

Mutation spectra induced by 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide in the *supF* gene of human XP-A fibroblasts

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1-Nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide are oxidative metabolites that are responsible for the mutagenicity of 1-nitropyrene. In this study, the mutation spectra induced by oxidative metabolites in human cells were determined using a shuttle vector assay. The mutation frequencies induced by 1-nitropyrene 9,10-oxide were 2-3 times higher than those induced by 1-nitropyrene 4,5-oxide. The base substitutions induced by 1-nitropyrene 4,5-oxide were G → A transitions, G → C transversions, and G → T transversions. In the case of 1-nitropyrene 9,10-oxide, G → A transitions, G → T transversions, A → G transitions and G → C transversions were observed. Most base substitution mutations induced by oxidative metabolites occurred at the guanine sites in the *supF* gene. These sequence-specific hot spots were commonly identified as 5'-GA sequences for both metabolites. On the other hand, the sequence-specific hot spots at the adenine sites were identified as 5'-CAC sequences for 1-nitropyrene 9,10-oxide. These results suggest that the oxidative metabolites of 1-nitropyrene induce sequence-specific DNA mutations at the guanine and adenine sites at high frequency. [BMB reports 2008; 41(8): 604-608]

INTRODUCTION

Nitropyrene, which is produced by diesel exhaust, coal fly ash, carbon black and toner, is a ubiquitous environmental pollutant (1, 2). The pollutant is also detected in foods, such as grilled chicken (3, 4). 1-Nitropyrene (1-NP), a representative of this class of compounds, and is the most abundant nitroaromatic compound in the environment. It has been well-established that 1-NP is mutagenic in bacteria, mammalian cells, and animals (1, 5-9). DNA sequence analysis of 1-NP-induced mutations in mice showed A → G transitions to be the most frequent mutations (5).

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Received 25 February 2008, Accepted 10 April 2008

Keywords: Mutagenicity, 1-Nitropyrene 4,5-Oxide, 1-Nitropyrene 9,10-Oxide, Shuttle vector plasmid pSP189, *supF* gene

1-NP can be metabolized by two pathways, nitroreduction or ring-oxidation. Alternatively, 1-NP can be metabolized by combinations of these two pathways (10-12). Nitroreduction yields unstable intermediates, 1-nitrosopyrene and *N*-hydroxyl-1-aminopyrene, which react mainly with guanine and to a much lesser extent with adenine (13-15). DNA sequence analysis of 1-nitrosopyrene-induced mutants in mammalian cells showed the preferential occurrence of G → A transitions and G → T transversions (7, 16, 17).

The second pathway is oxidation, which produces non-K-region phenols (1-nitropyrene-3-ol, 1-nitropyrene-6-ol, and 1-nitropyrene-8-ol) and K-region oxides (1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-oxide) (18, 19). The cytotoxicity of 1-nitropyrene-9,10-oxide has been reported to be higher than that of 1-nitropyrene-4,5-oxide (20-22). These oxidative metabolites can bind directly to guanine because K-region oxides contain an epoxide ring (20, 23, 24). Recent studies analyzing the mutation spectra in the *hprt* gene of Chinese hamster ovary cells reported that the predominant base substitutions induced by K-region oxides were A → G and G → A transitions (22). However, the sequence-specific mutations at the guanine and adenine sites were not examined.

Shuttle vectors carrying the *supF* suppressor tRNA gene were originally introduced in the mid-1980s for mutagenesis experiments (25). Since then, the *supF* gene has been used as a mutation reporter in other mammalian cells, yeast and transgenic mice. In order to determine the role of the oxidative metabolites in the mutagenicity of 1-NP in human cells, 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide were synthesized and their mutation spectra in the *supF* gene replicated in human XP-A fibroblasts were investigated using the shuttle vector pSP189. The results suggest that the DNA mutations produced by the oxidative metabolites of 1-NP are highly sequence-specific in human cells.

RESULTS AND DISCUSSION

Mutant frequencies induced by 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide

The SV40-based shuttle vector, pSP189, was treated with the

K-region oxides at concentrations of 2, 20, or 200 μM . The plasmids were transfected into XP-A human fibroblasts (XP12BE), which were then incubated for 72 h to allow DNA replication and mutation fixation to take place. The progeny shuttle vector was then rescued, and the DNA was cleaved with *DpnI* to remove the unreplicated plasmid. The rescued plasmids were electroporated into the bacterial strain MBM7070, which carries the *lacZ* gene with an amber mutation. The plasmids were isolated from the white, mutant colonies, and the *supF* gene was sequenced. The sibling plasmids that originated from the same mutational event were identified by the repeated appearance of a signature sequence adjacent to the *supF* gene (26). The siblings were excluded from the total number of white, mutant colonies.

The mutant frequencies were determined at various concentrations of K-region oxides (Table 1). The relative mutation frequencies induced by both K-region oxides were compared with those of the control. The mutant frequencies increased in a dose-dependent manner with increasing concentration of K-region oxides. There was a 3.25-fold increase in the mutant frequencies in the 1-nitropyrene 9,10-oxide-treated (200 μM) pSP189 vectors, and a 1.40-fold increase in the 1-nitropyrene 4,5-oxide-treated pSP189 vectors (200 μM) compared with the control. This suggests that the mutagenic activity of 1-nitropyrene 9,10-oxide is stronger than that of 1-nitropyrene 4,5-oxide. This differential mutagenic activity is consistent with previous studies (20-22). These results suggest the possibility that the DNA adducts induced by 1-nitropyrene 9,10-oxide might be more resistant to repair by excision repair enzymes than those induced by 1-nitropyrene 4,5-oxide (27).

Mutation spectra induced by 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide in the *supF* gene

The sequence of the *supF* gene was determined in more than 100 independently derived mutant plasmids. The plasmids obtained from a treatment with 200 μM of the K-region oxides were sequenced due to a large increase in mutant frequency over the background at those concentrations.

Table 2 and Fig. 1 gives a summary of the types of mutations

Table 1. *SupF* Mutation frequencies induced by 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide following replication in human XP-A fibroblasts

Treatment (μM)	Mutation frequency ^a ($\times 10^{-4}$)	Relative frequency ^b
Control	3.15	1.00
1-nitropyrene 4,5-oxide	2	1.03
	20	1.40
	200	1.40
1-nitropyrene 9,10-oxide	2	1.39
	20	2.42
	200	3.25

^aMutation frequency per 10^4 colonies.

^bRelative mutation frequency compared with control.

obtained. The mutation types for 1-nitropyrene 4,5-oxide were transitions (24/81), transversions (31/81), deletions (25/81), and insertions (1/81). In the case of 1-nitropyrene 9,10-oxide, transitions (43/99), transversions (31/99), deletions (20/99), insertions (3/99), and tandem mutations (2/99) were observed. Most mutants induced by both metabolites were base sub-

Table 2. Type of mutations in the *supF* gene of pSP189 plasmids treated with 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide following replication in human XP-A fibroblasts

Mutation	Number of plasmids with mutations (%)	
	1-Nitropyrene 4,5-oxide	1-Nitropyrene 9,10-oxide
Base substitutions	55 (67)	74 (75)
Transitions	24 (29)	43 (44)
A:T \rightarrow G:C	1 (1)	10 (10)
G:C \rightarrow A:T	23 (28)	33 (33)
Transversions	31 (38)	31 (31)
A:T \rightarrow T:A	0 (0)	4 (4)
A:T \rightarrow C:G	1 (1)	2 (2)
G:C \rightarrow T:A	13 (16)	16 (16)
G:C \rightarrow C:G	17 (21)	9 (9)
Frameshift	26 (33)	23 (23)
Deletions	25 (32)	20 (20)
One base deletions	2 (3)	3 (3)
> 2nt	23 (29)	17 (17)
Insertions	1 (1)	3 (3)
Tandem	0 (0)	2 (2)
Total	81 (100)	99 (100)

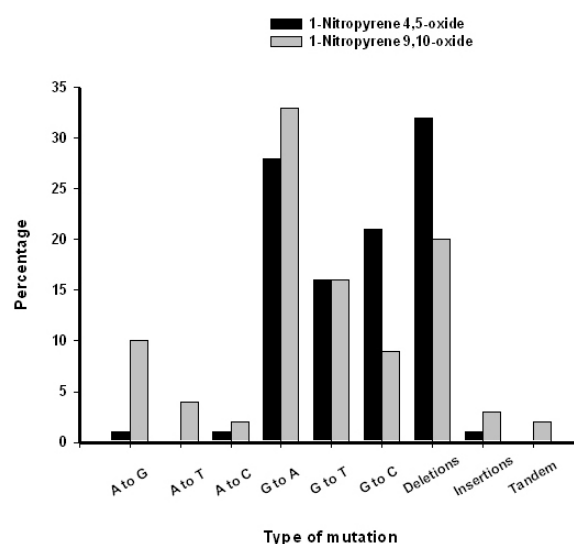


Fig. 1. Schematic diagram of the mutation spectra in the *supF* gene of the pSP189 plasmids treated with 1-nitropyrene 4,5-oxide (black bars) and 1-nitropyrene 9,10-oxide (gray bars).

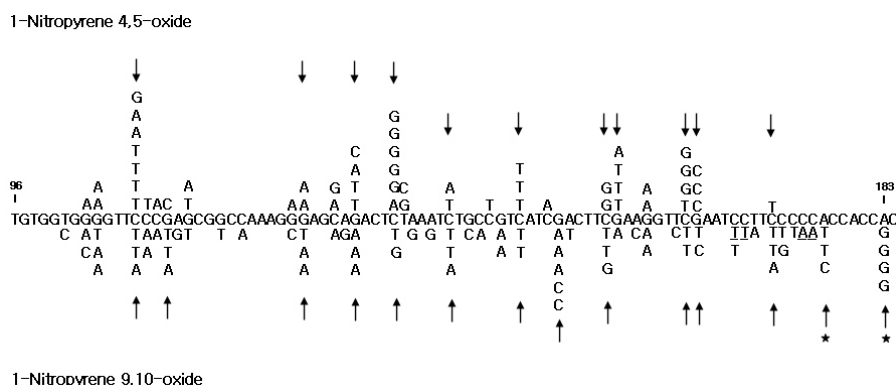


Fig. 2. Mutation spectra depicting the base substitutions induced by 1-nitropyrene 4,5-oxide (above) and 1-nitropyrene 9,10-oxide (below) in the *supF* gene. The original sequence shown in the middle represents the transcribed strand of the *supF* gene. The arrows indicate the guanine sites in the consensus 5'-GA sequence. The starred arrows indicate the adenine sites in the consensus 5'-CAC sequence. Tandem mutations are underlined.

stitutions that occurred predominantly at the G:C base pairs. The most common were G:C → A:T transitions, followed by G:C → T:A and G:C → C:G transversions. The major difference in base substitutions between the two metabolites was the A:T → G:C transition. The percentage of A:T → G:C transitions induced by 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide were 1% and 10% of all mutations, respectively. Fig. 2 shows the precise locations of the base substitutions within the suppressor tRNA sequence of the *supF* gene. Although base substitutions occurred at many sites within the target gene, several hot spots were observed at the adenine and guanine bases. These results are consistent with previous studies reporting that DNA adducts were formed at the guanine (major) and adenine (minor) bases (20, 23, 24). Sequencing analysis of the base substitutions revealed 5'-GA (5'-TC) and 5'-CAC (underline denotes the mutation site) to be consensus sequences at mutated guanine and adenine sites, respectively. Therefore, the base substitutions induced by oxidative metabolites were sequence-specific. It was reported that carbazone and its metabolites also induce sequence-specific mutations at high frequency (28). These sequence-specific mutations might result from sequence-specific DNA adduct formation.

In addition to base substitutions, a number of frameshift mutations occurred (Table 2). 1-Nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide induced frameshift mutations at frequencies of 33% and 23% of all mutations, respectively. Most frameshift mutations were deletions in which large regions of more than 2 nucleotides were predominantly deleted. These deletions might have been the result of adduct-induced template misalignment and slippage during plasmid replication. Two tandem mutations were also found (5'-CC → 5'-TT, 5'-CC → 5'-AA) but only in the mutants treated with 1-nitropyrene 9,10-oxide.

In conclusion, these findings have extended our prior work on the *hprt* gene of Chinese hamster ovary cells by showing that mutational events, or at least the predominant base substitutions, induced by K-region oxides occur mainly at the G:C and A:T sites. Such selectivity is consistent with the work from other laboratories, which also showed the selectivity of DNA

adduct formation at the guanine and adenine sites. It is also possible that DNA adduct formation at the adenine sites may be another source of the lethal lesions responsible for the mutagenic properties of these molecules. Future work will examine the sites of DNA adduct formation induced by 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide relative to the sites of mutagenesis.

MATERIALS AND METHODS

Materials

1-NP and 3-chlorobenzoic acid were purchased from Aldrich Chemical Co. The other materials were obtained from the following sources: fetal bovine serum, HyClone; DMEM media, trypsin-EDTA and penicillin-streptomycin, Gibco; isopropyl β-D-thiogalactopyranoside, X-gal and yeast extract, Duchefa Biochemie; Plasmid purification Maxi kit, Qiagen; Plasmid miniprep kit, Takara; and FuGene6 and RNase A, Roche. The primer was synthesized by cosmo4 Co. in Korea.

Cell lines and plasmids

SV40-transformed DNA repair-deficient human XP-A fibroblasts (XP12BE) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a 5% CO₂ humidified incubator. The pSP189 plasmid (including a randomly generated 8-base pair signature sequence at the 3'-end of the *supF* gene) was kindly provided by Michael Seidman (26). The bacterial strain, *E. coli* MBM7070, was cultured on agar containing ampicillin and isopropyl β-D-thiogalactoside, a β-galactosidase inducer, as well as X-gal as a color indicator. The colonies resulting from a transformation with a functional *supF* gene will appear blue, whereas those containing a mutated copy of the *supF* gene will appear white.

Synthesis of 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide

1-Nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide were

synthesized and purified by silica gel flash chromatography and HPLC as described elsewhere (29). Briefly, a reaction between 1-NP and 3-chloroperoxybenzoic acid in methylene chloride generated mixtures of 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide. The methylene chloride solution was concentrated *in vacuo* and applied to a silica gel flash chromatography column to generate a partially purified mixture of 1-nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide. The individual isomers were separated on a C₁₈ reverse-phase column by eluting with 65% methanol at 2 ml/min. 1-Nitropyrene 9,10-oxide eluted at 10.5 min, while 1-nitropyrene 4,5-oxide eluted at 12 min. Each compound was confirmed by IR and NMR analysis.

Treatments of pSP189 shuttle vector with K-region oxides *in vitro*

1-Nitropyrene 4,5-oxide and 1-nitropyrene 9,10-oxide were diluted with DMSO. The pSP189 plasmids were treated with various concentrations of the K-region oxides of 1-nitropyrene (final concentrations of 2, 20, and 200 μ M) in 50 mM potassium phosphate, pH 7.5, in total volume of 0.2 ml. The reaction was incubated for 3 h at 37°C followed by ethanol precipitation of the pSP189 plasmids to remove the unreacted excess K-region oxides. The control plasmid was treated with 1% DMSO only.

supF Mutagenesis assay

The pSP189 vectors were transfected into cultures of nucleotide excision repair-deficient human fibroblasts (XP12BE) using the transfection reagent, FuGene6. Enhanced mutant colonies can be selected compared with normal cells because the XP-A cells have a defect in DNA damage recognition and the binding protein, which is a component of nucleotide excision repair. After 72 h incubation, the plasmid was rescued from the cells using an alkaline lysis method. The cells were trypsinized, washed, and resuspended in a suspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 μ g/ml RNase A), mixed with 1 volume lysis buffer (0.2 M NaOH, 1% SDS), and incubated on ice for 5 min, followed by the addition of a neutralization buffer (3 M potassium acetate, pH 5.5). After 15 min incubation at room temperature, the mixture was centrifuged for 10 min at 16,000 \times g, and the supernatant was extracted with phenol-chloroform followed by ethanol precipitation. The recovered plasmid was digested with *DpnI*, which recognizes the bacterial adenine methylation patterns, to remove the unreplicated plasmid. The plasmids were then electroporated into the MBM7070 bacteria cells, which carry a *lacZ* gene with an amber mutation. The *E. coli* tyrosine amber suppressor transfer RNA gene, *supF*, enables read-through of a UAG stop codon in the *lacZ* gene, which results in the synthesis of functional β -galactosidase. The transformed bacteria were diluted in 2 ml of SOC medium and cultured on agar plates containing 50 μ g/ml ampicillin, 1 μ M isopropyl-1-thio- β -D-galactopyranoside, and 100 μ g/ml X-Gal. After incubation overnight at 37°C,

the white (mutant) and blue (wild-type) colonies were counted to determine the mutant frequency. Colonies containing a plasmid with a mutated *supF* gene were rescreened on agar plates containing ampicillin, isopropyl-1-thio- β -D-galactopyranoside, and X-Gal. The plasmids containing the mutated *supF* gene were purified using plasmid purification kits and sequenced using an automated DNA sequencer.

Acknowledgements

This study was supported financially by a Research Grant of Advanced Research Center in Yeungnam University (105096).

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