Biochemical and Biophysical Research Communications **280,** 48–52 (2001)
doi:10.1006/bbrc.2000.4095, available online at http://www.idealibrary.com on IDEAL®

Oxidative DNA Damage by a Metabolite of Carcinogenic 1-Nitropyrene

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Received November 28, 2000

Nitropyrenes are carcinogenic pollutants. Adduct formation following nitro-reduction is considered to be a major cause of nitropyrene-mediated DNA damage. We investigated the role of 1-nitrosopyrene, a metabolite of 1-nitropyrene, in causing oxidative DNA damage, using ^{32}P-5′-end-labeled DNA. 1-Nitrosopyrene was found to facilitate Cu(II)-mediated DNA damage in the presence of NADH. Catalase and a Cu(I)-specific chelator attenuated DNA damage, indicating the involvement of H_2O_2 and Cu(I). Typical $^{\cdot}OH$ scavenger did not have a significant effect. These results suggest that the main reactive species is probably a DNA-copper-hydroperoxo complex. We also measured 8-oxo-7,8-dihydro-2′-deoxyguanosine formation by 1-nitrosopyrene in the presence of Cu(II) and NADH, using an electrochemical detector coupled to a high-pressure liquid chromatograph. We conclude that oxidative DNA damage, in addition to DNA adduct formation, may play an important role in the carcinogenesis of nitropyrenes.

Key Words: **nitropyrene; nitrosopyrene; DNA damage; copper; hydrogen peroxide.**

Nitropyrenes (NPs) are widespread in the environment, due mainly to diesel engine emissions (1, 2). They are mutagenic and carcinogenic to experimental animals (1). The International Agency for Research on Cancer (IARC) has assessed that 1-nitropyrene (1-NP), 1,6-dinitropyrene (1,6-DNP) and 1,8-dinitropyrene (1,8-DNP) have been possibly carcinogenic to humans (group 2B) and 1-NP is the most abundant NP in the ambient atmosphere (1).

NPs are metabolized by both ring oxidation and nitro-reduction (3), the latter of which is believed to be essential for its tumorigenic activity (4). It has been reported that nitroso derivatives are more mutagenic than their parent NPs (5, 6). The DNA adduct formed upon nitro-reduction of 1-NP has been identified as *N*-(deoxyguanosine-8-yl)-1-aminopyrene (7). 1-Nitrosopyrene (1-NOP), a metabolic intermediate of the nitro-reduction of 1-NP, induced the same kind of DNA adduct formation more efficiently than 1-NP (5, 6, 8, 9). These findings indicate that DNA adduct formation upon nitro-reduction may constitute a mechanism of carcinogenesis by NPs.

Djuric *et al.* found not only DNA adducts but also oxidative DNA damage in rats treated with 1,6-DNP (10). There remains a possibility as oxidative DNA damage by nitro-reduced metabolites. In this study, we investigated the role of 1-NOP in causing oxidative DNA damage, using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene. We analyzed 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in calf thymus DNA by 1-NOP in the presence of Cu(II) and β-nicotinamide adenine dinucleotide (NADH).

MATERIALS AND METHODS

Materials. 1-NOP was synthesized by oxidation of 1-aminopyrene with *m*-chloroperbenzoic acid, according to the reference (11). Restriction enzymes (*Hin*dIII, *Sty*I, *Apa*I, *Ava*I and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was obtained from New England Nuclear. Alkaline phosphatase from calf intestine was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Piperidine, methionine and alanine were purchased from Wako Chemical Industries Ltd. (Osaka, Japan). Copper(II) chloride dihydrate was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased from Dojin Chemicals Co. (Kumamoto, Japan). Calf thymus DNA, superoxide dismutase (SOD) (3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were pur-

Abbreviations used: NPs, nitropyrenes; 1-NP, 1-nitropyrene; 1, 6-DNP, 1, 6-dinitropyrene; 1, 8-DNP, 1, 8-dinitropyrene; 1-NOP, 1-nitrosopyrene; 8-oxodG, 8-oxo-7, 8-dihydro-2′-deoxyguanosine; NADH, β-nicotinamide adenine dinucleotide (reduced form); O_2^-, superoxide; HPLC-ECD, an electrochemical detector coupled to a high performance liquid chromatography; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase.

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chased from Sigma Chemical Co. (St. Louis, MO, USA). Nuclease P_1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba, Japan).

Preparation of ^{32}P-5′-end-labeled DNA fragments obtained from the p53 gene and the c-Ha-ras-1 gene. DNA fragments were obtained from the human *p53* tumor suppressor gene (12) and the c-Ha-*ras*-1 protooncogene (13). A singly ^{32}P-5′-end-labeled double-stranded 118-bp *p53* fragment (*Hin*dIII* 13038-*Sty*I 13155) and 211-bp *p53* fragment (*Hin*dIII* 13972-*Apa*I 14182) were prepared from the pUC18 plasmid according to a method described previously (14). A 261-bp c-Ha-*ras*-1 fragment (*Ava*I* 1645–*Xba*I 1905) was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*H I chromosomal DNA restriction fragment. The asterisk indicates ^{32}P-labeling.

Detection of DNA damage. A standard reaction mixture (in a microtube; 1.5 ml) contained $CuCl_2$, NADH, a DMSO solution of 1-NOP, the ^{32}P-labeled double-stranded DNA fragments and calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37°C for 1 h, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min and treated as described previously (15, 16). The treated DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and an autoradiogram was obtained by exposing X-ray film to the gel. The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure (17) using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Analysis of 8-oxodG formation by 1-NOP. Calf thymus DNA fragment was incubated with 1-NOP, NADH and $CuCl_2$. After ethanol precipitation, DNA was digested to its component nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by HPLC-ECD, as described previously (18).

Analysis of NADH consumption. UV-visible spectrometry was used to follow the oxidation of NADH (UV-2500PC Shimadzu, Kyoto, Japan).

RESULTS

Damage to ^{32}P-labeled DNA. Figure 1 shows an autoradiogram of a DNA fragment treated with 1-NOP in the presence and absence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of both NADH and Cu(II), 1-NOP caused DNA damage in a dose-dependent manner. Piperidine treatment increased the number of oligonucleotides produced, suggesting that 1-NOP induces not only strand breakage but also base modification and/or liberation. Treatment with any two of the three agents studied (1-NOP, Cu(II), and NADH) alone did not result in DNA damage, nor did incubation with 1-NOP, Fe(II) and NADH or with 1-NP, Cu(II) and NADH (data not shown).

Effects of scavengers and bathocuproine on DNA damage. The effects of scavengers and bathocuproine on DNA damage by 1-NOP were investigated (Fig. 2). Mannitol, a typical ·OH scavenger, did not inhibit 1-NOP-induced DNA damage in the presence of NADH and Cu(II). Methionine inhibited DNA damage much more than other amino acid, possibly due to its unique thioether moiety. Bathocuproine, a Cu(I)-specific chelator, and catalase, but not SOD, were also found to reduce the amount of DNA damage.

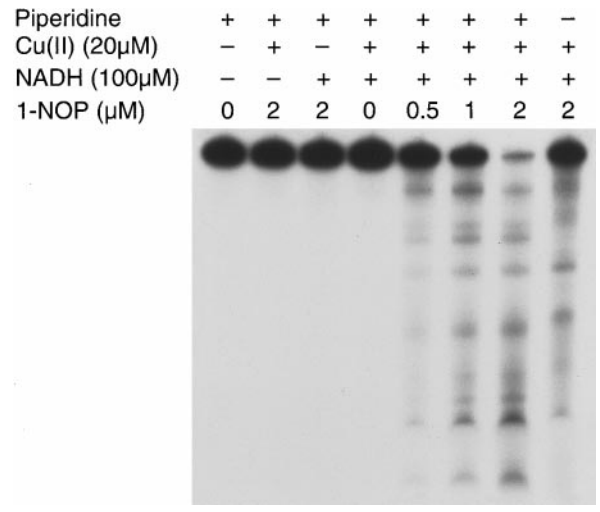

FIG. 1. Autoradiogram of ^{32}P-labeled DNA fragment incubated with 1-NOP in the presence of NADH and Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 211-bp DNA fragment, 50 μM per base of calf thymus DNA, the indicated concentrations of 1-NOP, 2% (v/v) DMSO, 100 μM NADH, and 20 μM $CuCl_2$ in phosphate buffer (pH 7.8). The reaction was incubated at 37°C for 1 h, followed by piperidine treatment, as described under Materials and Methods. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and an autoradiogram was obtained by exposing an X-ray film to the gel.

Site specificity of DNA damage by 1-NOP. An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensities of DNA cleavage products from the human *p53* tumor suppressor gene (Fig. 3A) and the c-Ha-*ras*-1 protooncogene (Fig. 3B). 1-NOP was observed to preferentially induce piperidine-labile sites at thymine and cytosine residues in the presence of NADH and Cu(II).

Formation of 8-oxodG in calf thymus DNA. Using HPLC-ECD, we measured the 8-oxodG content of calf thymus DNA treated with 1-NOP in the presence of NADH and Cu(II) (Fig. 4). The amount of 8-oxodG increased with the concentration of 1-NOP, as well as with DNA denaturation. In the absence of NADH, 1-NOP and Cu(II) together did not stimulate 8-oxodG formation above control levels (data not shown).

NADH consumption by 1-NOP. NADH consumption during 1-NOP oxidation was measured by observing NADH (reduced form) absorption at 340 nm. When 5 μM 1-NOP was incubated with 200 μM NADH at 37°C for 1 h, 59 μM NADH were consumed (data not shown).

DISCUSSION

The present study showed that 1-NOP, a metabolite of 1-NP, causes oxidative DNA damage in the presence

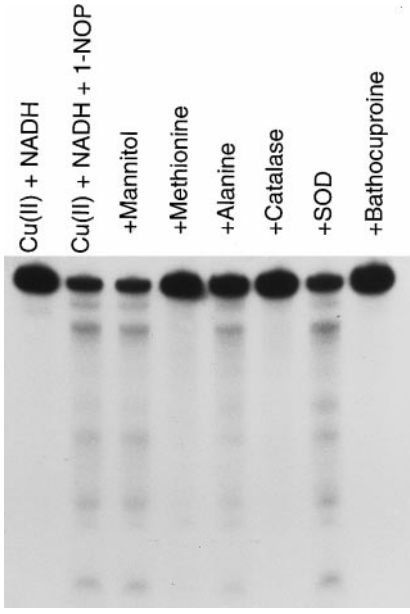

FIG. 2. Effects of scavengers and bathocuproine on DNA damage induced by 1-NOP in the presence of NADH and Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 118-bp DNA fragment, 50 μM per base of calf thymus DNA, 1.5 μM 1-NOP, 2% (v/v) DMSO and 20 μM $CuCl_2$ in phosphate buffer (pH 7.8). The reaction was incubated at 37°C for 1 h, followed by a piperidine treatment. The DNA fragments were analyzed as described in the legend to Fig. 1. The concentrations of scavengers and bathocuproine were as follows: 0.1 M mannitol; 500 μM methionine; 500 μM alanine; 30 units of catalase; 30 units of SOD; 50 μM bathocuproine.

of NADH and Cu(II). To clarify the reactive species involved in the process, we examined the effects of scavengers on DNA damage. Both catalase and bathocuproine were found to reduce DNA damage, indicating the involvement of H_2O_2 and Cu(I). Failure to see an effect of typical $^{\cdot}OH$ scavenger implies that free $^{\cdot}OH$ does not play a major role in DNA damage. Alternatively, the lack of effect of $^{\cdot}OH$ scavengers may be due to DNA damage induced by $^{\cdot}OH$ generated by metal ions bound in very close proximity to DNA. Indeed hydroxyl radical formation by copper ions bound to specific sites on DNA could explain the pattern of damage that was observed. The DNA-protective effect of methionine is probably due to the reactivity of thioether compounds with $^{\cdot}OH$ and less reactive species (19). Finally, without NADH-mediated reduction, 1-NOP did not cause DNA damage in the presence of Cu(II).

Based on these results, a possible mechanism could exist as follows: 1-NOP is reduced by an endogenous reductant, NADH, to a reactive intermediate, which is probably an *N*-hydroxy radical. Autoxidation of this intermediate to 1-NOP occurs, coupled with the generation of O_2^-. O_2^- is dismutated to H_2O_2 and reduces Cu(II) to Cu(I). H_2O_2, in turn, interacts with Cu(I) to form a DNA-copper-hydroperoxo complex, which causes DNA damage. DNA-copper-hydroperoxo complex has been reported in other studies (20, 21, 22). After 1-NOP is reduced by NADH to a reactive intermediate, it can be re-oxidized to 1-NOP. Very low concentrations of 1-NOP can effectively produce significant amounts of reactive oxygen species, through cyclic redox reactions. The concentration of NAD(P)H in certain tissue has been estimated to be as high as 100–200 μM (23). The biological importance of NADH and NADPH as nuclear reductants (24) has been demonstrated before (25, 26). This non-enzymatic reduction of 1-NOP by NADH, causing oxidative DNA damage, is yet another example of its relevance.

The majority of base substitutions in mutants generated from 1-NOP-treated plasmids were G · C → T · A transversions, although the mutational hot spots

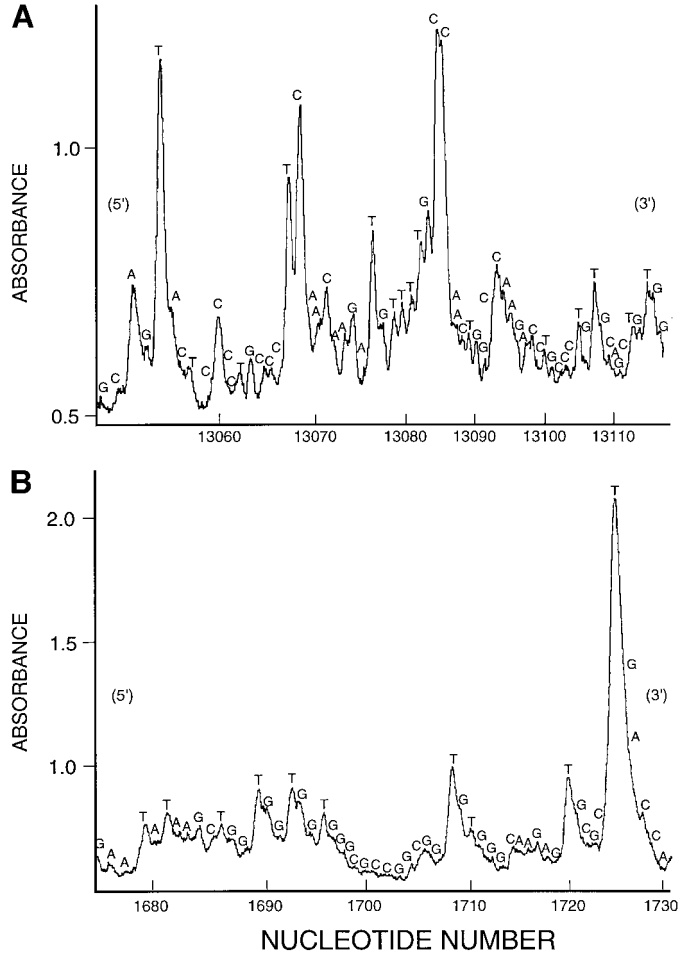

FIG. 3. Site specificity of DNA cleavage induced by 1-NOP in the presence of NADH and Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled DNA, 50 μM per base of calf thymus DNA, 1.5 μM 1-NOP, 2% (v/v) DMSO, 100 μM NADH and 20 μM $CuCl_2$, in phosphate buffer (pH 7.8). The reaction was incubated at 37°C for 1 h, followed piperidine treatment. The horizontal axis shows the nucleotide number. (A) 118-bp fragment (*Hin*dIII* 13038–*Sty*I 13155) of the *p53* tumor suppressor gene. (B) 261-bp fragment (*Ava*I* 1645–*Xba*I 1905) of the c-Ha-*ras*-1 protooncogene.

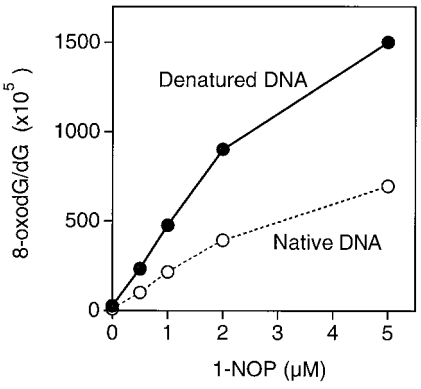

FIG. 4. Formation of 8-oxodG by 1-NOP in the presence of NADH and Cu(II). Calf thymus DNA (50 μM per base) was incubated with the indicated concentrations of 1-NOP, 100 μM NADH and 20 μM for 1 h at 37°C. After ethanol precipitation, DNA was enzymatically digested to individual nucleosides, and 8-oxodG content was measured by HPLC-ECD as described under Materials and Methods. Open circles indicate native DNA, and closed circles indicate denatured DNA.

and frequent sites of adduct formation do not strictly correlate (27). In this study, double-stranded DNA treated with 1-NOP plus NADH and Cu(II) induced piperidine-labile sites preferentially at thymine and cytosine residues. We also detected piperidine-inert oxidative damage at guanine, resulting in the formation of 8-oxodG. Shibutani *et al.* have reported that 8-oxodG causes DNA misreplication, which can lead to mutation, particularly G → T substitutions (28). The 8-oxodG formation may explain the discrepancy between mutational hot spots and observed sites of adduct formation by 1-NOP.

The present study has revealed that 1-NOP is nonenzymatically reduced by NADH resulting in oxidative DNA damage. It is reasonable to speculate that nitroso derivatives of other NPs can cause oxidative DNA damage following NAD(P)H reduction. Djuric *et al.* observed oxidative DNA damage, in addition to DNA adducts, in rats treated with 1,6-DNP (10). In the case of 1-NP, ring oxidation is predominant rather than nitro-reduction (3), while 1,6-DNP and 1,8-DNP are more efficiently nitro-reduced (29). Therefore, oxidative DNA damage by nitroso-derivatives may play more important role in the carcinogenesis of 1,6-DNP and 1,8-DNP than in that of 1-NP. Clearly, however, oxidative DNA damage is of comparable significance to DNA adduct formation in the carcinogenesis of NPs.

ACKNOWLEDGMENT

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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