Evaluation of Superoxide Anion Radicals Generated from an Aqueous Extract of Particulate Phase Cigarette Smoke by Electron Spin Resonance Using 5,5-Dimethyl-1-pyrroline-N-oxide

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The reactive oxygen species generated by an aqueous extract of the particulate phase of cigarette smoke were evaluated by an electron spin resonance (ESR) analysis, using spin-trapping agents, and by comparing with model reaction systems. The ESR signals of DMPO-OH were detected from the extract by using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). These signals were eliminated by adding superoxide dismutase, but hardly by catalase. These responses of the ESR signals to the scavengers were similar to those of a hypoxanthine-xanthine oxidase system. The results indicate that the signals of DMPO-OH from the extract were derived from a reaction product of DMPO with superoxide anion radicals and clarify the mechanism by which the extract generated superoxide anion radicals.

Key words: cigarette smoke; superoxide anion radical; hydroxyl radical; electron spin resonance

Such reactive oxygen species as superoxide anion radicals and hydrogen peroxide have been detected in an aqueous extract of cigarette smoke by a colorimetric method. These species have been associated with some biological activities of cigarette smoke. We have already established a method for quantitatively analyzing hydrogen peroxide which was applicable to the aqueous extract of cigarette smoke. Hydrogen peroxide is a stable compound under physiological conditions in the absence of metals; however, the quantitative results strongly depended on the procedure used for sample preparation, especially the extraction time. This dependence indicates that hydrogen peroxide was ‘generated’ during extraction with an aqueous solution.

Superoxide anion radicals and hydroxyl radicals, which are short-lived free radicals, are not directly detected by the analytical methods generally applied for stable compounds such as chromatography. The colorimetric method was used for the initial detection of superoxide anion radicals in an aqueous extract of cigarette smoke, using nitro blue tetrazolium. While the colorimetric method is widely used, it is necessary to consider that the cigarette smoke matrix contains more than 4,000 compounds, including such redox-active compounds as phenolics, which may affect the analytical results.

Electron spin resonance (ESR), using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trapping agent, has been widely used for detecting the free radicals generated in cigarette smoke. Many types of DMPO adducts were detected in an earlier study on an aqueous extract of the particulate phase of cigarette smoke; however, in the absence of EDTA, the intensity of the signals of DMPO-OH in the extract was only slightly different from the solution without the extract. Other reports have stated that the ESR signals of a DMPO adduct including DMPO-OOH were detected in the aqueous extract of particulate phase cigarette smoke and were eliminated by adding superoxide dismutase (SOD); however, the extract would have been modified in the presence of DMSO with a high concentration (7 m). Although the signals of DMPO-OH have been detected in the aqueous extract in the absence of DMSO, the effect of SOD without additional Fe2+ was not discussed. The signals of DMPO-OH have also been detected as evidence for hydroxyl radicals in an aqueous solution of the organic extract residue of whole smoke; however, the constituents of the extract would have been substantially modified during sample preparation. There was also the contradiction of detecting DMPO-OH as evidence for hydroxyl radicals regardless of the signals being eliminated by SOD and partly reduced by catalase; this contradiction was not discussed at all. The detection of DMPO-OOH has been reported when the whole smoke was bubbled into a DMPO solution (4 m), however, compounds in the vapor phase would have caused different results and the high concentration of DMPO could have acted as an organic solvent.

Although these reports suggest that the aqueous extract of cigarette smoke generated superoxide anion radicals and hydroxyl radicals, the results and discussions from independent studies completely differ from each other. The mechanism for generating superoxide anion radicals, hydrogen peroxide and hydroxyl radicals has thus remained unclear. The major reason for this is that the analytical techniques for detecting the super-
oxide anion radicals and hydroxyl radicals applicable to a smoke extract have not been well examined.

We have attempted in this present study to clarify the mechanism for generating superoxide anion radicals, hydrogen peroxide and hydroxyl radicals by cigarette smoke by analyzing an aqueous extract of the particulate phase of cigarette smoke by ESR using DMPO. Some scavengers were added to the extract, and the results were compared with those from a model reaction system generating superoxide anion radicals and hydroxyl radicals to clarify the relationship between the species and the detected DMPO adducts. Possible sources of the superoxide anion radicals will also be discussed.

Materials and Methods

Materials. DMPO (LM-2110) was obtained from Labotec (Tokyo, Japan). Hypoxanthine (HPX, >99%), xanthine oxidase (XOD, from buttermilk, 0.07 units/mg) and catalase (C10; from bovine liver, lyophilized powder, 13,500 units/mg) were obtained from Sigma (St. Louis, MO, USA). Superoxide dismutase (SOD; Cu/Zn, from bovine erythrocytes, 2,100 units/mg) was obtained from Wako Pure Chemicals (Osaka, Japan), and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). All other reagents were of guaranteed reagent grade. A 10 mM phosphate buffer (pH 7.4) was used as a buffering solution in all the experiments.

All the cigarettes used in this study were of the 3R4F reference type obtained from the University of Kentucky (Lexington, KY, USA). Each of the cigarettes generated approximately 10 mg of the particulate phase of cigarette smoke. All the cigarettes were conditioned according to ISO 3402, being maintained at 60 ± 2.5% relative humidity and 22 ± 1 °C for at least 48 h prior to being smoked.

Smoking conditions. Tar from three 3R4F cigarettes was collected on a 44-mm glass fiber filter (Borgwaldt, Hamburg, Germany) by using an RM20 smoking machine (Borgwaldt, Hamburg, Germany). Each cigarette was smoked according to ISO 3308 which requires a puff volume of each cigarette of 35 mL with a duration of 2 s and an interval of 60 s in an airflow surrounding the cigarette of 200 ± 30 mm/s.

Preparation of the aqueous extract of the particulate phase of cigarette smoke. The particulate phase of cigarette smoke that had been collected on a glass fiber filter was extracted with 15 mL of the buffer at 37 °C for 90 min. The extract was passed through a Minisart RC15 0.45-μm filter (Sartorius, Tokyo, Japan). The filtered extract was subjected to an ESR analysis within an hour after being kept on ice. No chelating agents were added to the extract so that the generation of reactive oxygen in the extract, which may have contained a small amount of metals, could be evaluated.

ESR analysis using DMPO. Approximately 100 mg of DMPO was diluted with 900 mL of the buffer. A 100 mM amount of the diluted DMPO was mixed with 250 μL of the extract and 250 μL of the buffer; the final concentration of DMPO was approximately 160 mM. An LLC-22/C6 smoking machine (Borgwaldt, Hamburg, Germany). Each of the cigarettes generated approximately 10 mg of the particulate phase of cigarette smoke by ESR using DMPO. Some scavengers were added to the extract, and the results were compared with those from a model reaction system generating superoxide anion radicals and hydroxyl radicals to clarify the relationship between the species and the detected DMPO adducts. Possible sources of the superoxide anion radicals will also be discussed.

Additional variety of scavengers to the extract and the model reaction systems. The experiments with SOD used 10 μL of the solution (1.050 units/mL) added instead of the same amount of the buffer in advance; the final concentration of SOD was 18 units/mL. With catalase, 50 μL of the solution (1.350 units/mL) was added instead of the same amount of the buffer; the final concentration of catalase was 112 units/mL. With mannitol, 100 μL of the solution (1 mM) was added instead of the same amount of the buffer in advance; the final concentration of mannitol was 170 mM. With diethylenetriamine pentaacetic acid (DTPA), 50 μL of the solution (1 mM) was added instead of the same amount of the buffer in advance; the final concentration of DTPA was 83 μM. With ascorbic acid, 10 μL of the solution (10 mM) was added instead of the same amount of the buffer in advance; the final concentration of ascorbic acid was 170 μM (for the extract only).

Quantification of hydrogen peroxide. Hydrogen peroxide in the extract was quantified by mixing 1 mL of the extract with another 1 mL of the buffer, leaving for 30 min at room temperature and subjecting to an analysis by the HPLC-ECD method. The effect of the scavengers was measured by adding 20 μL of SOD (21,000 units/mL) or 200 μL of catalase (1,350 units/mL) instead of the same amount of the buffer.

ESR analysis using DEPMPO. Approximately 47 mg of DEPMPO was dissolved in 200 μL of the phosphate buffer (pH 7.4). A 100 mM amount of diluted DEPMPO was mixed with 250 μL of the smoke extract and 250 μL of the buffer; the final concentration of DEPMPO was approximately 160 mM. The spectra were acquired 30 min after the extract had been finally added to all other agents, with the ESR instrument settings modified as follows: scan field, 335.3 ± 10 mT; scan time, 4 min; amplitude, 1,000. The Mn²⁺ marker was removed because its signals overlapped those of the adducts. The extract mixed with SOD was also analyzed in the same way as that with DMPO.

ESR spectra obtained from the aqueous extract of cigarette smoke with nitrogen gas bubbled. A 100 μL amount of diluted DMPO was mixed with 250 μL of the extract and 250 μL of the buffer. The spectra were obtained 30 min after the extract had been finally added to all other agents. Nitrogen gas was bubbled through the mixture until it was transferred to the quartz cell after approx. 28 min. The dial number of the Mn²⁺ marker was 450 which generated signals with approximately the same intensity as those obtained in other experiments before services of the instrument including the marker.

Generation of hydrogen peroxide from hydroquinone and catechol. Hydroquinone and catechol have been thought to be responsible for generating hydrogen peroxide from cigarette smoke, having been determined in cigarette smoke and found in fractionated solutions of the extract which generated hydrogen peroxide. The particulate phase of the smoke collected on the glass-fiber filter was predicted to contain 150 μg of hydroquinone and catechol. Aqueous solutions of these two compounds were prepared by dissolving 1.5 mg of each in 15 mL of the buffer to produce a ten times higher concentration of each compound than that predicted. Each solution was shaken for 90 min at 37 °C, and then subjected to the HPLC-ECD analysis to determine hydrogen peroxide.
Results

**ESR spectra obtained from the aqueous extract of the particulate phase of cigarette smoke and from the model reaction systems for generating superoxide anion radicals and hydroxyl radicals (Fig. 1)**

The ESR spectra obtained from the aqueous extract of the particulate phase of cigarette smoke are shown as A in Fig. 1. No signal from the sample was detected when the spectrum was obtained with an acquisition time of 1 min, but the signal gradually increased until 30 min. The obtained spectra agreed with those of DMPO-OH.15,16,19) The ESR spectra obtained from the HPX-XOD system are shown as B in Fig. 1. A combined spectrum for DMPO-OOH and DMPO-OH was obtained20) with an acquisition time of 1 min. The signal for DMPO-OOH disappeared, but that for DMPO-OH remained when the spectrum was obtained with an acquisition time over 5 min. The ESR spectra obtained from the H₂O₂–FeSO₄ system are shown as C in Fig. 1. The signal for DMPO-OH was detected when the spectrum was obtained with an acquisition time of 1 min 16) and remained until 30 min of acquisition.

**Comparison of the responses in the ESR spectra to the various scavengers (Fig. 2)**

To identify the chemical species detected by the ESR analysis using DMPO, various scavengers were added to the extract and the model reaction systems, and the responses in the ESR spectra were compared. SOD, catalase, and mannitol were respectively added as selective scavengers of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. DTPA was selected as a chelating agent, and ascorbic acid as a scavenger of reactive oxygen species.

The signals for DMPO-OH were eliminated in the presence of SOD in the experiment using the extract (A in Fig. 2). The signals were hardly changed when catalase, mannitol or DTPA was added to the sample. The signals were also eliminated in the presence of ascorbic acid (data not shown).

The concentration of hydrogen peroxide in the extract was analyzed to confirm that the amount of catalase added to the sample was adequate. Hydrogen peroxide in the extract was eliminated below the detection limit from 145 μM in the presence of catalase (135 units/mL). The concentration of hydrogen peroxide was hardly changed (194 μM from 208 μM) in the presence of SOD (210 units/mL).

In the experiment using the HPX-XOD system (B in Fig. 2), the mixed signals of DMPO-OOH and DMPO-OH, which were only detected with an acquisition time of 1 min, were eliminated in the presence of SOD, whereas the signals were hardly eliminated in the presence of catalase, mannitol or DTPA. The signals were eliminated in the presence of ascorbic acid with a half concentration added to the extract. The signals of DMPO-OH detected with an acquisition time over 5 min partially remained in the presence of SOD.

In the experiment using the H₂O₂–FeSO₄ system (C in Fig. 2), the ESR signals of DMPO-OH were eliminated in the presence of either catalase or mannitol. Catalase would have scavenged hydrogen peroxide with the H₂O₂–FeSO₄ system, whereas mannitol would have scavenged hydroxyl radicals generated with the H₂O₂–FeSO₄ system. The signals
were partially suppressed in the presence of SOD, whereas the signals were significantly enhanced in the presence of DTPA. The signals were eliminated in the presence of ascorbic acid with a half concentration added to the extract.

**ESR analysis using DEPMPO (Fig. 3)**

DEPMPO, which generated a more stable adduct of superoxide anion radicals, was used as the agent in an attempt to detect the superoxide adduct from the extract by the spin-trapping technique. The overlapped signals for DEPMPO-OOH and DEPMPO-OH were detected with an acquisition time of 30 min (A in Fig. 3). Only very weak signals for DEPMPO-OH were detected when the spectrum was acquired in the presence of SOD (B in Fig. 3).

**Contribution of oxygen to the generation of superoxide anion radicals (Fig. 4)**

It has been suggested that superoxide was generated by the reduction of oxygen by smoke components, possibly such phenolic components as hydroquinone and catechol. In order to investigate the effect of oxygen on generating superoxide anion radicals from the
extract, nitrogen gas was bubbled into the sample until conducting the analysis. The ESR signal was eliminated when nitrogen gas was bubbled into the sample (B in Fig. 4).

**Discussion**

We found when we started the study that the ESR signals for DMPO-OH detected from the extract of the particulate phase of cigarette smoke were hardly eliminated by adding catalase, although this was adequate for scavenging hydrogen peroxide in the extract. The ESR signals for DMPO-OH had been accepted as evidence for hydroxyl radicals, but the results here indicate that the signals for DMPO-OH were not derived from hydroxyl radicals. We therefore tried to clarify the origin of DMPO-OH detected from the extract. DMPO is widely used for detecting hydroxyl radicals because of the distinctive ESR signals for DMPO-OH. DMPO also reacts with superoxide anion radicals to generate DMPO-OOH; however, this adduct is known to be short-lived, for example, with a conformer lifetime of the order of 0.1 μs at room temperature. Therefore, the ESR signals of DMPO-OOH decayed in a very short time, and then the signals of DMPO-OH become predominant. The signals of DMPO-OH derived from the two origins could not be distinguished from each other. On the other hand, SOD and catalase are enzymes which are the selective scavengers of the target species. Mannitol is not an enzyme, but is widely used as a scavenger of hydroxyl radicals, possibly because mannitol does not generate by-products which produce other ESR signals affecting the results. We therefore tried to confirm the species trapped by DMPO by means of the selectivity of these scavengers. The signals of DMPO-OH detected from the extract wereeliminated by SOD, indicating that DMPO-OH detected from the extract was derived from superoxide anion radicals. The signals were hardly eliminated by catalase and mannitol, indicating that the detected DMPO-OH was not derived from hydroxyl radicals generated from hydrogen peroxide. The amounts of mannitol and catalase added in this study were enough to respectively scavenge hydroxyl radicals and H₂O₂, because the same amounts of the scavengers eliminated the signals of DMPO-OH derived from hydroxyl radicals generated by the Fenton reaction. The addition of DTPA to the extract hardly changed the signals of DMPO-OH, although DTPA enhanced the signals of DMPO-OH from the H₂O₂–FeSO₄ system. This result supports the notion that DMPO-OH detected from the extract was formed by mechanism other than the Fenton reaction.

A possible mechanism for forming DMPO-OH in the extract is that superoxide anion radicals were first generated in the extract, reacted with DMPO, and then finally generated DMPO-OH. In our experiments, however, no signals of DMPO-OOH were detected from the extract. We think it possible that the formation of DMPO-OOH in the presence of the smoke extract was much slower, apparently shown in A in Fig. 2, than the decay of DMPO-OH.

The detection of DMPO-OH was therefore not appropriate as evidence for hydroxyl radicals in the extract. DEPMPO, another spin-trapping agent which generates longer-lived adducts of superoxide anion radicals than DMPO, brought about mixed signals of DEPMPO-OOH and DEPMPO-OH. Both signals were eliminated in the presence of SOD, indicating that the two signals were both derived from superoxide anion radicals. The very weak signals of DEPMPO-OH that were detected in the presence of SOD could have been derived from hydroxyl radicals generated from hydrogen peroxide in the extract that were independent of superoxide anion radicals. Although we did not detect hydroxyl radicals by the ESR analysis when using DMPO, it has been reported that the extract formed 8-hydroxydeoxyguanosine (8-OHdG) when mixed with isolated DNA. DEPMPO-OH was a reaction product of deoxyguanosine with hydroxyl radicals, indicating that the extract would finally generate hydroxyl radicals. Our results indicate that oxygen was required for generating superoxide anion radicals. Oxygen would be reduced to superoxide anion radicals by smoke components in the aqueous extract of the particulate phase of cigarette smoke. The components include not only hydroquinone and catechol, but also other compounds. Superoxide anion radicals generated in the extract would be easily converted into hydrogen peroxide by disproportionation, in the absence of DMPO. Hydrogen peroxide in the extract could possibly generate hydroxyl radicals when catalyzed by metals. The proposed mechanism for generating reactive oxygen species in the aqueous extract of cigarette smoke is summarized in Fig. 5. This mechanism is strongly supported by the analytical results of the ESR spectra under the unified experimental conditions, including the time-course studies with several scavengers, and by the amount of hydrogen peroxide in the corresponding samples.

**Proposed Mechanism for Generating Reactive Oxygen Species from the Aqueous Extract of Particulate Phase Cigarette Smoke.**

![Fig. 5. Proposed Mechanism for Generating Reactive Oxygen Species from the Aqueous Extract of Particulate Phase Cigarette Smoke.](image-url)
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References