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ACTIVE OXYGEN CONTRIBUTES TO THE MAJOR PART OF CHROMOSOMAL ABERRATIONS IN V79 CHINESE HAMSTER CELLS EXPOSED TO N-HYDROXY-2-NAPHTHYLAMINE

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N-hydroxy-2-naphthylamine (NOH-2NA), an active metabolite of human occupational bladder carcinogens, induced, in V79 Chinese hamster cells, chromosomal aberrations which were suppressed in the presence of catalase and/or superoxide dismutase. The induction of the aberrations was more efficient in a more basic pH in parallel with the generation of hydrogen peroxide from NOH-2NA. The possible role of the oxidation product of NOH-2NA in the induction of the aberrations is discussed.

KEY WORDS: N-hydroxy-2-naphthylamine, chromosomal aberrations, active oxygen.

INTRODUCTION

There is increasing evidence that active oxygen species and free radicals might participate mainly in promotion steps of cell transformation as well as of tumorigenesis *in vivo*.[1] These radical species are known to induce DNA damage;[2] however, the role of DNA damage in carcinogenesis is unclear.

Generation of active oxygen species from several derivatives of naphthylamines and aminoazo dyes was found to be well correlated with the carcinogenicity of these compounds.[3] Actually N-hydroxy-2-naphthylamine (NOH-2NA), an active metabolite of the human occupational bladder carcinogen 2-naphthylamine, was found to induce oxidative DNA damage such as single-strand breaks[4] and thymine glycol[5] in cultured cells in addition to adduct formation at the C-8 and N^2-positions of guanine and the N^6-position of adenine.[6] Therefore, we have assumed that such oxidative DNA damage might in some way play a role in carcinogenesis, particularly in tumor promotion.

Although there is general consensus that gene mutation plays an important role in carcinogenesis, especially in initiation step,[2] active oxygen has little or no ability to induce gene mutations in microorganisms, except in plasmids in *Salmonella typhimurium*,[7] and in cultured mammalian cells.[8] On the other hand, chromosomal rearangement has been stressed as being important in both initiation and promotion steps of carcinogenesis.[1,9] Therefore, we assumed that oxidative DNA damage plays a role in carcinogenesis by inducing chromosomal mutations. Previously, we found that

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that hydrogen peroxide induces gene conversions efficiently in yeast cells at a non-toxic dose.[10] As a first step to investigate chromosomal mutations in mammalian cells, we tried to induce chromosomal aberrations in V79 Chinese hamster cells by exposing them to NOH-2NA. Preliminarily, we reported that a major part of abnormal metaphases in V79 cells exposed to NOH-2NA were suppressed by catalase and/or superoxide dismutase (SOD).[11] In the present study, we investigated the type of aberrations suppressed by these scavengers and determined the conditions under which active oxygen contributes to the generation of chromosomal aberrations and compared those conditions with those for hydrogen peroxide. We found that the contribution of active oxygen to induction of chromosomal aberrations by NOH-2NA was higher at basic pH and that the efficiency of NOH-2NA for induction of chromosomal aberrations was higher than that of a comparable concentration of hydrogen peroxide at neutral pH.

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MATERIALS AND METHODS

Cell Culture

V79 Chinese hamster cells were maintained in Eagle's minimum essential medium (MEM; GIBCO Laboratories, Gland Island, NY., USA) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and were incubated at 37°C in a humidified incubator in an atmosphere at 5% CO_2 in air.

Chromosome Studies

Inocula of 5×10^5 V79 cells were plated in plastic dishes (60 mm, Corning Glass Works, Corning, NY., USA). After 24 hr, the culture medium was replaced with HEPES-buffered Hanks' solution and the cells were treated with NOH-2NA dissolved in DMSO (Pierce Chemical Co., Rockfield, IL., USA) just before the treatment. For scavenging active oxygen, SOD (final concentration 50 μg/ml, Toyobo, Tokyo, Japan) and/or catalase (final concentration 50 μg/ml, from bovine blood; Sigma Chem. Co., St. Louis, Mo., USA) were added to the reaction medium at the time of addition of NOH-2NA. After 1 hr, the reaction was stopped by washing the cells twice with culture medium and then the cells were incubated in fresh culture medium containing 0.1 μg of colcemid (GIBCO) per ml for 1 hr to arrest the cells in mitosis. The second incubation after removal of NOH-2NA was performed when repairability of chromosomal damage was examined. Chromosome preparations were made by the usual flame-drying method[12] and stained with Giemsa solution. In each experiment, 100 metaphase chromosomes were examined for chromosomal aberrations.

Estimation of hydrogen peroxide

The concentration of hydrogen peroxide produced from 25 μM NOH-2NA in HEPES buffered Hanks' solution without cells at different pH after incubation for 1 hr at 37 °C was estimated by oxydation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS, Nakari Chem. LTD., Kyoto, Japan) in the presence of horseradish peroxidase after extraction by ethylacetate and ethylether.

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RESULTS

The inducibility of chromosomal aberrations in V79 cells by various concentrations of NOH-2NA is shown in Table I. The yield of aberrations increased in a dose-dependent manner. The condition of the treatment with NOH-2NA affected the yield of abnormal metaphases. The treatment in Hanks' solution in this study induced a higher yield of aberrations than that in the cultured medium used previously.[11] The types of aberrations induced were not affected by the conditions of the treatment and consisted mainly of chromatid gaps, ruffling (Figure 1a) and stickiness (Figure 1b). Ruffling and stickiness were more numerous than gaps at 15 μM NOH-2NA or higher. The yields of gaps, ruffling and stickiness were reduced after the second incubation, with a half-life of approximately 10 hr (data not shown). Thus, the chromosomal damage leading to these chromosomal aberrations was repairable.

In order to determine the contribution of active oxygen generated from NOH-2NA to the induction of various types of chromosomal aberrations, the effects of catalase and/or SOD were investigated (Table II). Table II shows that catalase or SOD alone suppressed the NOH-2NA-induced aberrations; however, the effect of the combination of the two scavengers was less than additive.

Previously we reported that hydrogen peroxide is produced from NOH-2NA more effectively at a more basic pH.[4] Therefore, if hydrogen peroxide generated from NOH-2NA contributes to the induction of chromosomal aberrations, the effect of catalase should be more marked at a basic pH. Figure 2 shows that the induced aberrations increased with increased alkalinity of the medium, in parallel with the formation of hydrogen peroxide and that of single-strand breaks.[4] Since there is a possibility that cellular defense activities against oxidative stress are diminished under basic conditions, the pH-dependent induction of aberrations by NOH-2NA was compared with that by hydrogen peroxide.[13]

Table III shows the effect of pH on the induction of aberrations by hydrogen peroxide and NOH-2NA. In contrast to NOH-2NA-induced aberrations, pH-dependent induction of aberrations by hydrogen peroxide was efficient at an acidic pH. In addition, the efficiency of the induction of aberrations by hydrogen peroxide at 50 μM was five times less than that of induction by NOH-2NA at 25 μM at pH 7.0, although

TABLE I
Induction of Chromosomal Aberrations by Various Concentrations of NOH-2NA

Concentration	Types of aberrations[a] (a)				Aberrant
of NOH-2NA (μM)	CG	CB	E	Ruf + Stc	metaphase (%)
0.0	7	1	0	0	8
7.5	10	2	0	12	24
10	24	0	0	16	36
15	18	4	2	32	44
20	20	2	0	51	65
25	26	0	0	70	72
30	16	4	0	84	90
40	8	0	0	92	98

a) Abbreviations of types of aberration are as follows: CG, chromatid gaps; CB, chromatid breaks; E, exchanges, Ruf, ruffling; Stc, stickiness.

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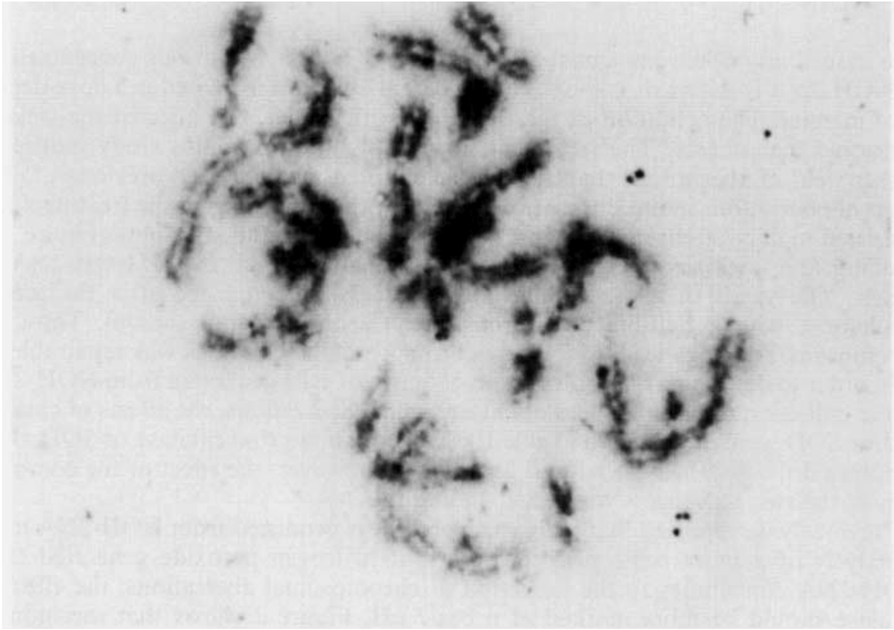

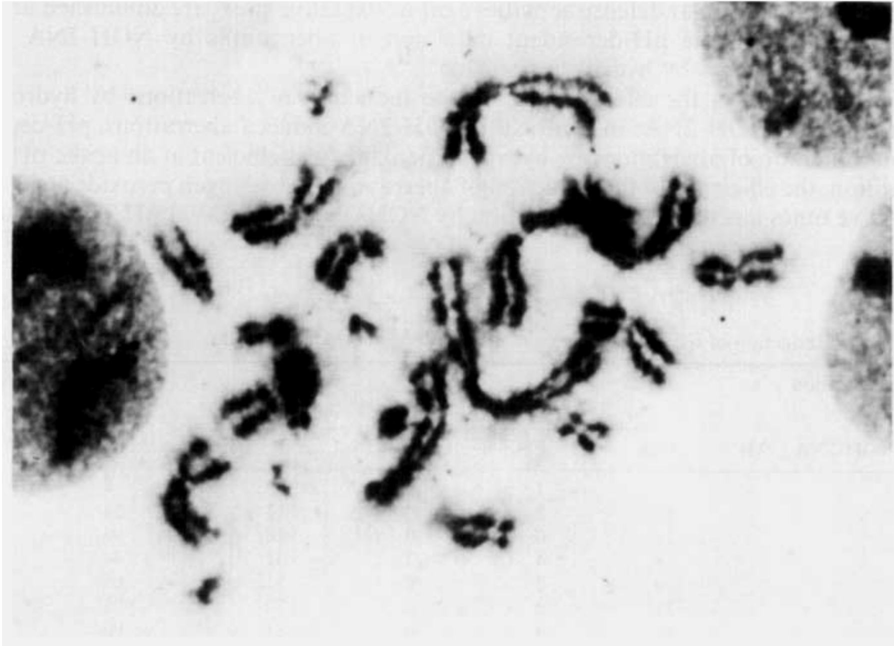

FIGURE 1 Chromosomal aberrations in V79 cells exposed to 25 μM NOH-2NA. The chromosomes in (a) show ruffling and those in (b) show stickiness.

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TABLE II
Effects of Catalase and Superoxide Dismutase on Chromosomal Aberrations

Treatment	Types of aberrations (%)				metaphase (%)
	CG	CB	E	Ruf + Stc	
0 μM NOH-2NA	3	3	0	0	6
+ catalase (50 μg/ml)	5	0	0	0	5
+ SOD (50 μg/ml)	2	2	0	0	4
+ catalase + SOD	4	2	0	0	6
20 μM NOH-2NA	13	2	0	7	21
+ catalase	8	3	0	0	10
+ SOD	5	1	0	2	9
+ catalase + SOD	10	0	0	0	10
30 μM NOH-2NA	19	14	0	65	84
+ catalase	21	4	0	27	58
+ SOD	15	1	0	38	54
+ catalase + SOD	30	3	0	10	42

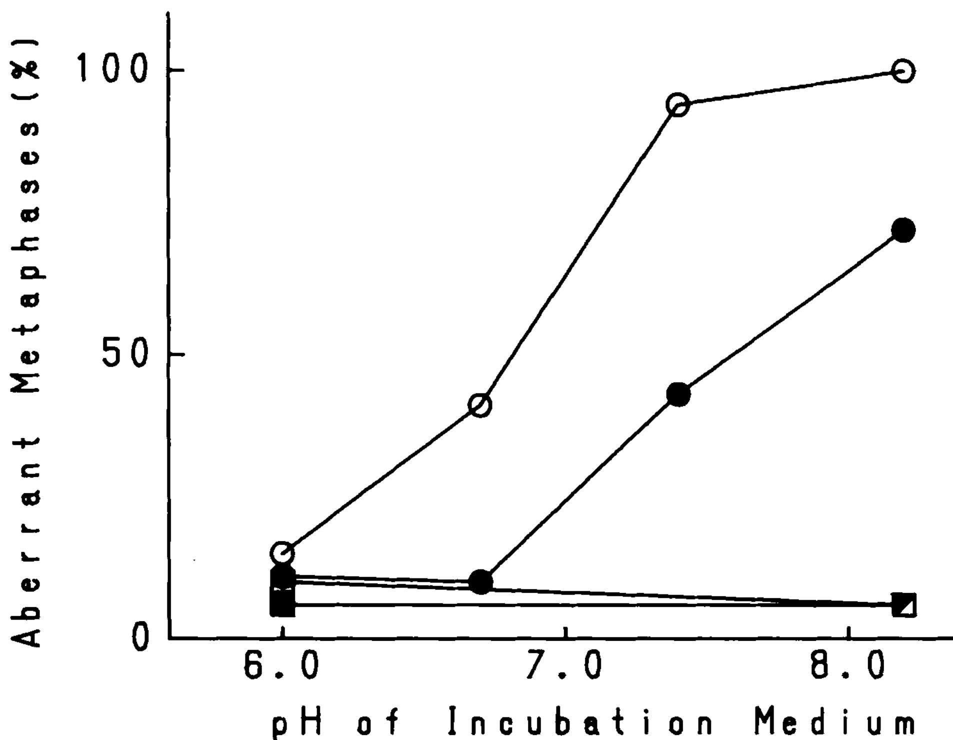

FIGURE 2 pH dependent induction of aberrant metaphases in V79 cells exposed to NOH-2NA and the effect of catalase and SOD: 25 μM NOH-2NA (○); 25 μM NOH-2NA plus catalase (50 μg/ml) and SOD (50 μg/ml) (●); mock-treated (□); catalase and SOD (■).

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TABLE III
Comparison of pH Dependent Induction of Chromosomal Aberrations by H_2O_2 and NOH-2NA.

Treatment	pH	Types of Aberrations (%)						Aberrant
		CG	CB	E	DC	EF[a]	Ruf + Stc	metaphase (%)
Control	6.0	8	0	0	0	0	0	8
	7.0	3	1	0	1	0	0	5
	7.8	6	0	0	0	0	0	6
H_2O_2 (50 μM)	6.0	45	14	5	0	2	0	52
	7.0	16	2	0	1	0	0	17
	7.8	11	3	0	2	0	0	16
H_2O_2 (100 μM)	6.0	38	18	6	2	43	0	80
	7.0	48	32	3	0	5	0	60
	7.8	36	18	3	2	3	0	48
NOH-2NA[b] (25 μM)	6.0	9	2	0	0	0	0	11
	7.0	39	19	0	2	0	52	75
	7.8	6	2	0	2	0	88	92

[a]EF shows the abbreviation of extensive fragmentation. [b]Concentration of H_2O_2 produced from 25 μM NOH-2NA in HEPES buffered Hanks' solution after incubation for 1 hr at 37°C was as follows; 1.1 μM (pH 6.0), 1.5 μM (pH 7.0) and 2.0 μM (pH 7.8).

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the concentration of hydrogen peroxide produced from 25 μM NOH-2NA in HEPES buffered Hanks' solution was 1.5 μM under this pH.

DISCUSSION

We found previously that oxidative DNA lesions such as single-strand breaks[4] and thymine glycol[5] are produced in a dose-dependent manner in cultured cells exposed to NOH-2NA, that their formation is suppressed by catalase and/or SOD and that single-strand breaks are produced more efficiently in a more basic pH. Induction of chromosomal aberrations by NOH-2NA paralleled the formation of DNA lesions in these points, in contrast to the reverse pH-dependent formation of covalent adducts between DNA and NOH-2NA in cultured cells exposed at various pH conditions.[14]

In spite of this good parallelism between the formation of DNA lesions and that of chromosomal aberrations, there was a discrepancy between them when the efficiency of formation of the abnormalities was compared. Contrary to the similar efficiency per concentration in the induction of single-strand breaks by NOH-2NA and by hydrogen peroxide,[13] the efficiency of induction of chromosomal aberrations by hydrogen peroxide at 50 μM was five time less than that by NOH-2NA at 25 μM at pH 7.0; at this concentration of NOH-2NA the concentration of hydrogen peroxide produced was 1.5 μM. The yield of production of hydrogen peroxide from NOH-2NA in HEPES buffered Hanks' solution was about one third of that in phosphate-buffered saline as previously reported.[4] Therefore, the induction of chromosomal aberrations by hydrogen peroxide was more than one order less efficient than that by NOH-2NA, normalizing to the concenration of hydrogen peroxide.

Considering the parallelism and the discrepancy between the formation of DNA lesions and that of chromosomal aberrations, we propose the following possibilities: in addition to $OH^{\cdot}$ radicals, which could be produced from hydrogen peroxide and superoxide anion by a metal ion-catalyzed Haber-Weiss type of reaction, further oxidation products from NOH-2NA might also participate in the induction of aberrations as in the induction of gene conversions in yeast cells exposed to NOH-2NA.[11] Furthermore, NOH-2NA-induced chromosomal aberrations could be induced mostly by oxidized DNA damage not detected by alkaline elution or by chromosomal protein damage, both of which were induced more efficiently by oxidation products from NOH-2NA than by $OH^{\cdot}$ radicals. These propositions are supported by the following observations: first, the most of the hydrogen peroxide-induced chromosomal aberrations wee not ruffling and stickiness but extensive fragmentations (Table III). Second, it has been proposed that chromosomal stickiness is induced by alteration of specific chromosomal nonhistone proteins.[15]

There is an additional interesting point regarding the pH dependency of hydrogen peroxide-induced aberrations, i.e. more aberrations were induced by hydrogen peroxide at an acidic pH. This phenomenon parallels the observations that toxicity of hydrogen peroxide for epithelial cells increases at an acidic pH. The latter observation is explained by the decreased activity of glutathione peroxidase at an acidic pH.[16] On the other hand, in the case of NOH-2NA, no increase in the aberrations was observed, probably because less hydrogen peroxide was produced at the acidic pH.

In conclusion, the major part of NOH-2NA-induced chromosomal aberrations were mediated by hydrogen peroxide and superoxide anions, but the efficiency of their induction by NOH-2NA was much higher than that by hydrogen peroxide, normaliz-

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ing to the concentration of hydrogen peroxide at neutral pH. Oxidized DNA damage or chromosomal protein damage by active oxygen species and reactive oxidation products of NOH-2NA migth contribute to the induction of chromosomal aberrations by NOH-2NA.

References

1. Cerutti, P.A., *Science*, **227**, 375–381, (1985).
2. Vuillaume, M., *Mut. Res.*, **186**, 43–72, (1987).
3. Nakayama, T., Kimura, T., Kodama, M. and C. Nagata, *Carcinogenesis*, **4**, 765–769, 1983.
4. Kaneko, M., Nakayama, T., Kodama, M. and Nagata, C. *Gann*, **75**, 349–354, 1984.
5. Kaneko, M. and Leadon, S.A., *Cancer Res.*, **46**, 71–75, 1986.
6. Kadulubar, F.F., Unruh, L.E., Beland, F.A., Straub, K.M. and Evans, F.E., *Carcinogenesis*, **1**, 139–150, 1980.
7. Levin, D.E., Holistein, M., Christman, M.F., Schweirs, E.A. and Ames, B.N., *Proc. Natl. Acad. Sci. USA*, **79**, 7445–7449, (1982).
8. Tsuda, H., *Jpn. J. Genet.* **56**, 1–8, (1981).
9. Yunis, J.J., *Science*, **221**, 227–235, (1983).
10. Kaneko, M., Leadon, S.A. and Ito, T., *Mut. Res.*, **207**, 17–22, (1988).
11. Kaneko, M., Ito, T. and Ochi, T., in "*Medical, Biochemical and Chemical Aspects of Free Radicals*" (ed. by E. Niki and T. Yoshikawa), Elsevier Sci. Pub., Amsterdam (in press).
12. Ochi, T., Mogi, M., Watanabe, M. and Ohsawa, M., *Mut. Res.*, **137**, 103–109, (1984).
13. Kaneko, M., Nagata, C. and Kodama, M., *Mut. Res.*, **143**, 103–108, (1985).
14. Oldham, J.W., Kadlubar, F.F. and Milo, G.E., *Carcinogenesis*, **2**, 937–940, (1981).
15. Gaulden, M.E., *Mutagenesis*, **2**, 357–365, (1987).
16. Link, E.M., in "*Free Radicals: A Search for New Methodology*" vol. 4, (ed. by C. Rice-Evans and B. Halliwell), Richelieu Pressm London (in press).

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