Spin Trapping of Nitric Oxide in Aqueous Solutions of Cigarette Smoke

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Nitric oxide, a gaseous free NO radical (•NO) generated in particulate-free gas-phase main-stream smoke of cigarettes, was observed with electrical spin resonance (ESR) using a spin trapping technique. N-Methyl-D-glucamine-dithiocarbamate $(MGD)_2$–Fe^{2+} complex was used for the NO radical spin trapper in aqueous solution. The intensity of the ESR signal of the spin adduct formed by bubbling smoke from one cigarette increased gradually with time over 2 hours at about 20°C and was constant for 2 days or longer. The time course of the production of the NO radical followed the rate equation $y = 1520(1 - e^{-0.018t})$ for the first-order reaction up to around 25 min after mixing of Fe^{2+} solution and then slowly approached the maximum value determined by the concentration of the spin adduct. These findings suggest that NO radical is produced slowly from NO radical donors such as amine •NO complexes, peroxinitrite ($ONOO^-$), and other reactants such as nitrogen oxides (NO_x), which are produced from the smoke of tobacco leaves, and suggest that its generation could be involved in the decomposition or cleavage of such substances. © 1998 Academic Press

Key Words: cigarette smoke; nitric oxide; spin trapping; radical; chemical kinetics.

Pryor and co-workers (1–15) investigated the radicals related to carcinogenic substances in particulate-free gas-phase cigarette smoke (1, 2, 4, 5, 8, 9) and tar (3, 6, 7, 10–15) using an ESR method. Cigarette smoke contains 3000 or more chemical compounds and many species of radicals (15). The semiquinone, hydroxyl, and superoxide radicals in cigarette tar and smoke have been observed with spin trapping techniques (3, 5, 6, 14, 15, 17, 18, 28–30). Smoking-related diseases such as emphysema and lung cancer have been considered to be closely connected with active oxygen species

and NO radical. Active oxygen species (7, 8, 10, 17–20) such as superoxide anion $O_2^{•-}$ have been found to break DNA strands in human cells with a high frequency ($\sim 10^4$ per cell) (15). On the other hand, NO radical (4, 6, 8, 11, 21) is known not to elicit such damages directly because it is comparatively stable (the lifetime is about 6 s *in vivo*) (27), but to produce cytotoxic radicals such as $ONOO^-$ (22–24) and hydroxyl radical •OH (6, 22), which have been thought to lead to carcinogenesis and mutagenesis (7, 12,13, 20, 24). Furthermore, the presence of heavy metals such as lead and cadmium in blood after smoking could be somewhat involved in generation of the radicals (6).

It is further known that cigarette smoke contains relatively high concentrations of NO and NO_2 radicals (16, 25–30). Pryor *et al.* (3) detected these radicals with the spin trapping technique using α-phenyl-N-*tert*-butylnitrone and 5,5-dimethyl-1-pyrroline-N-oxide, but they did not observe the ESR signals expected for the spin adducts of either NO or NO_2 in the smoke. Nitric oxide radical can be observed effectively by using MGD (28) as the spin trapper. In this paper, we describe investigations of the presence and time course of the generation of NO radical in particulate-free cigarette smoke by the spin trapping method and analyze the production of NO radical with chemical kinetics.

MATERIALS AND METHODS

A main-stream smoke sample of one cigarette (hi-lite, Nippon Tobacco, Japan), one of the most popular tobacco (*Nicotiana tabacum* L) brands with a filtered tip, was drawn by means of a smoking apparatus (Fig. 1) equipped with a suction pump (MP-2, Sibata Chemical Co., Ltd., Japan) at a smoking rate of about 600 ml/min and a sampling time of about 1 min per cigarette. These simulative conditions were chosen to represent those of moderate smokers. The length of a silicone rubber tube from a cigarette holder to the trapping solution was about 30 cm. NO radical is a hydrophobic gas, and therefore the trapping technique was performed in an open aqueous system. Cigarette smoke was passed through a glass fiber filter (Millipore AP 200 filter) and was led to the spin trapping solution containing $(MGD)_2$–Fe^{2+} complex (28–30) in phosphate-buffered solution (PBS) prepared at pH 7.4. MGD was purchased from Labotec Co. (Tokyo, Japan). Iron(II) sulfate ($FeSO_4$) and phosphate-
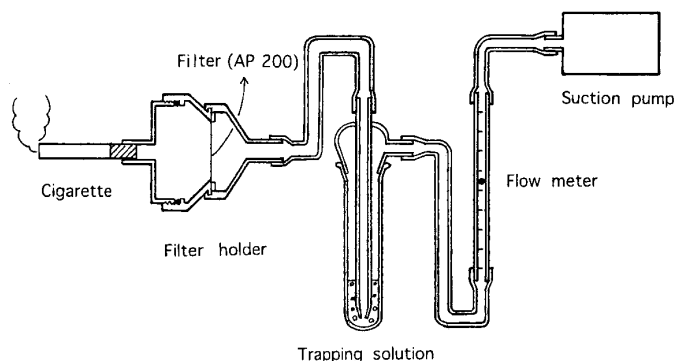
FIG. 1. Apparatus for smoking cigarettes (open system) and spin trapping the gas-phase radical. Smoking period or speed can be varied with the suction pump.

buffered powder were purchased from Wako Pure Chemical Industries Ltd. (Japan). S-Nitroso-N-acetylpenicillamine (SNAP) (Sigma Chemical Co., U.S.A.) was used as a standard donor of NO radical in preliminary experiments. The spin trapper, $(MGD)_2$–Fe^{2+} complex, was freshly prepared by adding 400 μl of 50 mM MGD and 400 μl of 50 mM $FeSO_4$ into 3 ml of pH 7.4 PBS. This is because the complex is unstable and gradually decomposes at a pH lower than 7.0 (29). Preliminary experiments (Fig. 2A) revealed that the optimum volume of 50 mM $FeSO_4$ solution was 200 μl or more, and so the volume giving the highest ESR spectrum was determined to be 400 μl of $FeSO_4$ solution for 20 μl of 10 mM SNAP added into the MGD–PBS mixture for safety. In actual experiments with cigarette smoke, we added twice 200 μl, a half volume of 400 μl of $FeSO_4$ solution before and after bubbling of the smoke into PBS, on the basis of experiments performed with cigarette smoke for the optimum order of addition of the reagents (Figs. 3a, b, and c).

We carried out control experiments on the air sucked through the cigarette alone without smoking under the same conditions as described above (Fig. 4a). We further examined the smoking of the cigarette paper ($\sim$330 mg/17 cigarettes) alone (Fig. 4b) and the fiber filter paper ($\sim$710 mg) alone (No. 7, Toyo Roshi Ltd., Japan), whose material is defined to be highly purified cellulose (Fig. 4c). After the smoke was bubbled, the $(MGD)_2$–Fe^{2+}–NO complex solution was

transferred to a flat quartz cell (LC25, 130 μl, JEOL, Japan), and the ESR signals of the spin adduct were recorded with an ESR spectrometer (TE-200, JEOL, Japan) from the mixing time of the latter half of the volume (200 μl) of 50 mM $FeSO_4$ solution for 90 min or longer at the well air-conditioned room temperature of 20°C. The signal height, which was measured as the peak to peak distance of the first signal (Fig. 2B), was read out and analyzed with personal computer PC 9801 β X 2 (NEC, Japan) using the program ESR Data Analyzer Ver. 2.0 TE (LABOTEC, Co. Ltd, Japan).

RESULTS

In preliminary experiments using SNAP as $\cdot$NO donor, a three-line spectrum characteristic of mononitrosyl–Fe^{2+} complex was observed at air-conditioned temperature (20°C) and the intensity remained unaltered for 2 or 3 days (Fig. 2B). We at first investigated the optimum order of the addition of $FeSO_4$ solution and the bubbling of smoking under the condition that the total volume of the $FeSO_4$ solution was 400 μl fixed (Fig. 3).

When the total volume of 400 μl of 50 mM $FeSO_4$ solution was added at one time before bubbling, the same three-line spectrum as SNAP was appreciably detected (Fig. 3a). When the same volume of the $FeSO_4$ solution was mixed after bubbling, the spectrum was fainter (Fig. 3b). When each 200 μl of $FeSO_4$ was added twice before and after bubbling, the spectrum was much larger (Fig. 3c). On the basis of these results, the procedure was executed in the following experiments. Furthermore, a change in the counteranion from $FeSO_4$ to $FeCl_2$ had little effect on the signal height (Fig. 3d).

In some of the control experiments (Fig. 4), sucked air alone without smoking gave no signal (Fig. 4a). However, the smoking of the cigarette paper alone gave a slight signal, as shown in Fig. 4b. A similar experi-
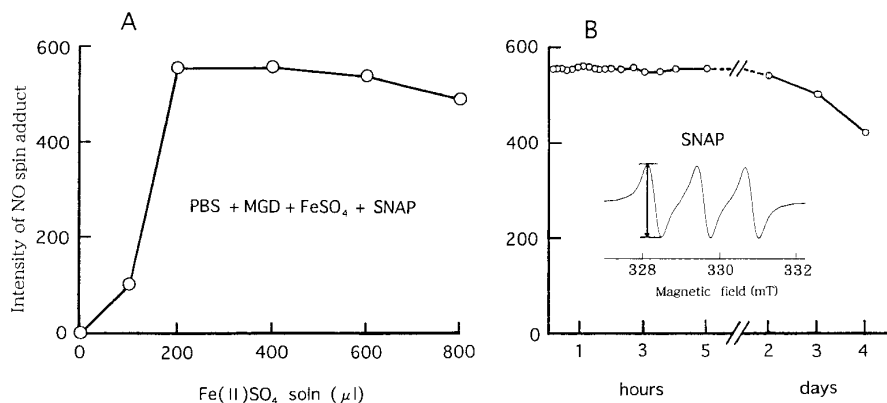

FIG. 2. The optimum mixing volume (μl) ratio of 50 mM $FeSO_4$ solution to (400 μl of 50 mM MGD + 3ml PBS) (A) and the stability of the spectrum of NO spin adduct, $(MGD)_2$–Fe^{2+}–NO complex, which is produced from SNAP (B). (A) The optimum volume of the $FeSO_4$ solution is 200 μl or more, and so we used 400 μl of the solution in the following experiments on cigarette smoke. (B) The complex produced immediately after the mixing is very stable for 2 or 3 days. The three-line spectrum for NO spin adduct is shown in the inset and its height was measured with the peak to peak distance of the first signal ($\updownarrow$). The recording conditions were magnetic field, 330$\pm$5 mT; frequency, 9.4154 GHz; microwave power, 4.08 mW; modulation frequency, 100 kHz; modulation width, 0.2 mT; amplitude, 200; time constant, 0.10 s; sweep time, 30 s. They were the same in the following experiments (Figs. 3–6).
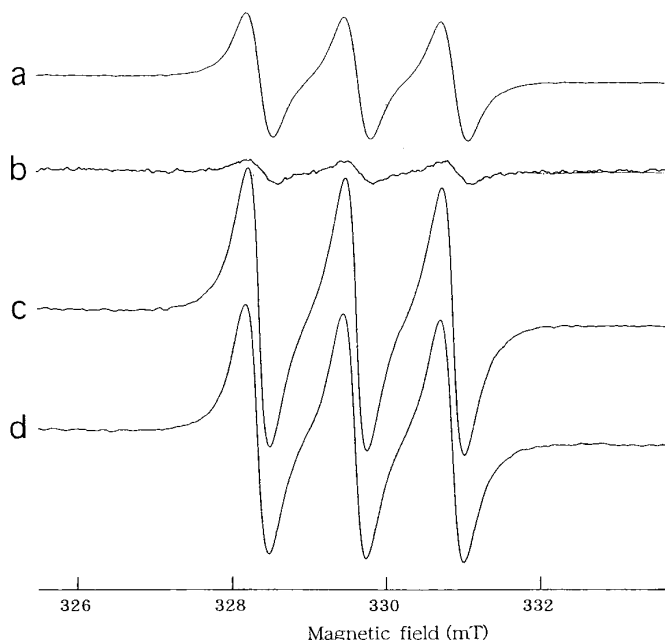
FIG. 3. The optimum order of addition of $FeSO_4$ solution and bubbling of smoke into PBS containing MGD (a, b, and c) and the effect of the counteranions, SO_4^{2-} and Cl^-, on the production of NO spin adduct (d). (a) The spectrum in the case where the total volume of 400 μl of $FeSO_4$ solution was added before bubbling. (b) The case where the same volume of the solution was added after bubbling. (c) A higher spectrum is observed 30 min later as a result of two additions of equally divided $FeSO_4$ solutions before and after bubbling. Therefore, this procedure has been used in our experiments. (d) The signal observed after a change from $FeSO_4$ to $FeCl_2$, which shows little or no effect on the spectrum compared with case c.

ment on the filter paper (No. 7) alone, a component of which is known to be pure cellulose, gave only a faint spectrum (Fig. 4c). A typical three-line spectrum at 30 min after mixing of the latter half (200 μl) of $FeSO_4$ solution is shown in Fig. 4d. These spectra show that the NO radical comes mainly from the tobacco smoke, not from the cigarette paper whose major component is cellulose.

The signal intensities observed in each sample of cigarette smoke, which were not detected at the start, increased rapidly for 25 min or so and slowly approached the maximum value determined by the amount of •NO spin adduct with a time lapse of 2 or 3 days (Figs. 5A and B).

The hyperfine coupling constant a_N was 1.27 mT and the g value was 2.042. The number of NO radicals produced from one cigarette was nearly 1.52×10^{17} spins for the maximum signal height obtained by comparison with the result calculated from that of 20 μl of 10 mM SNAP, the standard NO radical donor.

The time course of formation of the adduct was analyzed with chemical kinetics by numerical computation from a given pair of experimental points (Fig. 6). The rapidly increasing phase up to around 25 min could be approximated by a rate equation, $y = C_0(1-$

$e^{-kt})$ for the first-order reaction, and was given by $y = 1520(1-e^{-0.018t})$ ($0 \leqq t \leqq 25$). This rate equation agreed closely with observed values (Fig. 6a). For smoke from half a cigarette, the spectrum became larger with a similar time course, and thereafter the maximum height was about half that of one cigarette. The rate equation was approximated by $y = 830(1-e^{-0.018t})$ and the rate constant k was identical with that (0.018) from the smoke of one cigarette (Fig. 6b).

DISCUSSION

This study has revealed that main-stream cigarette smoke generated NO radical rapidly for about 25 min and that it gave the maximum spectrum for 2 or 3 days. The NO radical production from one cigarette follows the first-order reaction given by $y = 1520(1-e^{-0.018t})$. Because the NO radical is a hydrophobic gas and its lifetime is 6 s or shorter (27), the spin trapping technique was used with an open aqueous system, as shown Fig. 1. The optimum ratio MGD:Fe^{2+} for the measurement of the ESR signal seemed to be 1:1, as shown in Fig. 2A, although the ratio is 2:1 stoichiometrically. Excess Fe^{2+} precipitated $Fe(OH)_2$ in the basic solution. According to Lai and Komarov (29, 30), the molecular ratio MGD:Fe^{2+} was 5:1 *in vivo,* where such precipitations may not be formed. Preliminary experiments (Fig. 3) suggest that the presence of Fe^{2+} is essential to form $(MGD)_2$–Fe^{2+}–NO complex and to function as an anti-oxidant as well.

As shown in Fig. 3a, the lower spectrum observed when the total volume of the $FeSO_4$ solution was mixed once before bubbling means that the heated air that is sucked with the bubbling smoke oxidized Fe^{2+} to Fe^{3+}
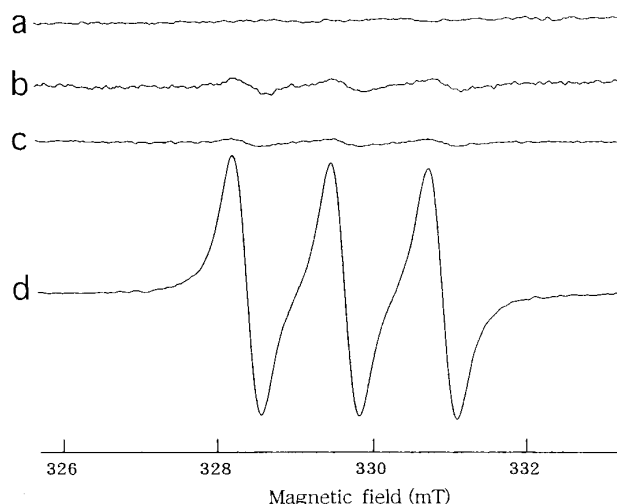

FIG. 4. (a) The control experiments on sucked air alone without smoking; (b) cigarette paper (~330 mg/17 cigarettes) alone; (c) filter paper No. 7 (~710 mg) alone, the material of which is pure cellulose; (d) and an ordinary spectrum from the smoke of one cigarette 30 min later.

and that the above complex was not formed so much. The fact that one-shot addition of the $FeSO_4$ solution after bubbling gave no signal indicates that the NO radical was hardly dissolved in the trapping solution because of the hydrophobicity. Therefore, the spin adduct was little produced (Fig. 3b). From these results, the two additions of Fe^{2+} solution before and after bubbling were necessary to obtain the higher spectrum of ${}^{\bullet}$NO spin adduct (Fig. 3c). It was found that the difference between the counteranions SO_4^{2-} and Cl^- causes little or no effect on the production of ${}^{\bullet}$NO spin adduct (Fig. 3d). This suggests that sulfate molecules forming the $(MGD)_2$–Fe^{2+}–NO complex result from those in MGD, but not from $FeSO_4$. The formation of the complex is much affected by transition metal ions such as Fe^{2+} with reducing power, but not by the counterions.

The variation in the spectra in Fig. 5B could be a result of the differences in temperature and the supply of oxygen due to the smoking period, the shaking times of the sample, and so on. The larger variations were observed with a change in species of cigarette (no figure). We have adopted the time elapsed from mixing of the second half volume of $FeSO_4$ solution as the abscissa, instead of the time from combustion of a cigarette. The initial increasing rate, which is given by kC_0 theoretically from the relation, $dy/dt = kC_0\, e^{-kt}$, is

FIG. 6. Time courses of spectra from the smoke of (a) one cigarette and (b) half a cigarette. Each broken line shows the theoretical curve fitted with the numerical computation by assuming that the generation of NO radical obeys a first-order reaction in the rapidly increasing phase up to about 25 min.

expected to be proportional to the maximum amount (C_0) of production of NO spin adduct which is stable in the reductive aqueous solution (Figs. 6a and b). We obtained 27.4/min for one cigarette and 14.9/min for half a cigarette as the initial rates.

From the results that the production of NO radical followed a first-order reaction and that the rate constants in both cases gave nearly the same values, we suggest that NO radical is produced by the decomposition of any of the NO radical donors such as amine ${}^{\bullet}$NO complexes, peroxinitrite ($ONOO^-$), and/or other reactants like NO_2, NO_2^-, and NO_3^-, which are produced from the combustion of tobacco leaves.

We should further elucidate the source of ${}^{\bullet}$NO generation.
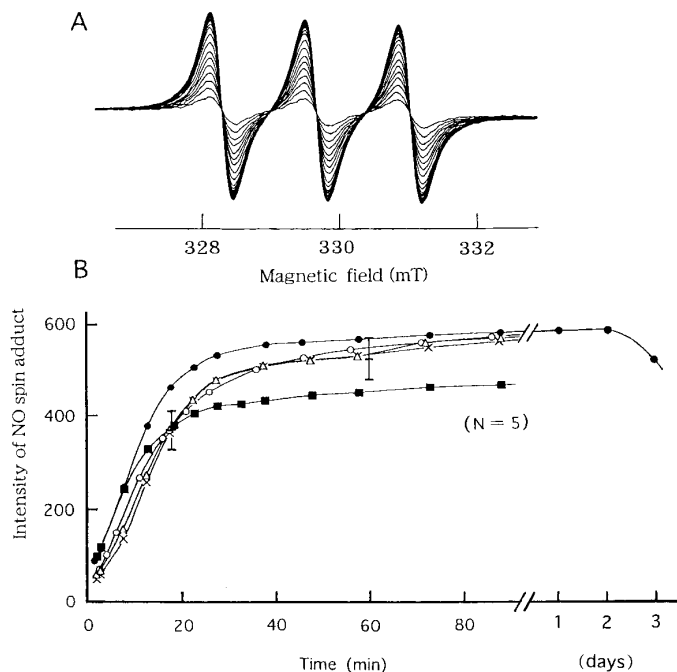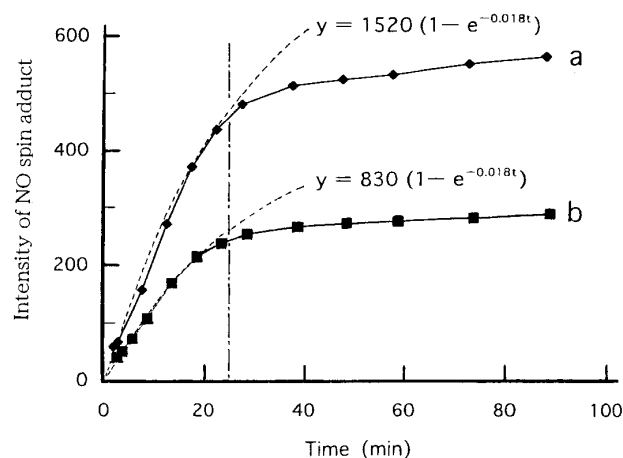

FIG. 5. Changes with time in the spectrum of NO radical for one cigarette. (A) An example of the growth of the spectrum of NO spin adduct. (B) The variation in the time course of signal intensities ($N=5$). Though indiscernibly small at the start, the spectra increased rapidly with time up to around 25 min and then slowly approached the maximum value. They were constant for 2 or 3 days. Symbols ($\bar{\top}$) at 20 and 60 min represent the means $\pm$ SD.

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