Mutations and oxidative DNA damage in phage M13mp2 exposed to N-nitrosomorpholine plus near-ultraviolet light

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Previously we reported that a direct-acting mutagen can be formed from N-nitrosomorpholine (NMOR) on exposure to near-ultraviolet light (UVA, 320–400 nm). We have now studied the spectrum of mutations caused by NMOR plus UVA. M13mp2 phages suspended in a sodium phosphate buffer were treated with NMOR under UVA irradiation and Escherichia coli NR9099 was then infected with the phage. Mutations induced in the phage DNA lacZα region were analyzed. The majority (~50%) of the induced sequence changes were G to T transversions. This suggested that modifications in guanine residues were responsible for these transversions. We explored the formation of 7,8-dihydro-8-oxodeoxyguanosine (8-oxodG) in the DNA. When the phage were treated with NMOR plus UVA, 8-oxodG/ dG in DNA increased up to 12-fold over the value in untreated control. When a mutM-deficient mutant of E.coli CSH50 was used as the host, the mutation level was higher than that observed with CSH50. We conclude that 8-oxodG may be involved in mutations induced by NMOR plus UVA.

Introduction

The cyclic nitrosouylamine N-nitrosomorpholine (NMOR*) has been reported to be carcinogenic in rodents and mutagenic in bacteria (1,2). NMOR is considered to be a potential health hazard for humans, since this compound has been found in workplaces and in cigarette smoke (3). In the rubber industry, human exposure to NMOR was reported to be as high as 11.2 mg/day (4). NMOR is formed in vivo in mice exposed by inhalation to NO₂ and by gavage simultaneously to morpholine, and the concentration of NMOR is highest in the skin (5). UVA (320–400 nm) is a major component of the solar irradiation that reaches the earth’s surface and is known to be able to penetrate the human skin (6,7). The possibility exists that NMOR and UVA may interact with each other. NMOR has been reported to be carcinogenic in rodents and mutagenic in bacteria (1,2). In addition, in the process of the mechanistic studies, we found that 2-nitro-3-methylimidazo[4,5-f]quinoline is formed on UVA irradiation of a mixture of 2-amino-3-methylimidazo[4,5-f]quinoline and N-nitrosodimethylamine, a phenomenon suggesting that oxidative species are produced in UVA plus nitrosamines (12). It is known that 8-oxodeoxyguanosine (8-oxodG) in DNA, a common lesion in oxidized DNA, results in mutations (13). Based on these considerations, we decided to explore the possibility that NMOR plus UVA might form 8-oxodG in DNA leading to mutations.

In this study, we treated M13mp2, a phage with single-stranded DNA, with NMOR plus UVA, and analyzed the spectrum of mutations induced in the lacZα region. We also measured the formation of 8-oxodG in the DNA of the treated phage. Furthermore, we have constructed a derivative of Escherichia coli defective in 8-oxodG DNA glycosylase activity (mutM) and investigated the effect of the repair deficiency toward the mutations caused by NMOR plus UVA.

Materials and methods

Bacteria and phage strains

Phage M13mp2, E.coli NR9099 (Δpro-lac), recA^, ara^, thi^-R' (proAB, lac^G^-Z^-ΔM155) and CSH50 (Δpro-lac), ara^, thi^-R'proD36, (proAB, lac^G^-Z^-ΔM155) were gifts from Dr T.A.Kunkel (NIHES, Research Triangle Park, NC) (14). E.coli YG5113 (ara^-, Δgpt-lac)5, mutM::cat^F'(lac378, lac^Z^-ΔM155, proAB) was a gift from Dr T.Nohmi (National Institute of Hygienic Sciences, Tokyo). Phage PI vir was obtained from the National Institute of Genetics, Shizuoka, Japan. E.coli MF67, a derivative of CSH50 carrying the mutM::cat allele, was constructed by P1 transduction.

Mutagenesis experiments

The irradiation was carried out using a National Black Light FL205 BL-B, which emits light of wavelength range 300–400 nm with a maximum centered at 350 nm. Mixtures of phage M13mp2 (10⁵ p.f.u.) and NMOR (Tokyo Kasei, Japan) in sodium phosphate buffer (20 mM, pH 7.0) were placed in wells of a sterile microtiter tray (Nunc, Denmark). The tray was covered with a 3 mm thick glass plate, which excludes light of wavelength <320 nm. The intensity of light at the surface of the suspension was 6 W/m², as measured by a black-ray UV intensity meter (Ultraviolet Products, San Gabriel, CA).

A portion of the irradiated phage solution was diluted and plated with E.coli strain NR9099, CSH50, or its mutM-deficient derivative strain. The plates were incubated at 37°C for one night to titrate the surviving phage (15). Based on the measured titers, the irradiated phage solutions were diluted to a concentration of 10 000–20 000 p.f.u./ml and 0.1 ml samples of the diluted solutions were plated with either of the E.coli strains. After incubation for 24 h at 37°C, the plates were examined to score the numbers of total plaques, and those of colorless or light blue plaques, which should contain phages defective in α-complementation due to a mutation in their lacZα region. The distinctive plaque color of the mutants was confirmed as described (16). DNA was prepared from the mutant phages and sequenced using an ABI DNA sequencer with the dye-primer method. For this purpose, we used a set of primers with four different dyes attached to the 5'-end. The primers were gifts from Dr TA.Kunkel (NIEHS, Research Triangle Park, NC) (14).

Regarding the reactive mutagen, we have isolated a product formed from N-nitrosopiperidine and UVA in phosphate buffer, and identified its structure as the phosphate ester of α-hydroxy-N-nitrosopiperidine (10). With NMOR plus UVA, a similar photoprocess will be formed. With respect to the mechanism of this photoreaction, a free radical involvement is implicated (11). In addition, in the process of the mechanistic studies, we found that 2-nitro-3-methylimidazo[4,5-f]quinoline is formed on UVA irradiation of a mixture of 2-amino-3-methylimidazo[4,5-f]quinoline and N-nitrosodimethylamine, a phenomenon suggesting that oxidative species are produced in UVA plus nitrosamines (12). When the phage were treated with NMOR plus UVA, 8-oxodG/ dG in DNA increased up to 12-fold over the value in untreated control. When a mutM-deficient mutant of E.coli CSH50 was used as the host, the mutation level was higher than that observed with CSH50. We conclude that 8-oxodG may be involved in mutations induced by NMOR plus UVA.

Abbreviations: NMOR, N-nitrosomorpholine; UVA, near UV (320–400 nm); 8-oxodG, 7,8-dihydro-8-oxodeoxyguanosine.
with UVA for 2 h under continuous nitrogen bubbling. Subsequent procedures were the same as those described above.

**Analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA**

The mixture was freeze-dried and the residue was solubilized in TE buffer (60 μg/ml) at 37°C for 2 h, and then with a mixture of alkaline phosphatase and a phenol/chloroform extraction followed by ethanol precipitation. The DNA was then dissolved and 0.1 ml samples were placed onto LB plates that contained rifampicin (100 μg/ml) and snake venom phosphodiesterase (60 μg/ml) in 20 mM Tris-HCl (pH 8.0). The phage DNA was isolated by centrifugation at 4°C for 10 min at 15 000 r.p.m. The supernatant was taken and the molar ratio of 8-oxoguanine (8-oxoG) to deoxyguanosine (dG) in each DNA sample was determined based on the peak heights, in comparison with those of known amounts of authentic 8-oxoG and dG (17). 8-oxoG was a gift from Dr H.Kasai (University of Occupational and Environmental Health, Kitakyusyu, Japan).

**P1 transduction and mutational specificity tests for E.coli**

Bacteriophage P1 transduction was carried out as described by Miller (18). A P1 lysate was prepared from E.coli YG5113 and then used to transduce E.coli CSH50. Derivatives of CSH50 carrying the mutM::cat allele were selected by chloramphenicol-resistance.

Independent cultures of CSH50 and its derivatives were incubated overnight, and 50 μl samples from individual cultures were placed onto LB plates containing rifampicin (100 μg/ml). After overnight incubation at 37°C, Rifr colonies were counted. For the measurement of survival, 10^9 and 50 (μl) samples from individual cultures were placed onto LB plates containing rifampicin (100 μg/ml). After overnight incubation at 4°C, colonies were counted. The results given in Figures 1 and 2 show that the viability of the phage decreased in a dose-dependent manner, and along with the loss, the induced mutation frequency increased. The dose-dependence was noted both for NMOR (up to 9 mM) and UVA. There was no loss of viability or increase of mutation frequency when the NMOR or light was omitted. At higher concentrations of NMOR (>9 mM), a recovery of survival and a decline of mutation frequency were observed: some secondary reactions between NMOR and UVA may be responsible for this phenomenon.

**Results**

**Lethality and mutagenesis**

Survival and mutation induction for single-stranded phage M13mp2 treated with NMOR and UVA were determined. The results given in Figures 1 and 2 show that the viability of the phage decreased in a dose-dependent manner, and along with the loss, the induced mutation frequency increased. The dose-dependence was noted both for NMOR (up to 9 mM) and UVA. There was no loss of viability or increase of mutation frequency when the NMOR or light was omitted. At higher concentrations of NMOR (>9 mM), a recovery of survival and a decline of mutation frequency were observed: some secondary reactions between NMOR and UVA may be responsible for this phenomenon.

**Mutation spectrum**

We collected 126 mutants resulting from the treatment with NMOR (36 mM) plus UVA (2 h) followed by proliferation in E.coli NR9099 as the host, and the DNA sequences were analyzed. Fifty of these mutants exhibited changes in nucleotide sequence. Phages (4x10^10 p.f.u./250 μl) were treated with NMOR plus UVA (2 h, 43 kJ/m^2) (●), or NMOR without UVA (○).

**Fig. 1. Viability and mutation frequency of M13mp2 phage treated with NMOR plus UVA, with E.coli NR9099 as host. Dependence on NMOR concentration. Phages (4x10^10 p.f.u./250 μl) were treated with NMOR plus UVA (2 h, 43 kJ/m^2) (●), or NMOR without UVA (○).**

**Fig. 2. Dependence on irradiation time. M13mp2 phages (4x10^10 p.f.u./250 μl) were treated with NMOR (36 mM) plus UVA (●) or UVA only (○).**

**Fig. 3. Spectrum of mutations generated by NMOR plus UVA in M13mp2 DNA. Single base substitutions are displayed below the wild-type sequence. Deletions and additions are indicated by – or +.**
DNA damage caused by NMOR plus UVA

Table I. 'NMOR + UVA' and 'UVA-alone'-induced mutations in M13mp2*

<table>
<thead>
<tr>
<th>Class of mutation</th>
<th>Mutants found</th>
<th>NMOR plus UVA</th>
<th>UVA</th>
</tr>
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<tbody>
<tr>
<td><strong>Base substitution</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G to T</td>
<td>26</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>C to A</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>G to C</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>A to C</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Transition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C to T</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>T to C</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>A to G</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>G to A</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Insertion</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Deletion</td>
<td>3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

*NMOR was 36 mM, and UVA irradiation was for 2 h. The host for phage was E.coli NR9099.

Fig. 4. Spectrum of mutations induced by UVA alone in M13mp2 DNA.

![Fig. 4. Spectrum of mutations induced by UVA alone in M13mp2 DNA.](image)

Fig. 5. Formation of 8-oxodG in M13mp2 phage treated with NMOR (36 mM) plus UVA (●) or UVA alone (○). Mutations were seen at many sites in the region sequenced, but there were some hot spots. Two obvious hot spots are -GG- at +29 to 30 and -GG- at +141 to 142. Of the 43 mutants obtained from treatment with UVA (2 h) alone, 23 exhibited changes in nucleotide sequence (Figure 4 and Table I). No hot spot can be seen in this mutation spectrum.

**Formation of 8-oxodG in DNA**
As several photosensitized reactions were reported to generate 8-oxodG in DNA (22–24), we explored the formation of 8-oxodG in phage M13mp2 treated with NMOR plus UVA. The treatment yielded extremely high amounts of 8-oxodG compared with the 8-oxodG in the NMOR alone or UVA alone to T (87%, 26/30). Mutations were seen at many sites in the region sequenced, but there were some hot spots. Two obvious hot spots are -GG- at +29 to 30 and -GG- at +141 to 142. Of the 43 mutants obtained from treatment with UVA (2 h) alone, 23 exhibited changes in nucleotide sequence (Figure 4 and Table I). No hot spot can be seen in this mutation spectrum.

**Table II. Formation of 8-oxodG in DNA, and effect of D2O on mutation frequency of phage M13mp2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>8-oxodG/10⁶ dG*</th>
<th>Mutation frequency (×10⁻⁴)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.6</td>
<td>2.4</td>
</tr>
<tr>
<td>NMOR plus UVA</td>
<td>18.6</td>
<td>14.1</td>
</tr>
<tr>
<td>NMOR only</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td>UVA only</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td><strong>In H₂O</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In D₂O</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Phage M13mp2 was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 36 mM NMOR. UVA irradiation was performed for 3 h. **UVA irradiation was for 2 h.

Table III. Mutation frequency of wild and mutM strains of E.coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rif frequency (×10⁻⁷)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSH50 (wild)</td>
<td>1.0</td>
</tr>
<tr>
<td>MF67 (mutM)</td>
<td>11.3</td>
</tr>
<tr>
<td>CC104*</td>
<td>0.5–1.0</td>
</tr>
<tr>
<td>CC104 mutM*</td>
<td>15</td>
</tr>
</tbody>
</table>

*Data from Michaels et al. (25).

Fig. 5. Formation of 8-oxodG in M13mp2 phage treated with NMOR (36 mM) plus UVA (●) or UVA only (○).

Fig. 6. The 8-oxoG DNA glycosylase assay for E.coli cell extracts. The assays were performed with a duplex 21mer oligonucleotide containing an 8-oxoG/C base-pair. The formation of a 10mer is the indication of the glycosylase activity.

(Figure 5 and Table II). After 1 hr of treatment with 36 mM NMOR plus UVA, an 8-oxodG content as high as one residue per 160 dG residues in DNA was obtained.
Characterization of a mutM mutant

It is known that the mutM protein in E.coli can excise 8-oxodG residues in cellular DNA (19). We attempted to study the role of mutM in the NMOR + UVA mutagenesis. If an E.coli strain that lacks the mutM gene was used as host for the phage proliferation, a higher level of mutation in treated phage may be expected to be observed. To construct the mutM strain, we used P1 phage transfer of a mutM::cat marker into the CSH50, the wild strain we used in this study. The spontaneous mutation rate of the constructed mutM strain, which we termed MF67, was 11-fold higher than the wild-type, as judged by the generation of Rif" colonies (Table III). This elevated mutation rate is similar to that found for E.coli CC104 mutM previously reported by Michaels et al. (25).

To confirm that the mutM activity is indeed deficient in MF67, we compared the 8-oxoG DNA glycosylase activities in crude extracts of mutM and wild-type cells. An 8-oxoG-containing 21mer oligonucleotide duplex was incubated with an extract of E.coli and the product was analyzed by electrophoresis. As Figure 6 shows, when the oligonucleotide was incubated with the cell extracts of NR9099 and CSH50, a cleavage product of chain length 10 was formed, which corresponded to the 5'-fragment of the 21mer incised at the 8-oxoG residue. In contrast, this activity was not detected in the extract of MF67, or that in YG5113, which is an authentic mutM-deficient E.coli. No such cleavage products were detected on incubation of the unmodified oligomer that contained a guanine residue in place of 8-oxoG with the extract of NR9099 or CSH50 (data not shown).

Effects of oxidative damage on the mutagenicity of phage M13mp2 treated with NMOR plus UVA

When phage M13mp2 treated with NMOR plus UVA was infected into the host cells deficient in mutM activity, a higher level of mutation was indeed observed, compared with that found with the use of the wild strain as the host (Figure 7 and Table IV). Lethality against the phage, however, was not elevated.

If the NMOR + UVA mutagenesis is mediated by singlet oxygen, the mutation frequency may be expected to increase in a D2O solution. However, this was not the case (Table II). When the treatment of phages with NMOR plus UVA was performed under nitrogen bubbling, however, an increase in the survival and a decline in the mutation rate were observed (Table V). Therefore, oxygen is required, but singlet oxygen is unlikely to be involved in this mutagenesis.

Discussion

The pathway of metabolic activation of N-nitrosodialkylamines involves a hydroxylation at carbon α to the amine nitrogen, followed by further degradation of the α-hydroxylated species to generate a carbonium ion that can alkylate DNA bases (3). For NMOR, it is known that the metabolically activated NMOR reacts with guanine, leading to the formation of adducts that have an extra cyclic ring attached to the guanine ring structure (26,27). Mutational events induced with metabolically activated NPYR were studied by Zielenska et al. (28). They showed that the majority of mutations occurring in the lact region of E.coli were GC to AT transitions. It is not known, however, whether cyclic guanine adducts that may be formed from activated NPYR can account for the transitions observed. In the case of the UVA activation of NMOR and its consequent mutational actions, which we report here, there are similarities and dissimilarities with the metabolic reactions. Regarding the products formed in the UVA-mediated conversion of NPYR and NPIP in aqueous solutions containing phosphate ion, we were able to identify the phosphate esters of α-hydroxylated NPYR and NPIP as the active principles (10,29). This phosphate ester is directly mutagenic to Salmonella typhimurium TA1535, a strain sensitive to mutagens causing base-pair

![Figure 7. Effect of mutM deficiency in the host upon the viability and mutation frequency of phage M13mp2. Treatment of phage was with NMOR (36 mM) plus or minus UVA, and the host for infection was E.coli wild or mutM: (●) NMOR + UVA, wild; (▲) NMOR + UVA, mutM; (○) NMOR only, wild; (△) NMOR only, mutM.]

Table IV. Mutation frequency of phage M13mp2 with different E.coli hosts

<table>
<thead>
<tr>
<th>Host strain (E. coli)</th>
<th>Mutation frequency of M13mp2 (×10^-4)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>MF67 (mutM)</td>
<td>3.5</td>
</tr>
<tr>
<td>NR9099 (wild)</td>
<td>3.9</td>
</tr>
<tr>
<td>CSH50 (wild)</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Phage M13mp2 was suspended in 20 mM sodium phosphate buffer (pH 7.0) containing 36 mM NMOR. UVA irradiation was for 2 h.

Table V. Effect of nitrogen bubbling on the survival and mutation of M13mp2 treated with NMOR + UVA*

<table>
<thead>
<tr>
<th>Host</th>
<th>Phage survival (%)</th>
<th>Mutation frequency (× 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>NMOR + UVA</td>
</tr>
<tr>
<td>CSH50</td>
<td>100</td>
<td>1.8</td>
</tr>
<tr>
<td>MF67</td>
<td>100</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*Conditions for phage treatment were the same as those given in Table IV.
substitutions. In the sense that the UVA activation leads to the formation of α-hydroxylated N-nitrosamines, the photoreaction is similar to the metabolic activation. However, as the results given above show, the base changes in the UVA-mediated mutagenesis are in major part GC to TA transversions (Table I). This mutation may be accounted for by the formation of 8-oxodG in the phage DNA by NMOR + UVA (Table II). It is reported that 8-oxoG can base-pair with either C or A, and as a result can cause transversions from GC to TA (30,31). The involvement of 8-oxoG in the ‘NMOR + UVA’ mutagenesis was further implicated by using a mutM-deficient host bacteria to the treated phage: a 2-fold increase in the mutation frequency was observed (Table V). The function of mutM protein is excision of 8-oxoG in DNA (19).

It is not clear how 8-oxodG is formed in this ‘NMOR + UVA’ reaction. The nitrogen bubbling experiment, in which strong inhibitions of the mutagenesis were observed (Table V), suggests that active oxygen species are involved, as might well be expected. Singlet oxygen, which is supposed to be involved in light-mediated pigment attack on DNA (23), is unlikely to be the active species in the mutation-causing reaction of NMOR + UVA (Table II).

Guanine is the most easily oxidizable among nucleic acid bases (32). The photooxidation by use of photodynamic agents such as methylene blue (33) and riboflavin (34) can generate 8-oxodG within DNA. Although singlet oxygen is possibly an essential intermediate in methylene blue + light-mediated formation of 8-oxodG (33), Kasai et al. (34) showed that with riboflavin + light electron transfer from the guanine moiety to triplet-excited riboflavin is likely to be the predominant reaction leading to the formation of a guanine radical cation, and that singlet oxygen is probably not involved. In the ‘NMOR + UVA’ reaction, a similar electron transfer from the guanine residue to an excited NMOR forming a guanine cation radical appears possible. Such a radical can react with a molecule of oxygen (35) to form 8-oxodG.

In this ‘NMOR + UVA’ mutagenesis, transitions, notably at C, take place (Table I). It is possible that base adduct formations are involved in causing these transitions.

It is almost an established fact that sunlight causes human skin cancer (6). UVC has been implicated to be the causative light because mutations in consecutive pyrimidines appear to be involved in sunlight-induced carcinogenesis (6). UVA, on the other hand, has been shown to be mutagenic in some organisms (7,36). N-Nitrosodialkylamines are carcinogenic to animals and possibly to humans as well (1,3). Experimental animals exposed to environmental nitrosamines are expected to maintain these compounds either in their metabolically altered form or in their original, unaltered form depending on the time that elapsed after the intake. Possible co-mutagenic organisms (7,36). /V-Nitrosodialkylamines are carcinogenic to action of NMOR and UVA forming 8-oxodG in DNA, as 7,8-dihydro-8-oxoguanine) in a rat-gene fragment. Nucleosides Nucleotides, 13, 1517-1534.

References


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