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ENHANCEMENT OF NITROGEN DIOXIDE-INDUCED LIPID PEROXIDATION AND DNA STRAND BREAKING BY CYSTEINE AND GLUTATHIONE

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Nitrogen dioxide less than 100 ppm in air induced lipid peroxidation of liposome composed of 1-palmitoyl-2-arachidonylphosphatidylcholine as assessed by thiobarbituric acid reactivity. The nitrogen dioxide-induced lipid peroxidation was enhanced by cysteine, glutathione and bovine serum albumin. While the activity of nitrogen dioxide in air to induce single strand breaks of supercoiled plasmid DNA was low, the breaking was remarkably enhanced by cysteine, glutathione and bovine serum albumin. ESR spin trapping using 5,5-dimethyl-1-pyrroline N-oxide showed that certain strong oxidant(s) were generated by interaction of nitrogen dioxide and cysteine. The spin trapping using 3,5-dibromo-4-nitrosobenzene-sulfonate suggested that sulfur-containing radicals were generated by interaction of nitrogen dioxide and cysteine or glutathione. Hence, certain sulfur-containing radicals generated by the interaction which could effectively induce lipid peroxidation and DNA strand breaks.

KEY WORDS: Nitrogen dioxide, cysteine, glutathione, sulfur-containing radical, lipid peroxidation, DNA breaking.

INTRODUCTION

Nitrogen dioxide, a free radical toxin in mainstream cigarette smoke (above 50 ppm), in smog (up to 1 ppm) and in polluted urban air (usually at 0.02–0.2 ppm), has been known to cause various disorders such as pulmonary edema, pulmonary fibrosis, bronchitis[1-3] and promotion and implantation of cancer.[4,5] In mammalian cells, nitrogen dioxide is probably formed as an oxidation product of nitric oxide formed via arginine pathway as a physiologically essential messenger molecule.[6,7] Exposure of nitrogen dioxide in air at low levels *in vivo* and *in vitro* causes an initiation of autoxidation of polyunsaturated fatty acids.[8-12] Our *in vitro* studies have demonstrated that the lipids in low density lipoprotein (LDL) was effectively oxidized by exposure to nitrogen dioxide in air, and the LDL thus oxidized whose apoprotein B was modified by lipid peroxidation products was readily taken up by macrophages to produce foam cells.[13] It has been shown that nitrogen dioxide in air can convert cysteine (Cys) into cystine,[14] and more efficiently damage Tyr and Trp residues in proteins.[15] It is also known that nitrogen dioxide in air induces chromosomal aberrations and sister chromatid exchanges *in vitro* and *in vivo*,[16,17] and is active as a

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tumor promoter.[18] Strand break of DNA molecule in cells by nitrogen dioxide has been demonstrated.[19,20]

It was found in the present investigation that nitrogen dioxide in air became more powerful free radical species that initiated lipid peroxidation and induced DNA strand breaking by interaction with Cys, glutathione (GSH) and bovine serum albumin (BSA). Generation of new free radical species by the interaction of nitrogen dioxide and these components was suggested by ESR spin trapping technique.

MATERIALS AND METHODS

Nitrogen dioxide (about 100 ppm) in air or in nitrogen gas was obtained from Nippon Sanso Ltd., Tokyo, Japan. The concentration of nitrogen dioxide was determined each time according to the method of Salzman[21,22] using a conversion coefficient of NO_2/NO_2^- : 0.84, the coefficient being adopted in Japan Industrial Standard.

L-α-Phosphatidic acid (PA), GSH and BSA were obtained from Sigma Chemical Company (St Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) and 1-palmitoyl-2-arachidonylphosphatidylcholine (PAPC) were obtained from Funakoshi Company (Tokyo, Japan). Cholesterol, tyrosine (Tyr), tryptophan (Trp) and thiobarbituric acid (TBA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Cys was from Nacalai tesque (Kyoto, Japan). Supercoiled plasmid pBR 322 DNA[23] (1 mg/ml in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) was obtained from Takara-shuzo Company (Kyoto, Japan). 5,5-Dimethyl-1-pyrroline N-oxide (DMPO) and 3,5-dibromo-4-nitrosobenzenesulfonic acid sodium salt hydrate (DBNBS) were obtained from Labotec Company (Tokyo, Japan).

Liposomes

DPPC and PAPC liposomes were prepared from the mixtures of DPPC or PAPC: PA: choleterol (6:1:3) according to the methods previously described.[24,25] After lyophilization, liposomes were hydrated in 10 mM Tris-HCl buffered saline (pH 7.5) for use. Concentration of liposomes was determined by measuring phosphorus according to the method previously described.[26]

Thiobarbituric Acid (TBA) Reactivity

The TBA reactivity was determined according to the method originally devised by Ohkawa *et al.*[27] Briefly, to a 0.8-ml solution of liposome (3 μmol/ml), 50 μl of 0.8% butylated hydroxytoluene solution in glacial acetic acid, 0.2 ml of 8.1% sodium dodecylsulfate solution, 1.5 ml of acetate buffer (pH 3.5), and 1.5 ml of 0.8% TBA solution were added. After keeping at 5°C for 60 min, the mixture was heated at 100°C for 60 min. Red pigment produced was determined by absorbance at 532 nm after extraction with 1-butanol/pyridine mixture.

DNA Strand Breaking Studies

Exposure of nitrogen dioxide was conducted in two ways. (i) Nitrogen dioxide in air or in nitrogen gas was passed into a 100-μl solution of supercoiled plasmid pBR 322 DNA in 50 mM phosphate buffer (pH 7.5) (10 μg/ml) containing the indicated concentration of each amino acid, GSH or BSA at room temperature at a flow rate of

10 ml/min for up to 20 h. (ii) Nitrogen dioxide in air was passed over the surface of the above DNA solution which was gently swirled throughout the exposure. After the treatment, a 10-μl aliquot of the mixture was subjected to agarose gel electrophoresis as described,[28] in order to detect conversion of the supercoiled form I DNA into the open circular relaxed form II DNA and/or the linear form III DNA.[29]

ESR Spin Trapping Studies

Nitrogen dioxide in air was passed into the solution of DMPO or DBNBS in 50 mM phosphate buffer (pH 7.5) containing each amino acid, GSH or BSA at room temperature at a flow rate of 15 ml/min for the indicated period. The ESR spectra were obtained on a Varian E-4 EPR spectrometer. The instrumental conditions were: field setting at 338.5 mT, scan range of 10 mT, microwave power of 10 mW and modulation amplitude of 0.1 mT.

RESULTS

Effect of Cys, GSH and BSA on Nitrogen Dioxide-Induced Lipid Peroxidation of Liposomes

Effect of various amino acids, GSH and BSA on the lipid peroxidation of liposomes induced by nitrogen dioxide in air was investigated. Nitrogen dioxide (60–80 ppm) in air was passed into a suspension of DPPC or PAPC liposome at room temperature for up to 5 h. Air was similarly passed into the suspension as a control experiment. The degree of lipid peroxidation was assessed by the TBA reactivity. The TBA reactivity of the nitrogen dioxide-exposed DPPC liposome did not increase during the experimetal periods. The TBA reactivity of the nitrogen dioxide-exposed PAPC liposome increased gradually (Figure 1, open circle), whearas the reactivity of the air-exposed PAPC liposome did not. The nitrogen dioxide-exposure to the suspension of PAPC liposome containing Cys dramatically increased the TBA reactivity (closed circle). The increased reactivity was similarly observed when nitrogen dioxide was exposed to the suspension of PAPC liposome containing GSH (open triangle). While most other amino acids did not affect the increase of the TBA reactivity of PAPC liposome induced by nitrogen dioxide, Trp and Tyr were found to be inhibitory (closed triangle and open square). When nitrogen dioxide was exposed to the suspension of PAPC liposome containing BSA, the TBA reactivity was extensively increased (closed square). The results indicate that Cys, GSH and BSA had stimulatory effect, and Trp and Tyr had inhibitory effect on the nitrogen dioxide-induced lipid peroxidation of PAPC liposome. The inhibitory effect of Trp and Tyr may be due to the consumption of nitrogen dioxide by these amino acids. It has been shown that nitrogen dioxide reacts swiftly with Tyr to form dityrosine and 3-nitrotyrosine and with Trp to form complex products.[15] It was found in the present experiment that dityrosine was similarly produced in the nitrogen dioxide-exposures to the mixture of PAPC and Tyr and to the solution of Tyr alone.

Effect of Cys, GSH and BSA on Nitrogen Dioxide-Induced DNA Strand Breaking

DNA breaking activity of nitrogen dioxide in air was examined by using a supercoiled plasmid pBR 322 DNA. It is known that the supercoiled (form I) DNA is converted

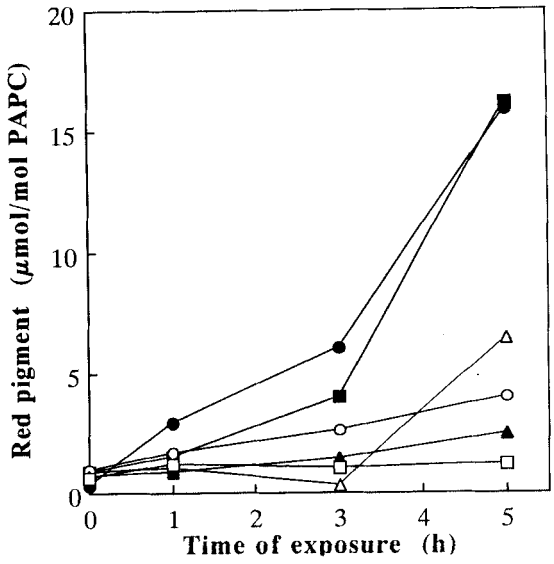

FIGURE 1 The TBA reactivity of PAPC liposome exposed to nitrogen dioxide in the presence of amino acids, GSH and BSA. Nitrogen dioxide (60–80 ppm) in air was passed into a 3.5 ml of 0.1 M Tris-HCl buffered saline (pH 7.5) containing 120 μmol PAPC liposome in the absence (○) and presence of 0.25 mM Cys (●), 1 mM GSH (△), 1 mM Trp (▲), 1 mM Tyr (□) and 1.5 mg/ml BSA (■) at room temperature at 10 ml/min. The TBA reactivity was determined as described in MATERIALS AND METHODS section, and expressed as the amount of red pigment produced.

into a nicked open circular form (form II) and/or into a linear form (form III) when the single strands are cleaved, and they can be separated by agarose gel electrophoresis.[29]

Introduction of nitrogen dioxide to the DNA solution was performed in two ways: passage of nitrogen dioxide in air into the DNA solution or passage over the surface of the DNA solution. When nitrogen dioxide (50–70 ppm) in air was passed into the DNA solution, form I DNA was slightly converted into form II DNA (Figure 2A, lane b). When the gas was passed into the DNA solution containing Cys at two different concentrations, form I DNA was effectively converted into form II DNA (lanes c and d). When the gas was passed into the DNA solution containing GSH at two different concentrations, form I DNA was similarly converted into form II DNA (lanes e and f). Passage of the gas into the DNA solution containing Trp, Tyr, Ala, His and Met did not show the breaking activity (data not shown). When the gas was passed into the DNA solution containing both Cys and Tyr, the breaking activity stimulated by Cys was abolished. When the gas at 30–40 ppm was passed into the DNA solution containing BSA, effective conversion of form I DNA into form II DNA was observed (lane g). In order to know the effect of oxygen in the gas, air in the gas was replaced by nitrogen gas. When nitrogen dioxide (70–80 ppm) in nitrogen gas was passed into the DNA solution containing Cys (lane h) and GSH (lane i), form I DNA was similarly converted into form II DNA. Hence, oxygen may not be involved in the enhancement of the nitrogen dioxide-induced DNA strand breaking by Cys and GSH. As control experiments, air was passed into the DNA solution containing Cys and GSH, but the form I DNA was little converted.

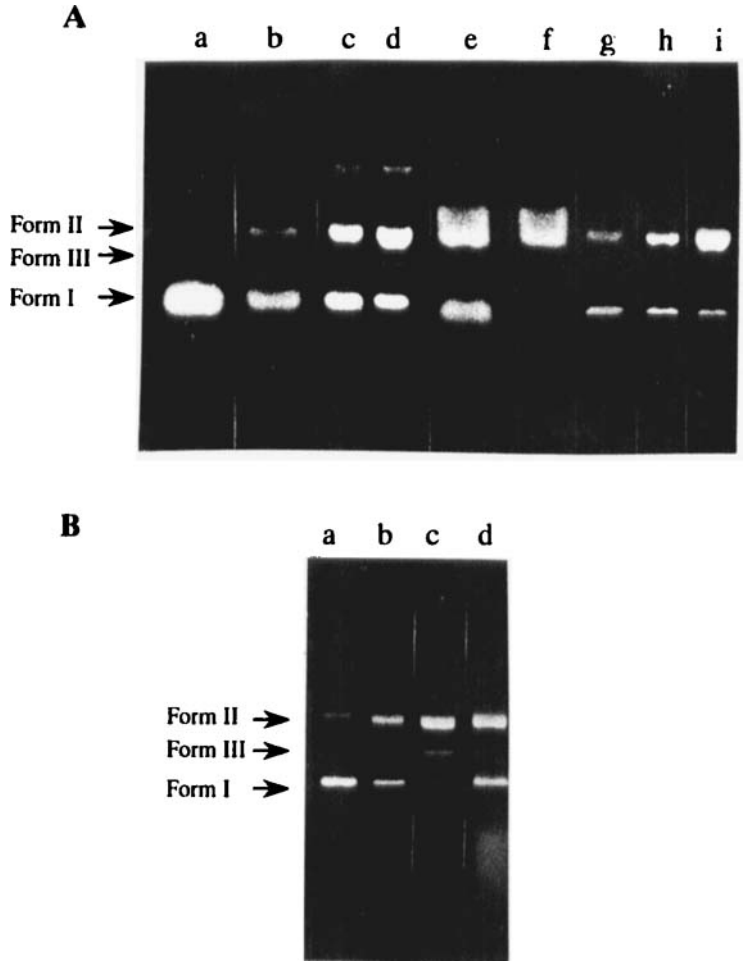

FIGURE 2 Agarose gel electrophoresis of supercoiled pBR 322 DNA treated with nitrogen dioxide in the presence of amino acids, GSH and BSA. A: Nitrogen dioxide (50–70 ppm) in air was passed into the DNA solution containing none (lane b), 0.5 mM (lane c) and 5 mM Cys (lane d), and 1 mM (lane e) and 10 mM GSH (lane f) for 10 h. Nitrogen dioxide (30–40 ppm) in air was passed into the DNA solution containing 10 mg/ml BSA for 5 h (lane g). Nitrogen dioxide (70–80 ppm) in nitrogen gas was passed into the DNA solution containing 10 mM Cys (lane h) and 10 mM GSH (lane i) for 20 h. B: Nitrogen dioxide (70–80 ppm) in air was passed over the surface of the DNA solution containing none (lane b), 10 mM Cys (lane c) and 10 mg/ml BSA (lane d) for 20 h. Lane a shows the control DNA. Positions of a supercoiled form (form I), a nicked open circular form (form II) and a linear form (form III) are indicated.

Nitrogen dioxide (70–80 ppm) in air was passed over the surface of the DNA solution containing Cys and BSA (Figure 2B). In this exposure, although nitrogen dioxide in air was active to convert form I DNA into form II DNA (lane b), the breaking activity was remarkably enhanced by Cys (lane c) and BSA (lane d). In the exposure in the presence of Cys (lane c), a slight amount of form III DNA was detected, indicating the effective cleavage of the single strands. It is difficult to compare the degrees of the single strand breaks obtained by the two series of experiments: nitrogen

dioxide passage into (Figure 2A) and over (Figure 2B) the DNA solution, because the concentrations of nitrogen dioxide and the effectors are different. From the results obtained above, it is likely that the interaction of nitrogen dioxide with Cys, GSH and BSA produced certain active components that would induce single strand breaks of the supercoiled plasmid DNA.

ESR Studies by Use of Spin Trapping Agents

When nitrogen dioxide in air was passed into the DMPO solution, no ESR spin signals were observable. In contrast, introduction of the gas into the DMPO solution containing Cys generated spin signals characteristic to those of 5,5-dimethyl-pyrrolidone-(2)-oxyl(1) (DMPOX)[30] with hyperfine splitting constant (hfsc) of $a_N = 0.725$ mT and $a_H = 0.40$ mT (Figure 3). DMPOX is a spin adduct produced by oxidation of DMPO.[30] Hence, certain strong oxidant(s) may be produced by interaction of nitrogen dioxide with Cys.

When nitrogen dioxide in air was passed into the DBNBS solution, weak spin signals composed of a triplet with hfsc of $a_N = 1.26$ mT appeared (Figure 4B). The signals may be due to the nitroxyl free radical spin adduct related to the interaction of DBNBS with nitrogen dioxide alone. Generation of the signals were not affected by the presence of Ala (Figure 4D). The signals were not observable in the presence of Tyr (Figure 4F), probably because Tyr trapped nitrogen dioxide by formation of 3-nitrotyrosine and dityrosine.[15]

When nitrogen dioxide in air was passed into the DBNBS solution containing Cys (Figure 4H) and GSH (Figure 4K), the intense signals due to the nitroxyl free radical with hfsc of $a_N = 1.26$ mT appeared. The signals were abolished in the presence of Tyr (Figure 4I). The results suggest that the reaction of nitrogen dioxide with Cys or GSH gave free radical species, *i.e.*, sulfur-containing radicals, which were in turn trapped by DBNBS to produce the nitroxyl free radical. The hfsc value of the nitroxyl free radical was indistinguishable from that of the spin adduct of DBNBS and sulfur trioxide anion radical ($a_N = 1.27$ mT).[31]

When nitrogen dioxide in air was introduced into the DBNBS solution containing

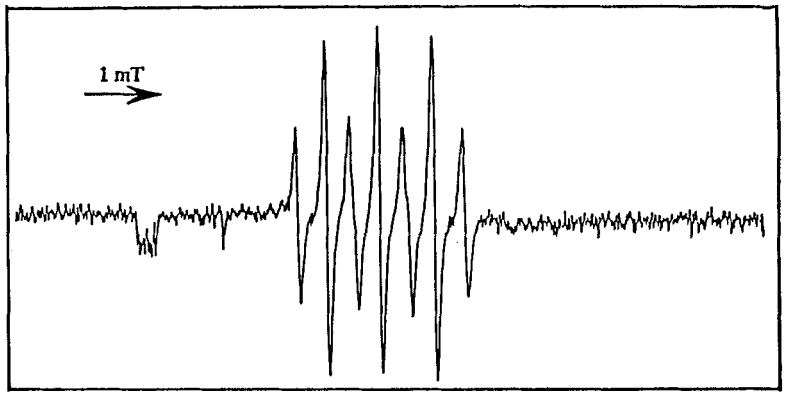

FIGURE 3 ESR spectrum of the mixture of 0.1 M DMPO and 1 mM Cys in 50 mM phosphate buffer (pH 7.5) into which nitrogen dioxide (60–70 ppm) in air was passed at a flow rate of 10 ml/min for 19 h. Receiver gain was set at 1250.

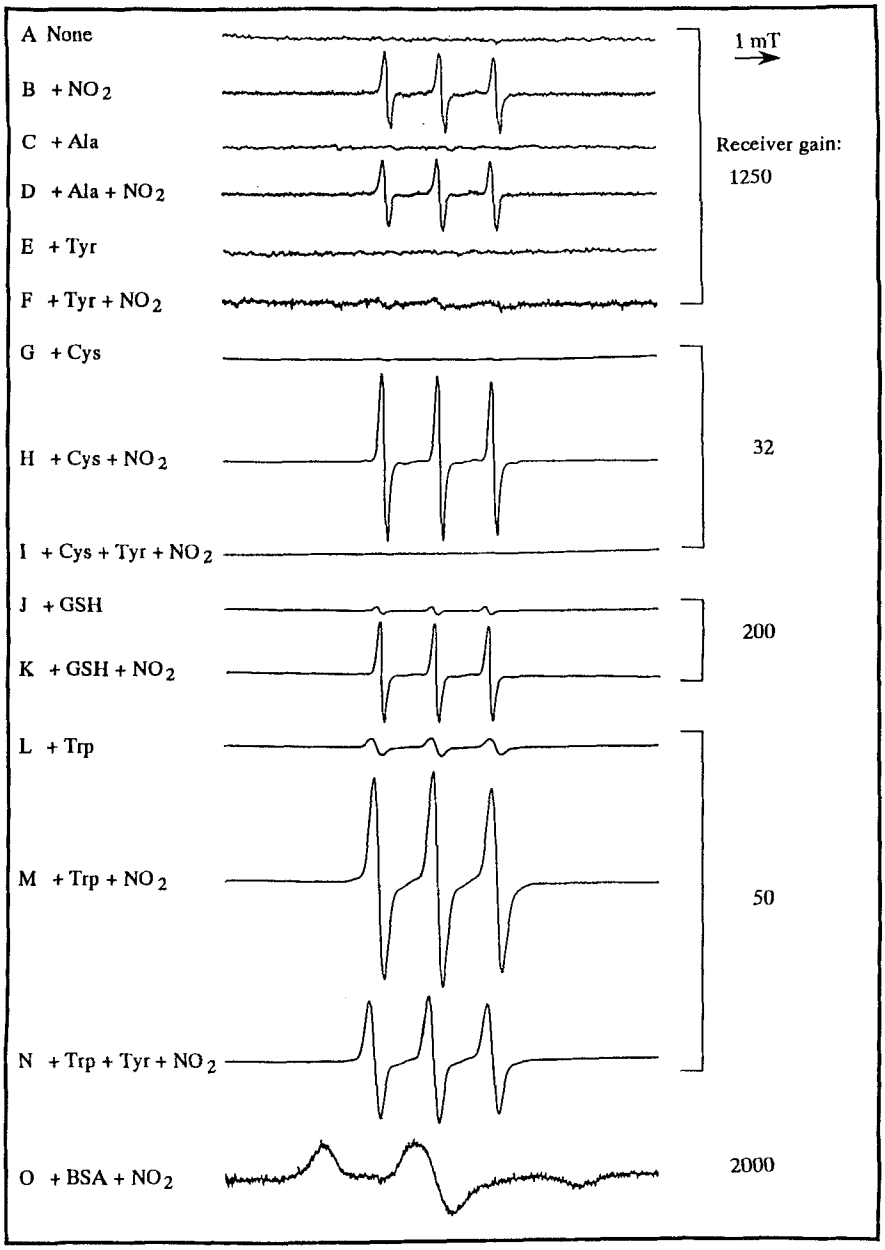

FIGURE 4 ESR spectra of the DBNBS solution containing amino acids, GSH and BSA exposed to nitrogen dioxide. Nitrogen dioxide (60–80 ppm) in air was passed into 0.1 ml of 0.1 M DBNBS solution in 50 mM phosphate buffer (pH 7.5) containing 10 mM each amino acid or 1% BSA at a flow rate of 10 ml/min for 3 h.

Trp, the intense signals due to the nitroxyl free radical spin adduct with hfsc of $a_N = 1.36$ mT appeared (Figure 4M). Tyr was little inhibitory to the appearance of the signals (Figure 4N). The hfsc of the signals indicates the formation of the nitroxyl free radical spin adduct formed by the reaction of aliphatic carbon-centered radicals with hfsc of $a_N = 1.25$–1.47 mT.[32] The results suggest that the reaction of nitrogen dioxide with Trp gave certain aliphatic carbon-centered free radicals, which were in turn trapped by DBNBS to produce the nitroxyl free radicals.

It was found that introduction of nitrogen dioxide into the DBNBS solution containing BSA gave broad spin signals, suggesting the formation of free radicals by interaction of nitrogen dioxide and BSA (Figure 4O).

DISCUSSION

It has been well-known that exposure of nitrogen dioxide in air at low concentrations to lipids causes an initiation of autoxidation of polyunsaturated fatty acids.[8–12] It is generally recognized that antioxidants including protein-SH groups protect lipids from *in vivo* autoxidation caused by nitrogen dioxide.[33] However, it was found in the present experiments that protein-SH groups including Cys, GSH and BSA enhanced the lipid peroxidation of liposome induced by nitrogen dioxide (less than 100 ppm) in air. This activity of enhancement may be derived from the interaction of nitrogen dioxide and these protein-SH groups. ESR spin-trapping studies indicated that strong oxidants including sulfur-containing radicals may be generated by the interaction of nitrogen dioxide with Cys and GSH. The radical oxidants may abstract hydrogen atom from polyunsaturated fatty acids to initiate lipid peroxidation in the presence of molecular oxygen. The present results suggest that nitrogen dioxide-induced lipid peroxidation can be enhanced by protein-SH groups under relatively severe conditions of nitrogen dioxide (below 100 ppm) just employed in the present study.

It has been demonstrated that exposure of nitrogen dioxide (up to 500 ppm) to Chinese hamster cells causes cellular DNA strand breaks.[19,20] In the present study, exposure of nitrogen dioxide in air to supercoiled plasmid DNA resulted in a slight strand breaking. To our surprise, Cys, GSH and BSA did not prevent but enhanced the DNA strand breaking. Oxygen may not be necessary for the enhancement. Both the strand breaking activity and the ESR spin signals generated by the interaction of nitrogen dioxide with Cys were abolished by Tyr, indicating that the the strand breaking activity was due to the radical species. As well as nitrogen dioxide alone, the radical species may abstract hydrogen atom from the deoxyribose moiety to cleave the phosphodiester bonds of DNA molecules. The present results indicate the possible enhancement of nitrogen dioxide-induced DNA strand breaks by these thiol compounds under relatively severe conditions.

It has been shown that nitrogen dioxide at low levels can cause damage of amino acids, most effectively Trp and Tyr.[15] Damage of Trp and Tyr is little prevented by Cys, indicating that Trp and Tyr are more susceptible to nintrogen dioxide than Cys.[15] In the present study, while the ESR spin signals from the interaction of nitrogen dioxide with Cys was abolished by the presence of Tyr, the signals from the interaction with Trp could not. Thus, Trp may be more susceptible to nitrogen dioxide than Tyr. The order of the susceptibility of these amino acids to nitrogen dioxide may be Trp, Tyr and Cys.

Our previous study has shown that the chemistry of a spin trapping agent

DBNBS[31,32] is very complex, and it gives weak ESR spin signals after long time incubation with amino acids including Cys, GSH, Trp and Pro.[34] In the present study, the more intense nitroxyl free radical spin signals were observed when DBNBS was added to the reaction mixture of nitrogen dioxide with Cys, GSH and Trp. The nitroxyl free radicals from Cys and GSH may be derived from sulfur-containing radicals, and that from Trp may be from carbon-centered radical, judging from the hfsc of the nitroxyl free radicals. While the sulfur-containing radicals generated from protein-SH groups were active to initiate lipid peroxidation and to induce DNA strand breaks, the carbon-centered radical generated from Trp was not. The precise structures of these radical species were now unclear.

In conclusion, protein-SH groups enhanced lipid peroxidation and DNA strand breaks induced by nitrogen dioxide at below 100 ppm in air by generating sulfur-containing radicals.

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