The role of Fpg protein in UVC-induced DNA lesions

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We previously demonstrated that reactive oxygen species (ROS) could be involved in ultraviolet-C (UVC)-induced DNA damage in *Escherichia coli* cells. In the present study, we evaluated the involvement of the GO system proteins in the repair of the 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG, GO) lesion, which is ROS-induced oxidative damage. We first found that the mutant strain Δfur , which produces an accumulation of iron, and the cells treated with 2,2'-dipyridyl, a iron chelator, were both as resistant to UVC-induced lethality as the wild strain. The 8-oxoG could be mediated by singlet oxygen ($^1O_2$). The Fpg protein repaired this lesion when it was linked to C (cytosine), whereas the MutY protein repaired 8-oxoG when it was linked to A (adenine). The survival assay showed that the Fpg protein, but not the MutY protein, was important to UVC-induced lethality and interacted with the UvrA protein, a nucleotide excision repair (NER) protein involved in UVC repair. The GC-TA reversion assay in the mutant strains from the '8-oxoG-repair' GO system showed that UVC-induced mutagenesis in the *fpg* mutants, but not in the MutY strain. The transformation assay demonstrated that the Fpg protein is important in UVC repair. These results suggest that UVC could also cause indirect ROS-mediated DNA damage and the Fpg protein plays a predominant role in repairing this indirect damage.

Keywords: *Escherichia coli*, Fpg protein, Reactive oxygen species, Singlet oxygen, UVC

Introduction

Despite the importance of the increased oxygen concentration on the Earth's surface to the maintenance of life, it induces some harmful effects through the formation of certain substances. This is due to the production of reactive oxygen species (ROS) during breathing,¹ which include the superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and others,² which can cause damage to DNA and other macromolecules such as proteins, lipids, and carbohydrates.³ This oxidative stress is an important agent of mutagenesis, carcinogenesis, aging, and a number of diseases.^{4,5}

The ultraviolet (UV) radiation from the Sun that reaches the Earth's surface consists of near-UV radiation, ultraviolet-A (UVA, $400 < \lambda < 320$ nm) and ultraviolet-B (UVB, $320 < \lambda < 290$ nm). Far-UV radiation, or ultraviolet-C (UVC, $290 < \lambda < 100$ nm), is filtered by the Earth's ozone layer and atmosphere.⁶

It is known that UV radiation changes several chemical and biological processes, leading to an

increased incidence of skin cancer, eye damage, and erythema.⁷ DNA is the main chromophore of UV radiation, and is the target of its impacts or effects.⁸ Therefore, exposing cells to UV radiation is one of the methods used to explore the biological consequences of damage to DNA, its repair, and tolerance.⁴ DNA damage by UV radiation is produced by direct and indirect effects. The direct effect results from the interaction of the energy from the radiation with DNA, leading to lesions such as cyclobutane pyrimidine dimers, pyrimidinehydrates, and adducts of pyrimidine. The indirect effects result from the interaction of radiation with intermediate-energy molecules, such as tryptophan, cysteine, riboflavin, and porphyrins, leading to the generation of ROS.⁴

The hydroxyl radical $\bullet OH$ causes oxidative DNA damage,^{9,10} and there is evidence to indicate that $^1O_2$ is also involved in this oxidative damage.¹¹ This ROS is produced by several biological systems¹² and can be formed by photosensitive reactions, which take place when chromophores are excited upon exposure to visible or UV light. This energy can be transferred to oxygen, converting it into $^1O_2$.²

It has been shown that $^1O_2$ reacts preferentially with guanine residues, whether they are free or composing

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the DNA molecule, leading to a variety of lesions at specific guanine sites. The most commonplace of these lesions is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG)^{13,14} which is mutagenic and lethal if not repaired.^{15,16}

Several studies show that near-UV (UVA and UVB) radiation may generate ROS.¹⁷ In contrast, it has been demonstrated that the lethality of far-UV (UVC) radiation is mainly caused by the formation of adducts between neighboring pyrimidines (direct lesions), which are lethal if not repaired.¹⁸

Aerobic organisms have developed complex defense and repair mechanisms to mitigate the harmful effects of ROS.¹⁹ Base excision repair and nucleotide excision repair (NER) are two mechanisms for DNA damage repair.

The UvrABC complex can undertake NER in a variety of lesions induced by ionizing radiation and oxidizing agents, such as AP sites, thymine glycols, pyrimidine dimers, and platinum adducts.²⁰

When 8-OxoG is misincorporated during DNA replication this causes GC to TA transversions. The removal of 8-oxoG, in *Escherichia coli*, involves a repair mechanism called the 'GO system'.²¹ The formamidopyrimidine glycosylase (Fpg) enzyme encoded by the *mutM* gene^{22,23} excises 8-oxoG from the 8-oxoG/C pairs with cytosine, whereas the MutY protein excises misincorporated A from 8-oxoG/A pairs. Meanwhile, in prokaryotes, direct UVC-induced damage is repaired by the UvrABC exonuclease, which carries out the entire process of damage recognition and excision.⁴

In contrast, some studies have shown that UVC causes oxidative damage, such as 8-oxoG,²⁴ 8-hydroxy-deoxyguanine (8-OHdG),²⁵ and thymine glycol.²⁶ Moreover, it has been suggested that UVC could generate ROS via lipid peroxidation in mammalian cells.²⁷ Our results corroborate this suggestion that UVC can also cause indirect DNA damage.²⁸

Materials and methods

Bacterial strains and plasmids

The *E. coli* strains and plasmids used in this work are described in Table 1. The DNA plasmid pUC 9.1, hosted by *E. coli* DH5aF'Iq (rec⁻), was extracted according to Sambrook and Russell.²⁹

Growth conditions

Cells were grown overnight (in a shaking incubator at 37°C) in LB (Luria-Bertani)-rich medium.³⁰ A starting inoculum was taken from these cultures and cells were grown in the same medium until the mid-exponential growth phase (2×10^8 cells/ml).

Survival experiments

The experiments were performed with cells in the mid-exponential growth phase. The cells grown in LB

Table 1 *E. coli* K-12 bacterial strains and plasmids used in this work

Strains	Relevant genotype	Source
AB1157	F ⁻ thr-1 leuB6 thi-1 argE3 his-4 proA2 lacY1 galK2 xyl-5 ara-14 rspL13 tsx-33 supE44	P. Howard-Flanders
BH20	As AB1157, but <i>fpg</i> ⁻	S. Boiteux
CC104	P90 C <i>ara</i> $\Delta(lac-pro)_{XIII}$ (<i>F</i> <i>lacZ</i> <i>proB</i> ⁺); GC-TA tester strain	Cupples and Miller
BH540	As CC104, but <i>fpg</i> ⁻	S. Boiteux
BH980	As CC104, but <i>mutY</i> ⁻	S. Boiteux
BH990	As CC104, but <i>mutY-fpg</i> ⁻	S. Boiteux
AB1886	As AB1157, but <i>uvrA</i> ⁻	P. Howard-Flanders
RJF624	As AB1886, but <i>fpg</i> ⁻	A. Leitão
GC4468	F ⁻ $\Delta(lac-argF)U169$ <i>rpsL</i> 179	D. Touati
QC1732	As GC4468, but Δfur	D. Touati
pUC 9.1	Amp ^R	Our laboratory
pFPG	<i>fpg</i> ⁺ Amp ^R	S. Boiteux

medium were centrifuged and resuspended in saline (NaCl 0.9%) to the same density, then irradiated with different doses of UVC (254 nm, G15T8, 15 W General Electric germicidal lamp, New York, NY, USA). Dosimetry was performed using an ultra-sensitive DC micro-ammeter (Mount Vernon, New York, NY, USA) with a dose rate of 0.5 J/m²s. 0.1 ml samples were collected, diluted in saline, and placed (in duplicate) in LB medium solidified with 1.5% agar. The colonies formed were counted after overnight incubation at 37°C. Additionally, cells grown to mid-exponential phase in LB medium were pre-treated for 20 minutes in the presence or absence of 1 mM dipyrindyl (Sigma), then centrifuged, resuspended in saline, and irradiated with different doses of UVC. Survival data were analyzed using the analysis of variance (ANOVA) test to detect significant differences ($P < 0.05$) between the tested strains. To identify the differences, the Tukey–Kramer multiple comparison test was employed for all the strains using the InStat statistical program version 4.0 (GraphPad Software, San Diego, CA, USA).

E. coli lacZ reversion mutation assay

This mutagenic assay was described by Cupples and Miller³¹ and very well explained by Josephy.³² Cells were grown overnight at 37°C with shaking in a minimal LB medium consisting of a C-salt³³ medium enriched with lactose supplemented with 0.5% glucose, 0.2% casamino acids, and 2 µg/ml thiamine. When the cultures reached the mid-exponential growth phase (2×10^8 cells/ml) they were irradiated with different doses of UVC (254 nm), centrifuged, and resuspended in the same volume of C-salts medium without glucose. 0.1 ml aliquots diluted to 10⁻⁶ were plated on LB plates and 0.1 ml aliquots were plated on C-salt plates enriched with 0.5% lactose and 2 µg/ml thiamine. Plates were incubated at 37°C and screened after 1 day for viable cells, or

after 2 days for Lac⁺ revertants. Only Lac⁺ revertants produce readily visible colonies on selective plates. Reversion frequencies were calculated by dividing the number of Lac⁺ revertants by the total number of viable cells. This assay was repeated at least five times and the data were analyzed using the ANOVA test, followed by Tukey's multiple comparison test ($P < 0.05$) using the InStat statistical program, version 4.0 (GraphPad Software, San Diego, CA, USA).

Bacterial plasmid pUC 9.1 transformation efficiency

The evaluation of bacterial transformation efficiency obtained from plasmid pUC 9.1 is determined by calculating the percentage of colony-forming units (CFUs) transformed by the plasmid (200 ng) by comparing the control sample and treated samples. The bacterial suspension (approximately 10^9 cells/ml) was mixed with pretreated plasmid DNA and incubated on ice for 20 minutes. Afterwards, the tubes were placed in a water bath at 42°C for 4 minutes followed by incubation on ice for 1 minutes, then 500 µl liquid LB was added. The cultures were then incubated for 1 hour at 37°C, and aliquots were plated onto LB-agar containing ampicilin (60 µg/ml) and incubated at 37°C overnight, after which time the number of CFUs was counted.²⁹ The sample control had a transformation efficiency of 100%. This assay was repeated at least five times and the collected data were submitted to the chi-square test (2), adopting 5% as the significance level.

Results and discussion

UVC radiation is one of the models used as a positive control in studies of direct DNA damage, such as pyrimidine dimers, which are generated by the interaction of this agent with the cellular genome. Whether UVC can generate indirect damage is as yet a moot point to the scientific community. However, our previous results indicate that ROS mediate the lethality induced by UVC in *E. coli* cells.^{28,34} Other studies have also shown that UVC (254 nm) radiation can induce the formation of oxidative DNA lesions.^{24,25}

ROS such as $O_2^{\bullet-}$ and $^1O_2$, are formed in cells during aerobic metabolism and during oxidative stress.² The generation of these species by endogenous and exogenous sources may result in DNA damage. Thus ROS may be mutagenic and carcinogenic.^{35,36}

However, under physiological conditions, $O_2^{\bullet-}$ does not produce DNA damage.^{37,38} Thus, the toxicity of this ROS is a result of its ion-catalyzed conversion into $\bullet OH$, a highly reactive radical.²

Likewise, the indirect damage mediated by ROS is mainly associated with $\bullet OH$ generation, through the Fenton reaction, which is dependent on transition

metals such as Fe and Cu, resulting in serious damage to DNA.²

Fur controls iron metabolism, repressing several genes involved in iron uptake by cells.³⁹ So, cells carrying a non-functional *fur* locus (Δfur mutant) present high levels of iron, which is sensitive to oxidative agents such as UVA.⁴⁰ 2,2'-dipyridyl, is a Fe^{2+} chelator, which diminishes $\bullet OH$ formation through the Fenton reaction.

Then, by checking the survival fraction, we verified the influence of the bacterial Fur protein and the chelator dipyridyl on UVC-induced lethality, and we showed that Δfur mutant and dipyridyl treatment cells were as resistant to UVC-induced lethality as the wild-type strain (Fig. 1; $P < 0.05$). These data suggest that the background iron level does not interfere in the effect of indirect UVC-induced damage, which indicates that $\bullet OH$ was not the main UVC-induced ROS.

Our previous results showed that the mutant strain *fpg* was more resistant to UVC-induced lethality. Zhang *et al.*²⁴ showed that UVC could induce 8-oxoG oxidative damage, which indicates the participation of the GO system in UVC-induced damage.

The GO system was repaired, mainly with the participation of two proteins, the Fpg or MutM protein and the MutY protein. The former protein removes 8-oxoG paired with cytosine, whereas the latter removes 8-oxoG paired with A.

In order to verify the GO system's participation in UVC-induced lethality, experiments were conducted which showed that the MutY strain did not seem to influence the repair of UVC-induced lesions, since their survival was similar to that of the wild strain (Fig. 2; $P < 0.05$). However, the MutM mutant strain was more resistant than the wild-type strain to the cytotoxic damage induced by UVC (Fig. 2; $P > 0.001$). Insertion of the pFPG plasmid in all the

Figure 1 Survival fraction of *E. coli* Δfur mutant QC1732 (squares), wild-type GC4468 (diamonds) and wild-type pretreated with dipyridyl 1 mM, a Fe^{2+} chelator (circles) irradiated with different doses of UVC (254 nm). Values are the mean of at least five experiments and were submitted to ANOVA test followed Tukey's comparison test ($P > 0.05$).

Figure 2 Survival fraction of *E. coli* wild-type CC104 (diamonds), *mutM*[−] mutant BH540 (squares), *mutY*[−] mutant BH980 (triangles), *mutM*[−] *mutY*[−] mutant BH990 (circles) original strains (filled symbols), or transformed with pFPG plasmid (open symbols) irradiated with different doses of UVC (254 nm). Values are the mean of at least five experiments and were submitted to ANOVA test followed Tukey's comparison test ($P > 0.05$, wild strain compared with *mutY*[−] mutant or *mutM*[−] *mutY*[−] mutant; $P < 0.001$ for all other comparisons).

transformed strains made the cells more sensitive to UVC (Fig. 2; $P > 0.001$). These results could be explained by the participation of Fpg in the repair mechanisms of UVC-induced damage, which involve the generation of many gaps, besides the ones generated by the NER pathway.⁴¹ As such, the *fpg*[−] mutant strains could produce fewer DNA breaks than the wild-type strain.

8-oxoG is a major lesion arising from oxidative stress in prokaryotes and eukaryotes, and is associated with cancer, aging, infertility, and others diseases⁴² and is therefore a lethal, mutagenic lesion. ¹O₂ is known to modify guanine residuals, and the major type of ¹O₂-induced DNA lesion is precisely 8-oxoG.⁴³ ¹O₂-induced mutagenicity damage mainly takes the form of base substitutions affecting G:C base pairs. The ability of 8-oxoG to mispair with dG causes GC→TA transversions.⁴⁴

To verify the participation of 8-oxoG in UVC-induced mutagenesis, the *lacZ* mutation assay was performed. We used the *E. coli* strains created by Cupples and Miller,³¹ which have a specific mutation in the *lacZ* gene that allows the rapid determination of GC→TA transversion, a transversion that could be induced by 8-oxoG.⁴⁵ The CC104 strain lost its ability to metabolize lactose (Lac[−] phenotype). The reversion to Lac⁺ recovered β-galactosidase synthesis.^{46,47}

We observed that UVC induced the 8-oxoG mutagenic lesion and the Fpg protein was important in repairing this lesion (Fig. 3; $P > 0.001$). However, despite being part of the GO system repair, the MutY protein does not seem as important as MutM in UVC-induced mutagenesis (Fig. 3, $P > 0.001$).

Boiteux *et al.*⁴⁸ suggest that mismatch repair mediated by MutY, acting synergistically with

Figure 3 Mutagenesis LacZ of *E. coli* wild-type CC104, *mutM*[−] mutant BH540 and *mutY*[−] mutant BH980 not treated (black column) or irradiated with 120 J/m² of UVC (254 nm) (gray column). Values are the mean of at least five experiments and were submitted to ANOVA test followed Tukey's comparison test ($P < 0.001$).

MutM, plays a major role in the prevention of 8-oxoG mutagenic effects. According to our results, the Fpg protein seemed to be very important in the repair of UVC-induced DNA lesions, indicating that UVC induces 8-oxoG in bacterial cells by generating ¹O₂, mediated by GC→TA transversion. However, the MutY protein would appear not to be so important in UVC-induced mutagenesis and lethality.

NER is able to repair a wide spectrum of DNA lesions. Proteins including UvrA, -B, -C, and -D are involved in the recognition of the lesion, cleavage at the 3' and 5' ends of that lesion, and the release of the patch of DNA including the damaged base.⁴⁹ Other researchers suggest that Fpg and UvrABC complement each other in the repair of lethal damage, 8-oxoG, induced by ¹O₂.^{20,50}

To confirm the importance of Fpg in the repair of UVC-induced lesions mediated by ROS, a survival assay was undertaken using *uvrA*[−] and *uvrA*[−] *fpg*[−] mutant strains transformed with pFPG plasmid. We observed that UVC damage induced nucleotide and base excision repair simultaneously, represented by Fpg and the UvrABC system (Fig. 4). In contrast, *uvrA*[−] *fpg*[−] mutant was more sensitive to UVC-induced lethality and the cells transformed with pFPG plasmid acquired significantly more resistance to this agent than the original strain (Fig. 4; $P > 0.001$).

Although the above results may seem controversial, we can explain them by the fact under this circumstance when the *uvrA* strain is far more UVC-sensitive than the wild strain, although the UvrABC system does not generate any gaps, there is a build-up of unrepaired lesions, and the presence of Fpg acts in the repair of the lesions, reducing the overall number of lesions in the DNA, ultimately helping the cell to survive (Fig. 4).

Still, from the level of protection acquired by the strains transformed with plasmid pFPG (Fig. 4; $P >$

Figure 4 Survival fraction of *E. coli uvrA*⁻ mutant AB1886 (diamonds), *uvrA*⁻ *mutM*⁻ mutant RJF624 (squares) original strains (filled symbols), or transformed with pFPG plasmid (open symbols) irradiated with different doses of UVC (254 nm). Values are the mean of at least five experiments and were submitted to ANOVA test followed Tukey's comparison test ($P > 0.05$, *uvrA*⁻ mutant compared with *uvrA*⁻ *mutM*⁻ mutant transformed with pFPG plasmid; $P < 0.001$ for all other comparisons).

0.001), we could conjecture that the Fpg protein may not just be mediating 8-oxoG repair by ¹O₂, but could also be involved in the repair of direct damage produced by UVC, such as pyrimidine dimers. As such, the UvrABC system could also repair 8-oxoG lesions.

Another approach to assess UVC-induced genotoxicity and UVC repair mechanism was the bacterial transformation assay, used to check the transformation capacity of *E. coli* AB1157 and *fpg*⁻ mutant (BH20) by pUC 9.1 DNA plasmid, which has a gene that is resistant to the antibiotic ampicillin. As such, we observed not only the capacity of the plasmid to penetrate cells, but also the capacity of the *E. coli* strain to repair a damaged plasmid. The transformation efficiency of the BH20 strain was significantly lower than that of the wild-type strain (Fig. 5, $P > 0.001$).

We therefore suggest that the repair of UVC-induced lesions was achieved with the participation

Figure 5 Efficiency of bacterial transformation with plasmid pUC 9.1 on strains AB1157 (black) and BH20 (gray) irradiated with 120 J/m² of UVC. Values are the mean of at least five experiments and were submitted to chi square, adopting 5% as the significance level ($P < 0.001$).

of the Fpg protein, and that this repair process could generate apyrimidinic sites⁴ which made DNA strand breaks in *E. coli* cells, leading to an increase in lethality. Meanwhile, the presence of this protein seen through the insertion of the plasmid pFPG reduces the mutagenesis induced by this agent.

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