Carcinogenesis vol.18 no.7 pp.1359–1363, 1997

Synergistic induction of DNA strand breakage by cigarette tar and nitric oxide

Yumiko Yoshie[1] and Hiroshi Ohshima[2]

Unit of Endogenous Cancer Risk Factors, International Agency for Research on Cancer, 150 Cours Albert Thomas, 69372, Lyon, Cedex 08, France
[1]Visiting Scientist at IARC from: Tokyo University of Fisheries, 4-5-7 Konan, Minato-ku, Tokyo 108, Japan

[2]To whom correspondence should be addressed

Cigarette smoking is a major cause of human cancer at a variety of sites, although its carcinogenic mechanisms remains unestablished. Cigarette smoke can be divided into two phases, gas phase and particulate matter (tar). Both phases contain high concentrations of oxidants and free radicals, especially nitric oxide (NO) and nitrogen oxides in the gas phase and quinone/hydroquinone complex in the tar. We have found that incubation of pBR322 plasmid DNA with aqueous extracts of cigarette tar and a NO-releasing compound (diethylamine NONOate) caused synergistic induction of DNA single-strand breakage, whereas either cigarette tar alone or NO alone induced much less strand breakage. This synergistic effect of cigarette tar and NO on DNA strand breakage was prevented by high concentrations of superoxide dismutase, carboxy-PTIO (an NO-trapping agent) or *N*-acetylcysteine, whereas hydroxyl radical scavengers such as dimethylsulfoxide, ethanol and D-mannitol did not show inhibitory effects. Possible mechanisms for this synergistic effect mediated by cigarette tar and NO are proposed, including involvement of peroxynitrite, which is a strong oxidant and nitrating agent formed rapidly by the reaction between NO and $O_2^{\cdot -}$. NO is present in the gas phase of smoke and may be formed by a constitutive or inducible NO synthase in the lung, whereas $O_2^{\cdot -}$ is generated by auto-oxidation of polyhydroxyaromatic compounds such as catechol and 1,4-hydroquinone present in cigarette tar. Thus, potent reactive species including peroxynitrite formed by the interaction between cigarette tar and NO may play an important role in smoking-related diseases including lung cancer.

Introduction

Epidemiological studies have shown that smoking is a major cause of human cancer at a variety of sites (1,2). However the mechanism by which cigarette smoking induces cancer remains unestablished. Cigarette smoke contains more than 3800 constituents, including a variety of tobacco-specific carcinogens, cocarcinogens and tumor promoters (1). Cigarette smoke can be also divided into two phases, gas phase and particulate matter (tar), both of which contain high concentrations of oxidants and free radicals. Freshly generated cigarette smoke contains up to 600 μg of nitric oxide (NO*) per cigarette in the gas phase (3). The NO concentration in main-stream smoke is correlated linearly with the amount of nitrate in a cigarette (3). However, up to 100 μg of NO in the smoke of a non-filter cigarette derives from oxidation of nitrogenous components in tobacco and, probably, from the oxidation of atmospheric nitrogen (4).

Cigarette tar contains high concentrations of radicals, which are sufficiently stable to be detected by electron spin resonance (5). The principal radical species have been identified as a quinone/hydroquinone (Q/QH_2) complex held in a tarry matrix (5–7). Pryor and his collaborators (8,9) have suggested that this Q/QH_2 polymer is an active redox system that can reduce molecular dioxygen to produce superoxide ($O_2^{\cdot -}$), leading further to the formation of hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$).

It has been demonstrated that cigarette smoke causes single-strand breaks in DNA from cultured human and rodent cells (8,10–14). Aqueous extracts of cigarette tar also induce strand breakage in supercoiled phage DNA (15). The induction of these strand breaks is inhibited by the addition of superoxide dismutase (SOD) or catalase, and therefore is presumed to be caused by active oxygen species such as $O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$ (8,10,11,14,15). The gas phase of cigarette smoke has recently been shown to cause single-strand breaks as well as modifications of DNA bases including formation of xanthine and hypoxanthine in human respiratory tract epithelial cells, suggesting that reactive nitrogen species may be involved in the induction of DNA damage (16).

There have been, however, no previous studies on cigarette smoke-induced DNA damage, using both the tar and gas-phase products in combination. We have recently found that concurrent incubation of plasmid DNA with an NO-releasing compound and a polyhydroxyaromatic compound, such as catechol or 1,4-hydroquinone, leads to synergistic induction of DNA strand breaks (Yoshie and Ohshima, submitted). This DNA breakage is inhibited by SOD and NO-trapping agents such as carboxy-PTIO or oxyhemoglobin, suggesting that simultaneous presence of both NO and $O_2^{\cdot -}$ is required to exert this synergistic effect on DNA damage. As cigarette smoke contains high concentrations of NO in the gas phase and an $O_2^{\cdot -}$-generating Q/QH_2 system in tar, we have examined the effects of combinations of cigarette tar and NO on the induction of DNA strand breakage. We report here that cigarette tar and NO act synergistically to induce more deleterious effects on DNA.

Materials and methods

Chemicals and biochemical reagents

Diethylamine NONOate (DEA-NO) and 1H-imidazol-1-yloxy, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-, 3-oxide, potassium salt (carboxy-PTIO) were obtained from Cayman Chemical Co. (Ann Arbor, MI). Superoxide dismutase (SOD), catalase, selenocystine, selenomethionine and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO). The plasmid pBR322 and desferrioxamine were obtained from Pharmacia (Uppsala, Sweden) and Ciba−Geigy Lab. (Rueil-Malmaison, France), respectively. All other chemicals were commercially available.

***Abbreviations:** NO, nitric oxide; Q/QH_2, quinone/hydroquinone; SOD, superoxide dismutase; DMSO, dimethylsulfoxide; DEA-NO, diethylamine NONOate; carboxy-PTIO, 1H-imidazol-1-yloxy, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-3-oxide, potassium salt.

Preparation of aqueous extracts of cigarette tar

Three cigarettes (non-filtered French cigarette; Gauloise) were smoked using a vacuum pump. Cigarette smoke was passed through a glass filter (Advantec GA-200) unit, purchased from Advantec (Tokyo, Japan). The tar on the filter was extracted with 6 ml of Chelex-100 (Sigma)-treated 0.01 mM sodium phosphate buffer, pH 7.4 and aliquots were stored at −80°C. The tar extract could be stored under these conditions without an apparent reduction in DNA-breaking activity for at least 3 months. The tar extract was further diluted five times with Chelex-treated water before use.

Reactions of plasmid DNA with NO and tar

All solutions used were treated with Chelex-100. The experiments were carried out by incubating plasmid pBR322 DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4 containing either 0–1 mM NO-releasing compound (DEA-NO) alone, various volumes (0–4 μl) of tar extract alone or the two compounds in combination at 37°C for 30 min (final volume was 10 μl). The influence of incubation time (up to 2 h) on strand breakage was studied similarly in 10 μl of 100 mM sodium phosphate buffer, pH 7.4, containing DEA-NO (0 or 0.1 mM) and tar extract (2 μl).

Effects of SOD, catalase, scavengers of NO and HO radicals, antioxidants and chelating agents on NO/tar-induced single-strand breakage

The effects of SOD and catalase (each 500 or 5000 units/ml), the NO-trapping agent (carboxy-PTIO at 1 or 10 mM), hydroxyl-radical scavengers (DMSO, ethanol and D-mannitol at 1 or 10 mM), anti-oxidants (ascorbic acid, *N*-acetylcysteine and selenomethionine at 1 or 10 mM: uric acid and selenocystine at 0.2 mM), sodium azide and desferrioxamine (at 1 or 10 mM) on DNA strand breakage induced by NO plus tar, were studied by incubating plasmid pBR322 DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DEA-NO plus 2 μl tar extract, at 37°C for 30 min.

Effects of Fe^{2+} or Cu^{2+} on NO/tar-induced single-strand breakage

The effects of ferrous ammonium sulfate or cupric sulfate (0–100 μM) on DNA strand breakage induced by NO alone (0, 0.1 or 1 mM DEA-NO), tar (2 μl) alone or NO plus tar were studied by incubating plasmid pBR322 DNA (100 ng) in 100 mM sodium phoshate buffer, pH 7.4 at 37°C for 30 min.

Analysis of DNA strand breakage

The conversion of the covalently closed circular double-stranded supercoiled DNA (form I) to a relaxed open circle (form II) and a linear form (form III) was used to investigate DNA strand breakage induced by NO plus tar. The reaction was terminated by the addition of 2 μl of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol). An aliquot (8 μl) of the mixture was loaded onto a 0.7% agarose gel prepared with 45 mM Tris–borate/1 mM EDTA (pH 8.0) containing 0.5 μg/ml ethidium bromide. We included non-treated PBR322 plasmid as a control in each run of gel electrophoresis, which was carried out at 8.5 V/cm for 90 min. Percentages of supercoiled (form I), relaxed (form II) and linear (form III) forms were calculated by an Imaging Densitometer Model GS-670, BIO-RAD (Hercules,CA). It was previously reported that ethidium bromide stains supercoiled DNA and relaxed open circular DNA differently, and a correction factor is sometimes applied to calculate percentages of the form I and II (17). However, in order to compare the present results with those published by other groups and ourselves (18–20), percentages of the form I and II were calculated without correction factors. Results are expressed as percentages of form II after subtracting background (non-treated pBR322 plasmid contained 5–15% form II, but no detectable form III) from the percentages calculated for the treated plasmid DNA, unless otherwise noted. All experiments were carried out in triplicate and statistical significance was calculated using Student's *t*-test.

Results and discussion

Figure 1 shows results obtained from a typical agarose gel electrophoresis of pBR322 plasmid DNA which was incubated with aqueous extracts of cigarette tar (2 μl) alone, an NO-releasing compound (DEA-NO) alone, or the two compounds in combination. Incubation of the plasmid DNA with cigarette tar alone resulted in a small increase in conversion of the covalently closed circular double-stranded supercoiled DNA (form I) to a relaxed open circle (form II) (16%; Figure 1, lane 2), compared with that of the non-treated plasmid (~12%; Figure 1, lane 1). DEA-NO at 0.1 mM did not increase the formation of form II significantly (12%; Figure 1, lane 3). However, when the plasmid was incubated in the presence of

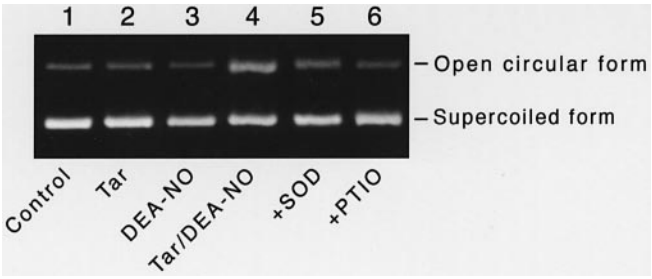

Fig. 1. A typical agarose-gel electrophoresis of pBR322 plasmid DNA incubated with either an NO-releasing compound (DEA-NO) alone, an aqueous extract of cigarette tar alone or the two compounds in combination and effects of NO and $O_2^{\cdot -}$-scavenging compounds on the DNA damage induced by cigarette tar and NO. The experiments were carried out by incubating pBR322 plasmid DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4, containing either 0.1 mM DEA-NO alone (**lane 2**), 2 μl of cigarette tar alone (**lane 3**) or the two compounds in combination (**lane 4**) at 37°C for 30 min (final volume was 10 μl). Effects of SOD (5000 U/ml) (**lane 5**) and carboxy-PTIO (10 mM) (**lane 6**) on the DNA damage induced by cigarette tar and NO were also studied.

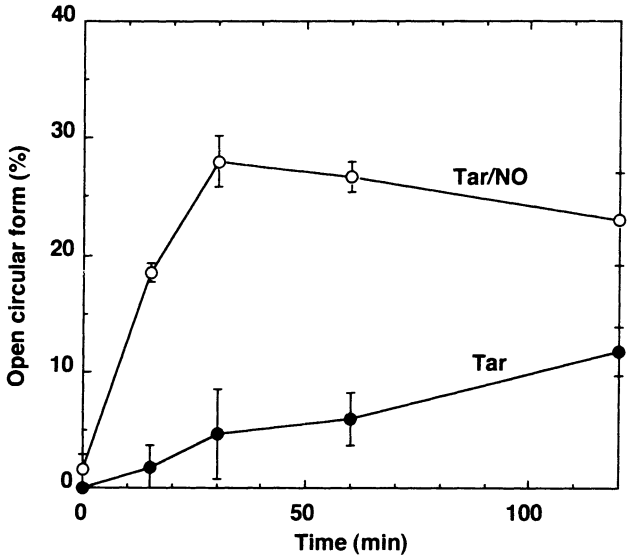

Fig. 2. Effect of incubation time on DNA strand breakage induced by cigarette tar in the presence or absence of DEA-NO. The experiments were carried out by incubating pBR322 plasmid DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4, containing either 2 μl of cigarette tar alone (●) or 2 μl of cigarette tar together with 0.1 mM DEA-NO (○) at 37°C for up to 120 min (final volume was 10 μl). Means ± SD (n = 3) are presented.

both cigarette tar and DEA-NO, significant conversion of form I to form II was observed (42%; Figure 1, lane 4). Although the linear form III could easily be detected by our method, there was no apparent formation of form III under the present experimental conditions. Figure 2 shows that the strand breakage induced by tar plus NO increased in the first 30 min and reached a plateau at 30 min, and no further increase was observed after this up to 120 min of incubation.

Figure 3A and B show the effects of concentrations of DEA-NO and cigarette tar on strand breakage. When the concentration of DEA-NO was constant at 0.1 mM, strand breakage (form II formation) was maximal with 1 μl of the tar extract; increased volumes of tar above 2 μl decreased induction of form II (Figure 3A). Cigarette tar alone did not induce strand breakage significantly under these conditions (Figure 3A). On the other hand, when the volume of tar added

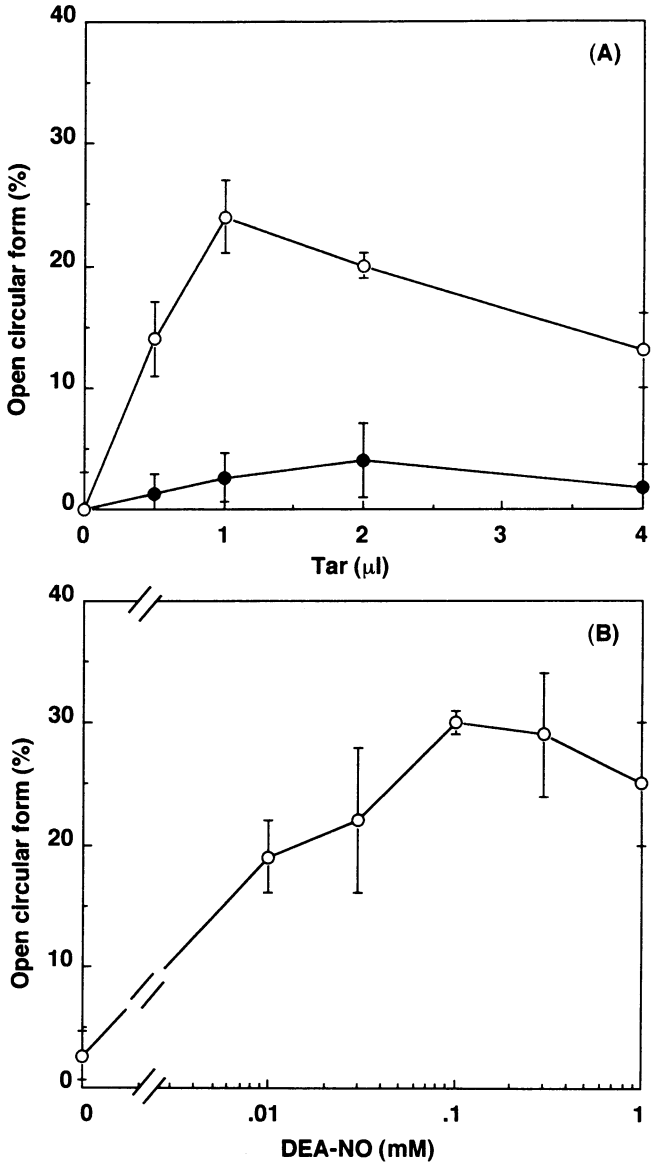

Fig. 3. Effect of concentrations of DEA-NO and aqueous extracts of cigarette tar on DNA strand breakage. The plasmid DNA was incubated with (**A**) various concentrations of cigarette tar in the presence (○) or absence (●) of DEA-NO (0.1 mM), and (**B**) various concentrations of DEA-NO (0–1 mM) in the presence of cigarette tar (2 μl). Means ± SD (n = 3) are presented.

to the reaction mixture was constant at 2 μl, strand breakage was maximal with 0.1 mM DEA-NO and increased concentrations of DEA-NO slightly decreased the form II formation (Figure 3B). No linear form III was observed even at higher concentrations of cigarette tar or DEA-NO alone or tar and NO in combination. DEA-NO alone did not induce strand breakage. Recent studies have shown that both hydroxylation of benzoate and oxidation of dihydrorhodamine caused by $O_2^{\cdot -}$ generated by hypoxanthine/xanthine oxidase and NO from spermine NONOate were maximal when NO and $O_2^{\cdot -}$ release was equimolar, whereas increased concentrations of one of the radical species, relative to the other radical, inhibited these hydroxylation/oxidation reactions (21). Similar inhibitory effects of NO on $O_2^{\cdot -}$-induced lipid peroxidation (22) as well as DNA strand breakage induced by polyhydroxyaromatic compounds such as catechol and 1,4-hydroquinone (Yoshie and Ohshima, submitted) have been reported.

In order to identify the compound(s) responsible for the tar/NO-induced strand breakage, we examined the effects on strand breakage of various $HO^{\cdot}$ scavengers (DMSO, ethanol, D-mannitol), antioxidants (ascorbic acid, uric acid, *N*-acetylcysteine) and other trapping agents. As Figure 1 shows, the addition of SOD (5000 U/ml) reduced the formation of an open circular DNA (form II) induced by tar and NO from 42 to 26% (lane 5). Similarly 10 mM carboxy-PTIO [NO trapping agent, (23)] reduced breakage from 42 to 20% (lane 6). Among the tested compounds, only higher concentrations of carboxy-PTIO, SOD and selenomethionine significantly ($P < 0.05$) inhibited the strand breakage induced by tar plus NO. The presence of both carboxy-PTIO and SOD also inhibited the formation of form II by ~70%. *N*-Acetylcysteine also tended to inhibit strand breakage, although the difference was not statistically significant (Figure 4A and B). We have recently found that the strand breakage induced by peroxynitrite is effectively inhibited by high concentrations of ascorbate, desferrioxamine, carboxy-PTIO and uric acid. On the other hand, the breakage induced by pyrogallol and DEA-NO was less effectively inhibited by ascorbate and desferrioxamine, but strongly inhibited by carboxy-PTIO and uric acid. The same scavengers and antioxidants (ascorbate, desferrioxamine, carboxy-PTIO and uric acid) were, however, less effective against the strand breakage induced by cigarette tar and NO (Figure 4). The $HO^{\cdot}$ scavengers (DMSO, ethanol, D-mannitol) showed no inhibitory effects against the breakage induced by cigarette tar plus NO (Figure 4), peroxynitrite or pyrogallol plus NO (Yoshie and Ohshima, submitted).

Metal ions have been reported to be involved in $HO^{\cdot}$ generation by aqueous extracts of cigarette tar (24). We examined the effects of Fe^{2+} or Cu^{2+} on the DNA strand breakage induced by cigarette tar alone, NO alone and cigarette tar and NO in combination. As Figure 5A shows, increasing concentrations of ferrous ion up to 100 μM dose-dependently enhanced DNA strand breakage. There was no synergistic effect of Fe^{2+} on the breakage induced by tar alone, NO alone or tar plus NO in combination. On the other hand, low concentrations (0.1–10 μM) of Cu^{2+} did not show any effect on the breakage induced by tar alone or tar plus NO, whereas a high concentration (100 μM) enhanced markedly the breakage induced by these agents. In the absence of cigarette tar, 100 μM Cu^{2+} did not enhance strand breakage.

We have demonstrated that DNA strand breakage is induced synergistically when plasmid DNA is incubated in the presence of both an NO-releasing compound and aqueous extracts of cigarette tar (Figure 1). Either an NO-releasing compound or a tar extract alone induced much fewer strand breaks. Scavenger studies indicate that the breakage induced by NO plus cigarette tar can be inhibited by carboxy-PTIO (an NO-trapping agent), SOD and selenomethionine, and weakly by *N*-acetylcysteine, but not by $HO^{\cdot}$ scavengers such as DMSO, ethanol and D-mannitol. These results suggest that the free $HO^{\cdot}$ is not involved; rather a new oxidant(s) formed by the reaction between NO and cigarette tar could be responsible for causing DNA strand breakage. One possible compound is peroxynitrite ($ONOO^-$), which is formed rapidly by the reaction between NO and $O_2^{\cdot -}$ (25,26). The latter compound ($O_2^{\cdot -}$) can be generated from auto-oxidation of polyhydroxyaromatics such as catechol and 1,4-hydroquinone, both present in high concentrations in cigarette tar (1). Peroxynitrite is a strong oxidant and nitrating agent that can initiate reactions characteristic of $HO^{\cdot}$, nitronium ion (NO_2^+) and nitrogen dioxide radical ($^{\cdot}NO_2$)

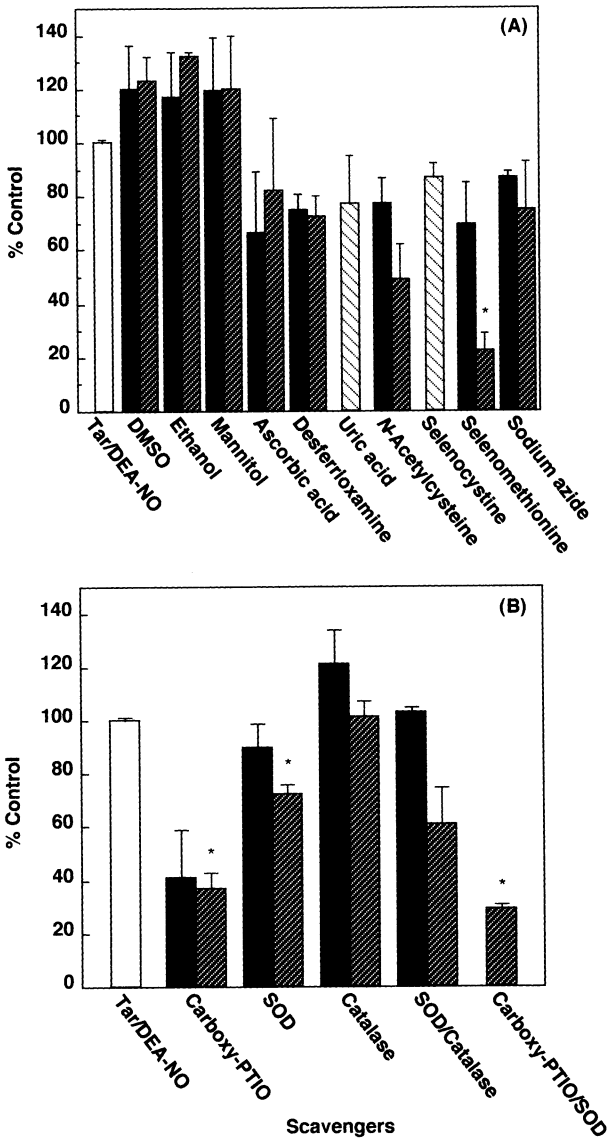

Fig. 4. Effects of various scavengers of free radicals, antioxidants, antioxidant enzymes and a metal ion chelator on DNA strand breakage induced by cigarette tar and NO. The experiments were carried out as described in Figure 1, using 2 μl of cigarette tar together with 0.1 mM DEA-NO at 37°C for 30 min. (**A**) Concentrations of scavengers were 1 mM (filled bar) or 10 mM (dark hatched bar), except for uric acid and selenocystine, whose concentration was 0.2 mM (light hatched bar) (**B**). Both SOD and catalase were examined at either 500 or 5000 U/ml. Carboxy-PTIO was examined at 1 mM (filled bar) or 10 mM (dark hatched bar). When the combination of carboxy-PTIO and SOD was examined, their concentrations were 10 mM and 5000 U/ml, respectively. Means ± SD ($n = 3$) are presented. * Significantly different from control (tar plus DEA-NO) ($P < 0.05$).

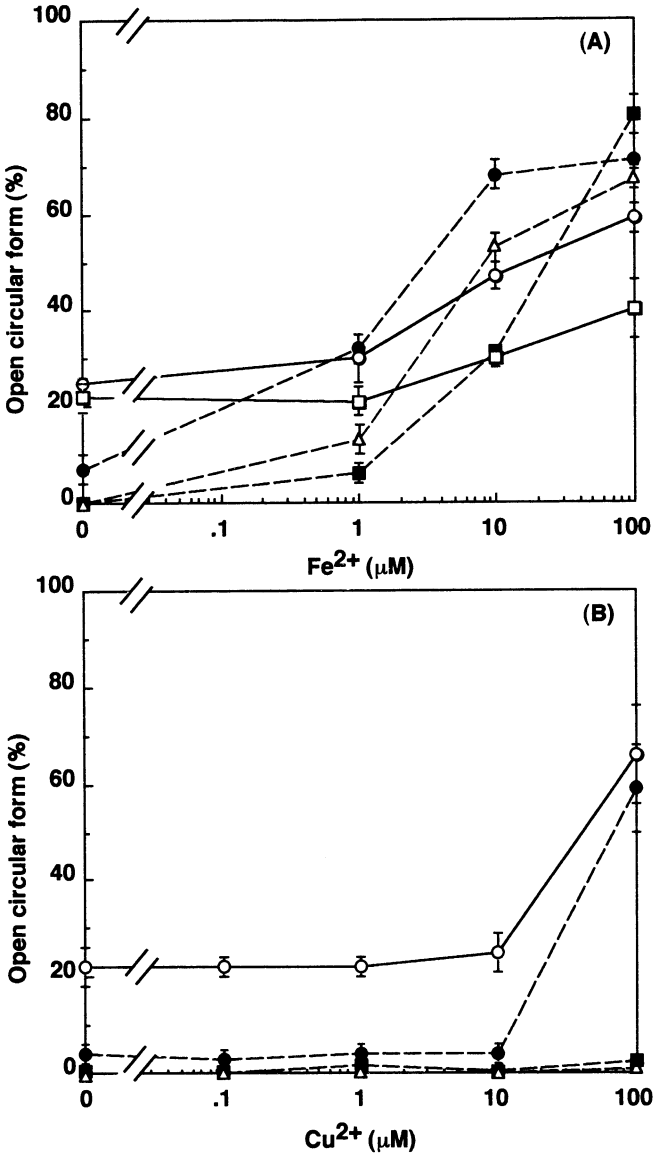

Fig. 5. Effects of Fe^{2+} and Cu^{2+} ions on DNA strand breakage induced by cigarette tar with or without NO. The experiments were carried out as described in Figure 1, using 2 μl of cigarette tar together with either 0.1 or 1 mM DEA-NO in the presence of ferrous ammonium sulfate (A) or $CuSO_4$ (B) at 37°C for 30 min. △: only metal ion; ●: tar plus metal ion; ■: 0.1 mM NO plus metal ion; ○: tar plus 0.1 mM DEA-NO and metal ion; □: tar plus 1 mM DEA-NO and metal ion. Means ± SD ($n = 3$) are presented.

(25,26). Peroxynitrite can induce DNA strand breaks in plasmid DNA *in vitro* (19,27,28), which, however, cannot be inhibited by $HO^{\cdot}$ scavengers such as D-mannitol and DMSO [(19), Yoshie and Ohshima, submitted]. Similarly, the strand breakage induced by cigarette tar plus NO was not inhibited by $HO^{\cdot}$ scavengers (DMSO, ethanol and D-mannitol) (Figure 4). It has recently been reported that selenomethionine can inhibit the induction of single-strand breaks caused by peroxynitrite (18). Selenomethionine at high concentrations effectively inhibited the DNA breakage caused by tar and NO. Taken together, our data suggest that new oxidants, possibly including peroxynitrite, could be generated by the reaction between cigarette tar and NO, thus supporting the proposal of Pryor and his collaborators that cigarette smoke may form peroxynitrite (9,29). They have reported that cigarette smoke as well as peroxynitrite can inactivate α1-protease inhibitor (30,31), which may lead to excessive proteinase activation and subsequent destruction of the connective tissue in the lower respiratory tract. Such connective tissue destruction has been associated with emphysema observed in smokers (32). In addition to peroxynitrite, however, the reaction between NO and cigarette tar (including both water-soluble and lipophilic components) may also yield other types of compound (e.g. nitroso/nitro derivatives of polyaromatic hydrocarbons), which could cause DNA damage.

NO is present in the gas phase of smoke at high concentra-

tions (see Introduction). NO can also be formed endogenously by a constitutive or inducible NO synthase in the lung. Recent studies have demonstrated that an inducible form of NO synthase is expressed constitutively in human airway epithelial cells in the lung (33–35). Therefore NO may react directly with polyhydroxyaromatic compounds such as catechol and 1,4-hydroquinone present in cigarette tar, or with $O_2^{\cdot-}$ generated by an active Q/QH_2 redox system to form peroxynitrite. Thus, reactive species, including peroxynitrite and unidentified new compounds, formed by the interaction between cigarette tar and NO may play an important role in smoking-related diseases including lung cancer.

Acknowledgements

The authors thank Dr T. Nakayama (University of Shizuoka, Japan) for his advice on cigarette tar collection, Dr J.Cheney for editing the manuscript, and Mrs P.Collard for secretarial assistance. Y.Y. is a recipient of a fellowship from the Japan Society for the Promotion of Science.

Note added in proof

The paper described in the text as 'Yoshie and Ohshima, submitted' has now been published: Yoshie,Y. and Ohshima,H. (1997) Nitric oxide synergistically enhances DNA strand breakage induced by polyhydroxyaromatic compounds, but inhibits that induced by the Fenton reaction. *Arch. Biochem. Biophys.* **342**, 13–21.

References

1. International Agency for Research on Cancer (1986) *Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans. Vol. 38. Tobacco Smoking*. IARC, Lyon, France.
2. Parkin,D.M., Pisani,P., Lopez,A.D. and Masuyer,E. (1994) At least one in seven cases of cancer is caused by smoking. Global estimates for 1985. *Int. J. Cancer*, **59**, 494–504.
3. Brunnemann,K.D. and Hoffmann,D. (1982) Pyrolytic origins of major gas phase constituents of cigarette smoke. *Recent Adv. Tob. Sci*., **8**, 103–140.
4. Norman,V., Ihrig,A.M., Larson,T.M. and Moss,B.L. (1983) The effect of some nitrogenous blend componets on NO/NOx and HCN levels in mainstream and sidestream smoke. *Beitr. Tabakforsch*., **12**, 55–62.
5. Pryor,W.A., Prier,D.G. and Church,D.F. (1983) Electron-spin resonance study of mainstream and sidestream cigarette smoke: nature of the free radicals in gas-phase smoke and in cigarette tar. *Environ. Health Perspect*., **47**, 345–355.
6. Pryor,W.A., Hales,B.J., Premovic,P.I. and Church,D.F. (1983) The radicals in cigarette tar: their nature and suggested physiological implications. *Science*, **220**, 425–427.
7. Church,D.F. and Pryor,W.A. (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect*., **64**, 111–126.
8. Stone,K.K., Bermudez,E. and Pryor,W.A. (1994) Aqueous extracts of cigarette tar containing the tar free radical cause DNA nicks in mammalian cells. *Environ. Health Perspect*., **102 (**Suppl. 10), 173–178.
9. Pryor,W.A. (1992) Biological effects of cigarette smoke, wood smoke, and the smoke from plastics: the use of electron spin resonance. *Free Radic. Biol. Med*., **13**, 659–676.
10. Nakayama,T., Kodama,M. and Nagata,C. (1984) Generation of hydrogen peroxide and superoxide anion radical from cigarette smoke. *Gann*, **75**, 95–98.
11. Nakayama,T., Kaneko,M., Kodama,M. and Nagata,C. (1985) Cigarette smoke induces DNA single-strand breaks in human cells. *Nature*, **314**, 462–464.
12. Fielding,S., Short,C., Davies,K., Wald,N., Bridges,B.A. and Waters,R. (1989) Studies on the ability of smoke from different types of cigarettes to induce DNA single-strand breaks in cultured human cells. *Mutat. Res*., **214**, 147–151.
13. Leanderson,P. and Tagesson,C. (1992) Cigarette smoke-induced DNA damage in cultured human lung cells: role of hydroxyl radicals and endonuclease activation. *Chem. Biol. Interact*., **81**, 197–208.
14. Stone,K., Bermudez,E., Zang,L.Y., Carter,K.M., Queenan,K.E. and Pryor,W.A. (1995) The ESR properties, DNA nicking, and DNA association of aged solutions of catechol versus aqueous extracts of tar from cigarette smoke. *Arch. Biochem. Biophys*., **319**, 196–203.
15. Borish,E.T., Cosgrove,J.P., Church,D.F., Deutsch,W.A. and Pryor,W.A. (1985) Cigarette tar causes single-strand breaks in DNA. *Biochem. Biophys. Res. Commun*., **133**, 780–786.
16. Spencer,J.P., Jenner,A., Chimel,K., Aruoma,O.I., Cross,C.E., Wu,R. and Halliwell,B. (1995) DNA damage in human respiratory tract epithelial cells: damage by gas phase cigarette smoke apparently involves attack by reactive nitrogen species in addition to oxygen radicals. *FEBS Lett*., **375**, 179–182.
17. Fisher,G.R. and Gutierrez,P.L. (1991) Free radical formation and DNA strand breakage during metabolism of diaziquone by NAD(P)H quinone-acceptor oxidoreductase (DT-diaphorase) and NADPH cytochrome c reductase. *Free Radic. Biol. Med*., **11**, 595–607.
18. Roussyn,I., Briviba,K., Masumoto,H. and Sies,H. (1996) Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. *Arch. Biochem. Biophys*., **330**, 216–218.
19. Salgo,M.G., Stone,K., Squadrito,G.L., Battista,J.R. and Pryor,W.A. (1995) Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem. Biophys. Res. Commun*., **210**, 1025–1030.
20. Yermilov,V., Yoshie,Y., Rubio,J. and Ohshima,H. (1996) Effects of carbon dioxide/bicarbonate on induction of DNA single-strand breaks and formation of 8-nitroguanine, 8-oxoquanine and base-propenal mediated by peroxynitrite. *FEBS Lett*., **399**, 67–70.
21. Miles,A.M., Bohle,D.S., Glassbrenner,P.A., Hansert,B., Wink,D.A. and Grisham,M.B. (1996) Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J. Biol. Chem*., **271**, 40–47.
22. Rubbo,H., Radi,R., Trujillo,M., Telleri,R., Kalyanaraman,B., Barnes,S., Kirk,M. and Freeman,B.A. (1994) Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J. Biol. Chem*., **269**, 26066–26075.
23. Akaike,T., Yoshida,M., Miyamoto,Y., Sato,K., Kohno,M., Sasamoto,K., Miyazaki,K., Ueda,S. and Maeda,H. (1993) Antagonistic action of imidazolineoxyl N-oxides against endothelium-derived relaxing factor/·NO through a radical reaction. *Biochemistry*, **32**, 827–832.
24. Cosgrove,J.P., Borish,E.T., Church,D.F. and Pryor,W.A. (1985) The metal-mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. *Biochem. Biophys. Res. Commun*., **132**, 390–396.
25. Beckman,J.S., Chen,J., Ischiropoulos,H. and Crow,J.P. (1994) Oxidative chemistry of peroxynitrite. *Methods Enzymol*., **233**, 229–240.
26. Pryor,W.A. and Squadrito,G.L. (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am. J. Physiol*., **268**, L699−L722.
27. King,P.A., Anderson,V.E., Edwards,J.O., Gustafson,G., Plumb,R.C. and Suggs,J.W. (1992) A stable solid that generates hydroxyl radical upon dissolution in aqueous solutions: reactions with proteins and nucleic acid. *J. Am. Chem. Soc*., **114**, 5430–5432.
28. Rubio,J., Yermilov,V. and Ohshima,H. (1996) DNA damage induced by peroxynitrite: formation of 8-nitroguanine and base propenals. In Moncada,S., Stamler,J., Gross,S. and Higgs,E.A. (eds) *The Biology of Nitric Oxide. Part 5*. Portland Press, London. p. 34.
29. Stone,K. and Pryor,W.A. (1994) Cigarettes: then and now. *J. Lab. Clin. Med*., **124**, 313–314.
30. Evans,M.D. and Pryor,W.A. (1992) Damage to human alpha-1-proteinase inhibitor by aqueous cigarette tar extracts and the formation of methionine sulfoxide. *Chem. Res. Toxicol*., **5**, 654–660.
31. Moreno,J.J. and Pryor,W.A. (1992) Inactivation of alpha 1-proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol*., **5**, 425–431.
32. Evans,M.D. and Pryor,W.A. (1994) Cigarette smoking, emphysema, and damage to alpha 1-proteinase inhibitor. *Am. J. Physiol*., **266**, L593−L611.
33. Kobzik,L., Bredt,D.S., Lowenstein,C.J., Drazen,J., Gaston,B., Sugarbaker,D. and Stamler,J.S. (1993) Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am. J. Respir. Cell Mol. Biol*., **9**, 371–377.
34. Asano,K., Chee,C.B., Gaston,B., Lilly,C.M., Gerard,C., Drazen,J.M. and Stamler,J.S. (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. *Proc. Natl Acad. Sci. USA*, **91**, 10089–10093.
35. Guo,F.H., De Raeve,H.R., Rice,T.W., Stuehr,D.J., Thunnissen,F.B. and Erzurum,S.C. (1995) Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*. *Proc. Natl Acad. Sci. USA*, **92**, 7809–7813.

Received on November 26, 1996; revised on February 20, 1997; accepted on April 23, 1997