemical Research, Hirosawa, Wako-shi, Saitama, Japan;* [b] *College of Medical Technology, Hokkaido University, Sapporo, Japan;* [c] *Tsukuba Materials Information Laboratory, Sapporo Branch, 1-45-510, S1W27, Chuoh-ku, Sapporo 064-0801, Japan*

Accepted by Prof. E. Niki

(Received 26 November 1997; In revised form 1 April 1998)

Generation of free radicals in the reaction of ozone with blood samples and related salt solutions was investigated *in vitro* by using ESR spin-trapping technique with DMPO. In the reactions of low levels of ozone, a carbon-centered radical was spin-trapped with DMPO, giving rise to the 6-line ESR signal in both whole blood and blood plasma. In the blood plasma, DMPO-spin adduct of hydroxyl radical (DMPO-OH) was detected together with the spin adduct of carbon-centered radical. The present spin-trapping study demonstrates that, when exposed to ozone, 0.9% NaCl solution in the presence of DMPO gives rise to the formation of DMPO-OH. The addition effects of ethanol, which is a $^{\bullet}OH$ scavenger, into the NaCl solution reveal that DMPO-OH is produced by the reaction of DMPO with both $^{\bullet}OH$ and unidentified oxidants originated from the reaction of Cl^- and ozone. Based on these observations, we consider that $^{\bullet}OH$ is generated similarly in the blood plasma exposed to ozone. The ESR study of DMPO-spin adducts in the ozone-exposed aqueous solution of NaOCl indicates that Cl^- reacts with ozone to give ClO^-. Presumably, further oxidation of ClO^- by ozone leads to the formations of $^{\bullet}OH$ and the unidentified oxidants.

Keywords: Ozone, human blood, carbon-centered radical, hydroxyl radical, ESR spin-trapping, Cl^- effect

Ozone is known as a highly reactive oxidant to be hazardous for the life.[1] Indeed, many reports published so far indicated toxic effects of ozone on the experimental animals with induction of various diseases, especially in respiratory systems.[2–4] However, since more than 40 years ago low levels of ozone have been used as a therapeutic reagent for patients suffering from various chronic diseases in European countries.[5] In those therapeutic usages (autohemotherapy), a small volume of blood drawn from a patient was exposed to a dose of ozone gas *ex vivo*, and then, the ozone-treated blood was reinfused into the blood-stream of the donor. In our previous *in vitro* experiments human blood specimens

*Corresponding author. Research Institute for Biosciences, Science University of Tokyo, 2669 Yamazaki, Noda-city 278-0022, Japan. Tel.: +81-471-23-9772. Fax: +81-3-3938-4953.

were treated with ozone gas in the same manner as the European autohemotherapy. We found that ozone was completely consumed by the blood plasma constituents, mainly ascorbic acid, uric acid and α-tocopherol, but, these antioxidants in the plasma still remained after exposure to ozone gas.[6] No significant loss of the enzymatic functions was observed in the erythrocytes, in which a large amount of glutathione remained in the reduced form.[6] These observations consisted of reports on the interactions between ozone and tissue constituents.[3,7,8] We speculated that in the autohemotherapy ozone or ozone-derived free radicals stimulated some blood components *ex vivo*, and the stimulated components further activated cytokine network after returning back into the blood-stream of patients.

Recently, Bocci and his group reported the induced formation of interferon γ in human blood specimens *in vitro* after giving a low dose of ozone under the similar conditions as those used in the autohemotherapy.[9] This report supports our concept that ozone indirectly gives its effects on the human immune system in the autohemotherapy.

Regulation of cytokine gene expression by active oxygen species has been reported in the whole blood.[10] Chemically, ozone is a strong oxidant with generation of $^{\bullet}OH$ which exhibits radiomimetic toxicity in living cells.[11–15] Therefore, it is quite possible that free radicals are generated in the blood under exposure of ozone. We hypothesized as follows: first, free radicals are generated in the blood under exposure of ozone and the free radicals stimulate blood cells *ex vivo*, and second, the stimulated blood cells promote cytokine production of the surrounding cells after returning back to the blood-stream *in vivo*. Based on these hypotheses, we investigated, as the first step, generation of free radicals in the blood specimens under treatment of ozone by using the spin-trapping ESR technique. Here we show generation of carbon-centered and hydroxyl radicals in the blood samples together with requirement of Cl^- for the hydroxyl radical generation in aqueous solutions when they are treated with low levels of ozone *in vitro*.

MATERIALS AND METHODS

Ozone Generation

Ozone was generated from oxygen gas (99.99%) by using an ozone generator equipped with an UV (253.7 nm) monitor and a display of ozone concentration (μg of O_3/ml gas) (MEZONE M-10, Nippon Ozone Co., Tokyo, Japan). Ozone–oxygen gas mixture containing an adequate concentration of ozone was drawn from a nozzle attached to the generator by a microsyringe.

Methods

A fresh human blood sample collected in the presence of 3.8% trisodium citrate (9 : 1, v/v) was treated with ozone gas in the presence of 5,5-Dimethyl-1-pyroline-1-oxide (DMPO, Labotec Co., Tokyo, Japan) as follows: Ten μl DMPO (final concentration, 0.09 M) and 1 ml of the anti-coagulant-containing blood sample (designated as blood) were sequentially put in a glass tube (10 ml, Venoject tube, Termo Co., Tokyo, Japan). The tube was sealed with a silicone rubber cap. After drawing 1.2 ml of the air from the tube by using an injection syringe, 1 ml of the ozone–oxygen gas mixture quickly drawn from the ozone generator into a microsyringe was immediately injected into the tube. The ozone and blood in the tube were quickly mixed avoiding the foam formation by horizontal rotation of the tube for 30 s. In the quantitative experiments conducted previously,[6] we confirmed that the ozone concentrations displayed on the ozone generator agreed well to those quantitated by the KI method[16] in a neutral phosphate buffer with a regression coefficient of 1.010. Complete consumption of 100 μg of ozone in 1 ml of blood by the 30 s mixing adopted here was also confirmed by the KI method[16] in the previous experiments. Thus, we adjusted the ozone

concentration to 20–100 μg (0.416–2.08 μmol/ml of the ozone–oxygen gas mixture in the ozone generator, and mixed 1 ml of the blood with 1 ml of the ozone–oxygen gas mixture to give 20–100 μg of ozone/ml blood in the tube. In some experiments, DMPO was added after the treatment of blood with ozone. Samples of blood plasma, phosphate buffered saline (PBS), NaCl and NaOCl were treated with ozone in the same manner as in the case of the blood. The sample thus treated with ozone was transferred to a flat quartz ESR cuvette (160 μl), which set to the cavity of the ESR spectrometer (JEOL FE-1X with 100 kHz field modulation, X-band). Recordings of the spectra started 1 min after the addition of ozone–oxygen gas to the sample in the glass tube at 24°C. Each scan took 2 min. The ESR spectra of the DMPO-spin adducts were identified based on the hyperfine splitting constants reported previously.[17,18]

RESULTS

Each 1 ml of blood sample was mixed with ozone gas for 30 s in a sealed tube containing 0.09 M DMPO followed by transferring the mixture to the ESR cuvette. Recordings of the ESR spectra started just 1 min after the addition of ozone to the blood with 2 min sweep time. Figure 1 shows an ESR spectrum of the blood mixed with 40 μg (0.83 μmol) of ozone. Main signal of DMPO-spin adduct composed of six lines with hyperfine splitting constants of $A_N = 15.6$ G and $A_{\beta H} = 23.3$ G. The blood sample without ozone treatment showed no signal in the presence of DMPO (data not shown). All the blood samples mixed with varied doses from 10 to 100 μg (0.208–2.08 μmol) of ozone showed the same six lines as observed in Figure 1 but with varied intensities (data not shown).

In order to investigate the correlation between the dose of ozone and generation of the DMPO-spin adduct, intensities of the first (extreme left-hand peak) signal of the ESR spectra, recorded for the first 2 min, were plotted against the dose of ozone. As shown in Figure 2, the intensity of DMPO-spin adduct increased with the dose of ozone until at 20 μg of ozone/ml blood, followed by almost constant level thereafter, to 80 μg of ozone. These data suggest that a specific component(s) present in the whole blood reacts with ozone or ozone-derived radical to give the carbon-centered radical(s), leading to the

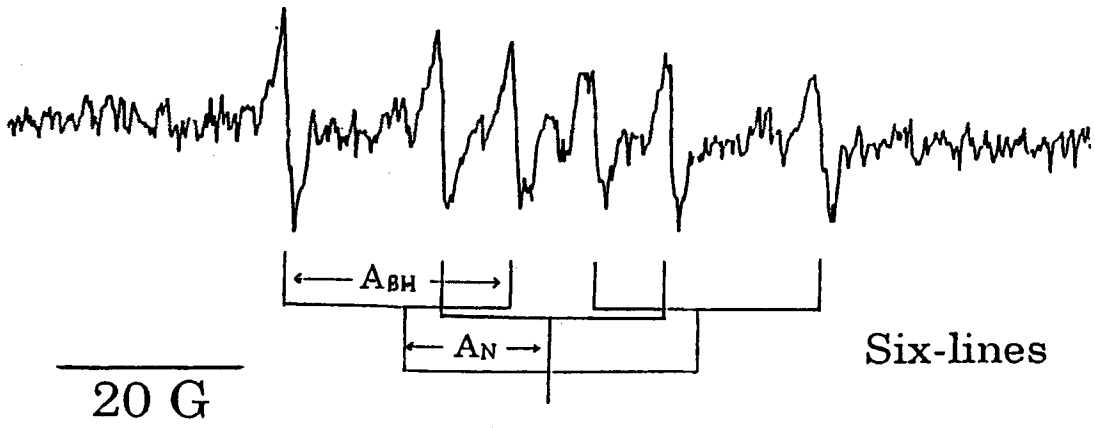

FIGURE 1 ESR spectrum of DMPO-spin adduct formed in whole blood mixed with ozone gas in the presence of DMPO. One ml of blood was mixed with 40 μg of ozone in the presence of 10 μl of DMPO (final concentration, 0.09 M) in a sealed tube (10 ml) for 30 s. Then, the sample was transferred to a quartz flat ESR cuvett (160 μl), which was fixed in the cavity. Recording of the ESR spectrum started 1 min after mixing blood with ozone and took 2 min. Other experimental conditions were noted in the Methods.

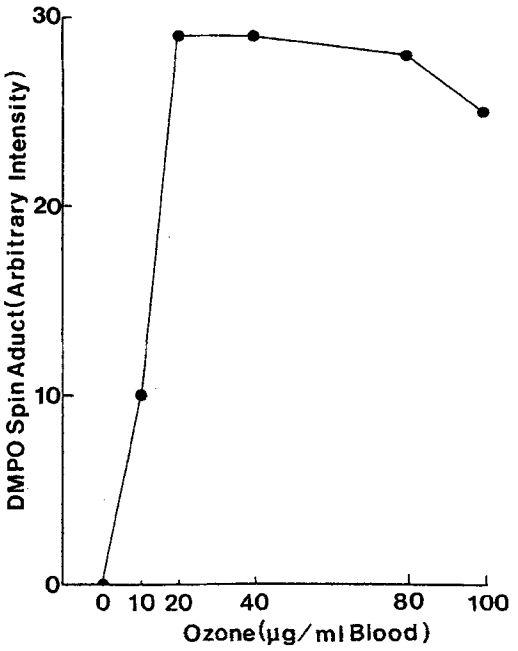

FIGURE 2 Effect of the dose of ozone on the formation of DMPO-spin adduct in the whole blood. Each 1 ml of blood was mixed with ozone (20, 40, 60, 80, or 100 μg) in the presence of 0.09 M DMPO, as done in Figure 1. After recording the ESR spectra of each samples intensities of the first signal of each spectra were plotted against the dose of ozone.

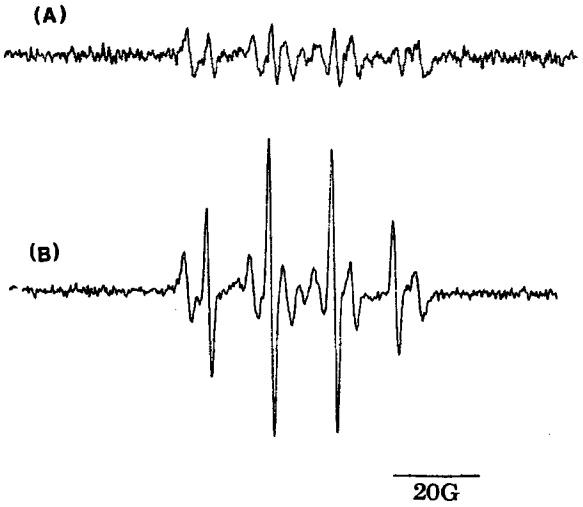

FIGURE 3 ESR spectra of DMPO-spin adducts formed in the blood plasma mixed with different amounts of ozone gas in the presence of DMPO. Each 1 ml of blood plasma was mixed with either 40 μg (A) or 60 μg (B) of ozone in the presence of DMPO. Other experimental conditions were same as in Figure 1.

production of DMPO-spin adduct with the 6-line signal. These data also indicated that the reactive component(s) counterbalanced with 20 μg of ozone and excess ozone consumed by other antioxidants without formation of radicals. When ozone (100 μg) was first mixed with blood in the absence of DMPO and then DMPO was added 2 or 15 min thereafter, ESR spectrum showed the 6-line signal as in the case of mixing ozone with blood in the presence of DMPO (data not shown). We presumed that in the reaction between blood component(s) and ozone, some long-lived ozonide(s) or radical(s) was generated and after decomposition of the ozonide(s) to radical, it was trapped by DMPO with production of DMPO-spin adduct having the 6-line signal. From the hyperfine splitting constants of the signal ($A_N = 15.6$ G, $A_{\beta H} = 23.3$ G) we assigned the 6-line signal to the DMPO-spin adduct of carbon-centered radical.

In order to make clear whether the radicals trapped by DMPO in the whole blood were generated in the plasma fraction of the blood, we examined ESR spectra of DMPO-spin adducts formed in the plasma each mixed with 40 and 60 μg of ozone in the presence of DMPO. As shown in Figure 3A and B, in both cases two spectra, each composed of six lines ($A_N = 15.6$ G, $A_{\beta H} = 23.3$ G) and four lines ($A_N = A_{\beta H} = 15.0$ G), were observed in the ESR spectra. However, at 60 μg of ozone the 4-line signal was more prominent. These data indicated that when plasma was treated with ozone the same radical as that trapped in the whole blood was generated. However, another radical species which showed the 4-line signal was possibly generated.

In Figure 4, the intensities of the first signal of the two DMPO-spin adducts were plotted against the dose of ozone. In the doses less than 40 μg of ozone, the intensities of both spectra were similar to each other, however, in the doses at 60 μg or higher, the intensity of the 4-line signal increased with the rate twice larger than that of the 6-line signal intensity. Thus, in the blood plasma some

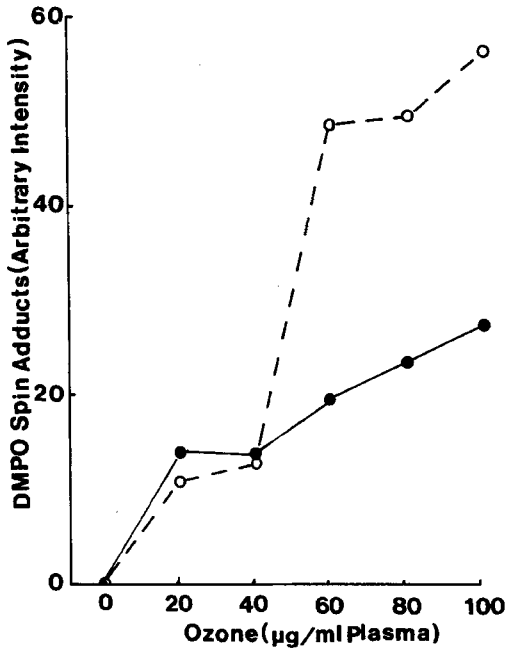

FIGURE 4 Correlation between the formation of DMPO-spin adducts in the blood plasma and the dose of ozone. Each 1 ml of blood plasma was mixed with ozone (20, 40, 60, 80 or 100 μg) in the presence of 0.09 M DMPO. After recording the ESR spectra of each samples, intensities of the first signals of each spectra were plotted against the ozone dose. (•) DMPO-spin adduct of carbon-centered radical. (○) DMPO-OH.

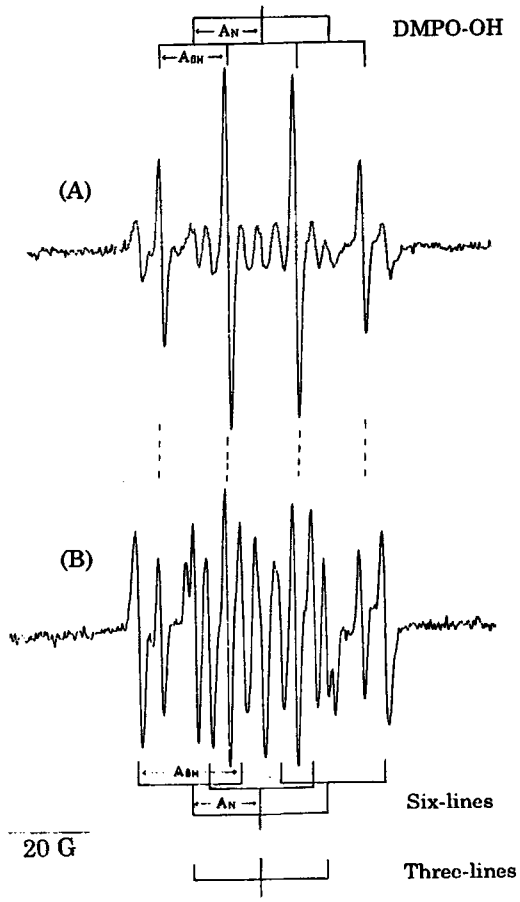

FIGURE 5 ESR spectra of DMPO-spin adducts formed in the saline solutions exposed to ozone gas in the presence or absence of ethanol together with DMPO. One ml of 0.9% NaCl solution added with (A) or without (B) ethanol (final concentration, 0.35 M) was exposed to 1 ml of ozone gas (90 μg) in the presence of DMPO. Other experimental conditions were as same as in Figure 1.

chemical component which gave the 4-line signal in ESR spectra was generated together with the carbon-centered radical in the reaction with ozone. The component with 4-line signal significantly increased with increasing ozone dose. When the plasma sample was treated with ozone in the absence of DMPO, and then DMPO was added, the ESR spectrum showed only the 6-line signal (data not shown), indicating a short life-time of the component which formed DMPO-spin adduct with the 4-line signal. Because the DMPO-spin adduct showed the ESR spectrum consisted of the 1:2:2:1 quartet signal with hyperfine splitting constants ($A_N = A_{\beta H} =$ 15.0 G), we assigned the adduct to DMPO-OH.

In order to find out the mechanism of generations of the radicals observed in the ozone-mixed plasma fraction, PBS solution, instead of blood plasma, was treated with ozone in the presence of DMPO. A prominent signal of DMPO-OH appeared as in Figure 3B (data not shown). When 0.9% NaCl solution was used, instead of PBS, the same 4-line signal of DMPO-OH was observed together with small signals probably composed of 6 and 3 lines, respectively (Figure 5A). These data indicated that in all the Cl^--containing solutions DMPO-OH was generated from DMPO in the presence of ozone.

However, detection of DMPO-OH does not necessarily give the proof of the formation of •OH.[18,19] It is well known that •OH reacts with ethanol by hydrogen atom abstraction to form α-hydroxy ethyl radical which reacts with DMPO yielding spin adduct of the carbon-centered radical with the 6-line ESR signal.[18,20] Therefore, in order to verify that the DMPO-OH was generated in the spin-trapping reaction of •OH with DMPO, that is, •OH was surely generated in the reaction, ozone gas was mixed with the NaCl solution in the presence of 0.35 M ethanol together with DMPO. As shown in Figure 5B, in the presence of ethanol the 6-line signal was higher than that in the absence of ethanol in Figure 5A, indicating that at least a part of DMPO-OH observed in the NaCl solution (Figure 5A) was formed from •OH by the spin-trapping reaction; generation of •OH was confirmed in the ozone-treated NaCl solution, though the signal height of DMPO-OH was lowered only a little (Figure 5B). DMPO-containing water showed no ESR signals by the addition of ozone (data not shown). Thus, in the presence of Cl^-, •OH was generated in the aqueous solutions treated with ozone.

Bernofsky *et al.*[21] reported DMPO-OH formation in the reaction of 7×10^{-2} M NaOCl and DMPO. We assumed that ozone oxidized NaCl to NaOCl even though the rate constant is very low ($3 \times 10^{-3}\,M^{-1}\,s^{-1}$),[22] and the produced NaOCl was responsible for generation of the DMPO-spin adducts observed in Figure 5A. To prove this hypothesis, NaOCl solution was mixed with ozone in the presence of DMPO.

As shown in Figure 6, the ESR spectrum observed in the ozone-treated NaOCl solution (0.0006%, 8×10^{-5} M) was almost identical to that obtained in the ozone-treated NaCl solution (0.9%, 0.154 M) (Figure 5A). It consists of a marked signal of DMPO-OH and signals of six and three lines. In the absence of ozone, the solution containing NaOCl and DMPO showed no significant signals (data not shown), indicating that DMPO-OH was formed in the ClO^- solution at the concentration far lower than that of NaCl, and requirement of further oxidation of ClO^- by ozone for the formation of DMPO-OH.

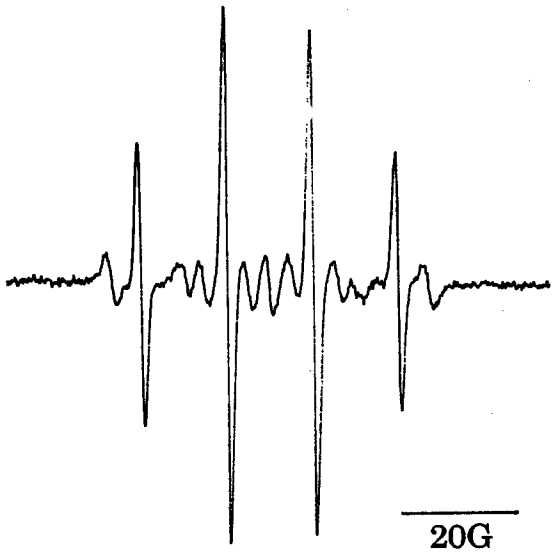

FIGURE 6 ESR spectra of DMPO-spin adducts formed in the NaOCl solution exposed to ozone gas in the presence of DMPO. One ml of 0.0006% NaOCl solution was exposed to 1 ml of ozone gas (90 μg) in the presence of DMPO. Other experimental conditions were same as in Figure 1.

DISCUSSION

When ozone was given to the whole blood or its plasma fraction in the presence of DMPO, DMPO-spin adduct with a spectrum composed of 6 lines ($A_N = 15.6$ G, $A_{\beta H} = 23.3$ G) was detected by ESR. The 6-line signal was very similar to that reported by Makino *et al.*[23] in the rabbit serum incubated at 37°C in the presence of DMPO. They also demonstrated formation of the 6-line signal by incubating linolenic acid with DMPO. The same 6-line signal was also reported in the UV-irradiated linolenic acid by Fujita *et al.*[24] These data indicated that, when certain unsaturated fatty acids were oxidized, intermediarily produced carbon-centered radicals were able to be detected by ESR spin-trapping technique. In the case of ozone exposure, generation of free radical was also detected

by ESR measurement in linoleic acid by Goldstein *et al.*[25] In their report at least 3 h was taken before the appearance of free radical, suggesting ozonization of the fatty acid followed by decomposition of the ozonide to free radical. Free radical production from ozonized unsaturated fatty acids was also reported by Pryor *et al.*[13] Blood plasma contains more than hundred nmol/ml of unsaturated fatty acids. We presumed that some plasma component(s), probably unsaturated fatty acid(s), reacted with ozone to form ozonide(s), which then decomposed to free radical(s). Because the 6-line signal of the spin adduct was detected even DMPO was added 15 min after mixing of ozone to the plasma, we supposed that the ozonide(s) or free radical of the unsaturated fatty acid(s) kept itself stably for a while in the blood plasma. Based on these presumptions we assigned the ESR spectrum with 6-line signal obtained in our plasma experiments to the DMPO-spin adduct of carbon-centered radical(s).

It has been reported that superoxide anion and hydroxyl radicals are generated in the ozonated water.[26] In the present experiments, neither the spin adduct of superoxide anion nor that of hydroxyl radical was detected in the whole blood in the presence of ozone, instead, DMPO-spin adduct of carbon-centered radical(s), was detected, as described above. However, when plasma fraction was used, instead of whole blood, DMPO-OH was formed together with the DMPO-spin adduct of the carbon-centered radicals. In higher doses of ozone larger amounts of DMPO-OH were generated. Presumably, the major amount of DMPO-OH in the ozone-treated plasma is produced by the reaction of DMPO and unidentified oxidants generated from ClO^- and ozone, as will be discussed later.

According to Makino *et al.*[23] $^{\bullet}OH$ was generated in the process of lipid peroxidation and trapped by DMPO as DMPO-OH. However, DMPO-OH formations were reported in various reactions of DMPO other than spin-trapping of $^{\bullet}OH$.[18] In the present experiments not only in the blood plasma but also in PBS and saline, all of them contained Cl^-, DMPO-OH was formed when they were treated with ozone in the presence of DMPO. We verified the generation of $^{\bullet}OH$ in the Cl^--containing solutions by showing appearance of DMPO-spin adduct of carbon-centered radical together with decreased formation of DMPO-OH in the ethanol added saline solution. We concluded that in the blood plasma at least a part of ozone decomposed with production of $^{\bullet}OH$, which was trapped by DMPO with formation of DMPO-OH.

In the whole blood treated with ozone, DMPO-OH was not detected as mentioned above. As demonstrated in the previous experiments[6] as well as other reports[27,28] very high concentrations of antioxidants including ascorbic acid, uric acid and α-tochopherol, are present in the blood plasma. No significant damage of the erythrocyte functions were observed. As described in the methods, ozone less than 100 μg (2.08 μmol) was completely consumed by 1 ml of whole blood by 30 s mixing. It is known that plasma components such as proteins, unsaturated fatty acids, nucleotides and amino acids have antioxidant properties. They are all highly reactive with ozone and their total amounts (more than 2.9 μmol[28]) in 0.5 ml of the plasma fraction (corresponding to 1 ml of the whole blood) are enough to react with 100 μg (2.08 μmol) of ozone. Therefore, when ozone gas comes into the blood major part of the ozone reacts first with those plasma antioxidants without generation of radicals. Among the rest of ozone, certain part reacts with unsaturated fatty acids to form ozonides, which decompose to peroxide radicals, as detected as the 6-line signal of DMPO-spin adduct. Some part directly decomposes to $^{\bullet}OH$ in the plasma. Lacked observation of the $^{\bullet}OH$ in the whole blood might be due to the immediate reaction of $^{\bullet}OH$ with other organic substances on the surface of the platelet and/or cells. After these reactions if ozone still remains it reacts with platelet and/or cells. In the present study, the dose of ozone given to the whole blood is almost identical with that utilized in

the autohemotherapy. Even though the concentration of the dosed ozone was higher than that usually used in the ozone exposure experiments, only a very minor amount of ozone passed through the antioxidant-defense system might react with the platelet and/or cells.

We observed predominant formation of DMPO-OH in the blood plasma when treated with a rather high dose of ozone (60 μg/ml of plasma). From the observed formation of $^{\bullet}OH$ in the NaCl solution exposed to ozone (Figure 5) we estimate that at least some part of the DMPO-OH detected in the blood plasma reflects the $^{\bullet}OH$ formation in the blood plasma. Under exposure to ozone DMPO-OH was formed not only in the blood plasma, PBS and saline, but also in the diluted NaOCl solution. These observations suggest that oxidation of Cl^- by ozone contributes to the formation of DMPO-OH. Janzen *et al.*[29] postulated HOCl as a source of DMPO-OH produced in the respiratory burst of neutrophils and Bernofsky *et al.*[21] demonstrated the DMPO-OH formation through non-radical reactions between NaOCl and DMPO. Our present experiments indicated further oxidation of OCl^- to some radical such as $ClO^{\bullet}$ is required to form DMPO-OH. This observation is supported by the report of $BrO^{\bullet}$ formation in the reaction of BrO^- and $^{\bullet}OH$ derived from ozone.[30] Thus, in the blood plasma, PBS, and saline solutions exposed to ozone, DMPO-OH might also be produced in a reaction of DMPO with some unidentified Cl-containing radical such as $ClO^{\bullet}$ through the formation of ClO^-. In all the Cl^--containing solutions a small signal with three lines was detected in ESR spectra. It was not identified in the present experiments, however.

In the present experiments, the 6-line signal was also observed in PBS and NaOCl solutions without adding organic compounds other than DMPO in the presence of ozone. Similar spin adduct with the 6-line signal was reported in dimeric structure of DMPO or DMPO condensed with its derivative.[31] The 6-line signal was not observed in the simple aqueous DMPO solution without NaCl in the presence of ozone (data not shown), and therefore, the signal observed in the NaCl-containing solutions seems to be due to the compound produced from DMPO by dimerization and/or decomposition in the presence of NaCl.

In summary, the present work demonstrated the generation of two radical species in the whole blood exposed to ozone. One is carbon-centered radical(s) which is detected as DMPO-spin adduct with an ESR signal composed of six lines both in the whole blood and its plasma fraction. This carbon-centered radical is likely to be generated in the reaction of plasma components, probably unsaturated fatty acids, with ozone. Another is $^{\bullet}OH$ which is detected as DMPO-OH in the blood plasma. The detection of $^{\bullet}OH$ in the saline exposed to ozone indicates that Cl^- is essential for the generation of $^{\bullet}OH$ in the ozone-exposed aqueous solutions and blood plasma.

References

[1] M.A. Mehlman and C. Boreck (1987) Toxicity and biochemical mechanisms of ozone. *Environmental Research*, **42**, 36–53.

[2] B.D. Goldstein, B. Pearson, C. Lodi, R.D. Buckley and O.J. Balchum (1968) The effect of ozone on mouse blood *in vivo*. *Archives of Environmental Health*, **16**, 648–650.

[3] R.D. Buckley, J.D. Hackney, K. Clark and C. Posin (1975) Ozone and human blood. *Archives of Environmental Health*, **30**, 40–43.

[4] M. Yanai, T. Ohrui, T. Aikawa, H. Okayama, K. Sekizawa, K. Maeyama, H. Sasaki and T. Takishima (1990) Ozone increases susceptibility to antigen inhalation in allergic dogs. *Journal Applied Physiology*, **68**, 2267–2273.

[5] R. Viebahn (1994) The use of ozone in medicine, 2nd revised edition, translated by A. Lee. Karl F. Haug Publishers, Heidelberg.

[6] N. Shinriki, T. Suzuki, K. Takama, K. Fukunaga, S. Ohgiya, K. Kubota and T. Miura (1998) Susceptibilities of plasma antioxidants and erythrocyte constituents to low levels of ozone. *Haematologia*, **29**(3) (in press).

[7] D.B. Menzel and R. Wash (1971) Oxidation of biologically active reducing substances by ozone. *Archives of Environmental Health*, **23**, 149–153.

[8] J.N. Roehm, J.G. Hadley and D.B. Menzel (1972) The influence of vitamine E on the lung fatty acids of rats exposed to ozone. *Archives of Environmental Health*, **24**, 237–242.

[9] V. Bocci, and L. Paulesu (1990) Studies on the biological effects of ozone: 1. Induction of interferon γ on human leucocytes. *Haematologica*, **75**, 510–515.

[10] D.G. Remick and L. Villarete (1996) Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. *Journal Leukocyte Biology*, **59**, 471–475.

[11] W.A. Pryor and D.F. Church (1991) The reaction of ozone with unsaturated fatty acids: aldehydes and hydrogen peroxide as mediators of ozone toxicity. In: *Oxidative Damage and Repair; Chemical, biological and medical aspects* (ed. K.J.A. Davies) Pergamon Press, Oxford, pp. 496–504.

[12] National Research Council. Committee on Medical and Biologic Effects of Environmental Pollutant (1977) Ozone and other photochemical oxidants, National Academy of Sciences, Washington, DC, pp. 340–387.

[13] W.A. Pryor, D.G. Prier and D.F. Church (1981) Radical production from the interaction of ozone and PUFA as demonstrated by electron spin resonance spin-trapping techniques. *Environmental Research*, **24**, 42–52.

[14] W.A. Pryor, D.G. Prier and D.F. Church (1983) Detection of free radicals from low-temperature ozone-olefin reactions by ESR spin trapping: evidence that the radical precursor is a trioxide. *Journal of American Chemical Society*, **105**, 2883–2888.

[15] W.A. Pryor, M.M. Dooley, D.F. Church and F. Daniel (1983) Mechanisms for the reaction of ozone with biological molecules: the source of the toxic effects of ozone. *Advanced Modern Environmental Toxicology*, **5**, 7–19.

[16] B.E. Saltzman and N. Gilbert (1959) Iodometric microdetermination of organic oxidants and ozone; Resolution of mixtures by kinetic colorimetry, *Analytical Chemistry*, **31**, 1914–1920.

[17] B.E. Britigan, M.S. Cohen and G.M. Rosen (1987) Detection of the production of oxygen-centered free radicals by human neutrophils using spin trapping techniques: A critical perspective. *Journal of Leukocyte Biology*, **41**, 349–362.

[18] E. Finkelstein, G.M. Rosen and E. Rauckman (1980) Spin trapping of superoxide and hydroxyl radical: practical aspects. *Archives of Biochemistry and Biophysics*, **200**, 1–16.

[19] W. Chamulitrat, H. Iwahashi, D.J. Kelman and R.P. Mason (1992) Evidence against the 1:2:2:1 quartet DMPO spectrum as the radical adduct of the lipid alkoxyl radical, *Archives of Biochemistry and Biophysics*, **269**, 645–649.

[20] G.E. Adams and P. Wardman (1977) The pulse radiolysis approach. In: *Free Radicals in Biology*, Vol. 3, (ed. W.A. Pryor). Academic press, New York, pp. 53–95.

[21] C. Bernofsky, B.M.R. Bandra and O. Hinojosa (1990) Electron spin resonance studies of the reaction of hypochlorite with 5,5-dimethyl-1-pyrroline-N-oxide. *Free Radical Biology and Medicine*, **8**, 231–239.

[22] J. Hoigné, H. Bader, W.R. Haag and J. Staehelin (1985) Rate constants of reactions of ozone with organic and inorganic compounds in water – III. Inorganic compounds and radicals. *Water Research*, **19**, 993–1004.

[23] K. Makino, H. Imaishi, S. Morinishi, T. Takeuchi and Y. Fujita (1986) An ESR study on lipid peroxidation process. Formation of hydrogen atoms and hydroxyl radicals. *Biochemical and Biophysical Research Communications*, **141**, 381–386.

[24] Y. Fujita (1987) Radicals observed in the rat brain mitochondria by spin trapping method. *Free Radicals in Clinical Medicine*, **2**, 39–49 (in Japanese).

[25] B.D. Goldstein, O.J. Balchum, H.B. Demopoulos and P.S. Duke (1868) Electron paramagnetic resonance spectroscopy. Free radical signals associated with ozonization of linoleic acid. *Archives of Environmental Health*, **17**, 46–49.

[26] H.D. Grimes, K. Perkins and W.F. Boss (1983) Ozone degrades into hydroxyl radical under physiological conditions. *Plant Physiology*, **72**, 1016–1020.

[27] C.E. Cross, P.A. Motchnik, B.A. Bruener, D.A. Jones, H. Kaur, B.N. Ames and B. Halliwell (1892) Oxidative damage to plasma constituents by ozone. *FEBS Letters*, **298**, 269–272.

[28] Constituents of body fluids In: *Biochemical Data Book I*, (ed. editorial committee of Japanese Biochemical Society), Kagaku Dojinn, Tokyo, 1979, pp. 1541–1565.

[29] E.G. Janzen, L.T. Jandrisits and D.L. Barber (1987) Studies on the origin of the hydroxyl spin adduct of DMPO produced from the stimulation of neutrophils by phorbol-12-myristate-13-acetate. *Free Radical Research Communications*, **4**, 115–123.

[30] U. von Gunten and J. Hoigné (1994) Bromate formation during ozonation of bromide-containing waters: Interaction of ozone and hydroxyl radicals. *Environmental Science and Technology*, **28**, 1234–1242.

[31] E. Finkelstein, G.M. Rosen, E. Rauckman and J. Paxton (1979) Spin trapping of superoxide. *Molecular Pharmacology*, **16**, 676–685.