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Hydrogen peroxide induces a specific DNA base change profile in the presence of the iron chelator 2,2' dipyridyl in *Escherichia coli*

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Abstract

Pretreatment of *Escherichia coli* cultures with the iron chelator 2,2'-dipyridyl (1 mM) protects against the lethal effects of low concentrations of hydrogen peroxide (<15 mM). However, at H_2O_2 concentrations equal to or greater than 15 mM, dipyridyl pretreatment increases lethality and mutagenesis, which is attributed to the formation of different types of DNA lesions. We show here that pretreatment with dipyridyl (1 mM) prior to challenge with high H_2O_2 concentrations (≥15 mM) induced mainly G:C→A:T transitions (more than 100X with 15 mM and more than 250X with 20 mM over the spontaneous mutagenesis rate) in *E. coli*. In contrast, high H_2O_2 concentrations in the absence of dipyridyl preferentially induced A:T→T:A transversions (more than 1800X and more than 300X over spontaneous mutagenesis for 15 and 20 mM, respectively). We also show that in the *fpg nth* double mutant, the *rpoB* gene mutation (Rif^S-Rif^R) induced by 20 mM H_2O_2 alone (20X higher) was increased in 20 mM H_2O_2 and dipyridyl-treated cultures (110X higher), suggesting additional and/or different lesions in cells treated with H_2O_2 under iron deprivation. It is suggested that, upon iron deprivation, cytosine may be the main damaged base and the origin of the pre-mutagenic lesions induced by H_2O_2.

Key words: Hydrogen peroxide; Dipyridyl; Mutagenesis; Iron; Fenton reaction

Introduction

In *Escherichia coli*, it has been demonstrated that H_2O_2 induces cell death by two different modes of action as a function of H_2O_2 concentration, both of which are accompanied by enhanced mutagenesis (1,2). Oxidative DNA damage induced by hydrogen peroxide is thought to occur through the Fenton reaction in the Haber-Weiss cycle. Our group and others have demonstrated (2-4) that pretreatment with the iron chelators 2-2'dipyridyl, 1-10 phenanthrolene or deferoxamine protects *E. coli* cells against the lethal effect of H_2O_2, suggesting that ionic iron is the main transition metal that mediates its genotoxicity (5). Dipyridyl also protects the cells against the oxidative lesions produced by UV-B radiation (6). Asad and Leitão (3) have proposed a different pathway for DNA damage induced by H_2O_2 when cells are depleted of iron because DNA lesions produced under these conditions were repaired even in an exonuclease III (*xthA*)-deficient mutant (3). It was thought that this pathway would occur through the Fenton reaction mediated by transition metals other than iron. Indeed, copper ions have been implicated as candidates to mediate this pathway. It was suggested that copper plus H_2O_2 would generate different types of DNA lesions or a higher number of the same lesions that are generated through the iron-mediated Fenton reaction (7). Following this characterization, Asad et al. (8) detected the increased sensitivity of *fpg* and *uvrA* mutants to H_2O_2 challenge when iron deprived. Based on the fact that oxidation involving copper generates a significant amount of 8oxo-G and that Fpg and UvrA play an important role in repairing this lesion, Almeida et al. (7) have suggested that 8oxo-G should be the most important DNA lesion produced by H_2O_2 challenge in iron-depleted cultures (9,10). The SOS response is also believed to play an important role in cell

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survival after treatment with H_2O_2 in the presence of iron chelators (9).

In the present study, we investigated H_2O_2-induced mutagenesis under iron deprivation using a reversion test with a series of *E. coli* mutant strains (11) as well as a forward mutation test (12) using base excision repair mutant strains.

Material and Methods

Bacterial strains

The bacterial strains used are derived from *E. coli* K-12 and are listed in Table 1.

Table 1. Strain description.

Escherichia coli strains		
Designation*	Relevant genotype	Reversion event
CC101	*ara*⁻. Δ(*lac - proB*)$_{XIII}$ [F' *lacI*⁻ *Z*⁻.*proB*⁺]	A:T→C:G
CC102	*ara*⁻. Δ(*lac - proB*)$_{XIII}$ [F' *lacI*⁻ *Z*⁻.*proB*⁺]	G:C→A:T
CC103	*ara*⁻. Δ(*lac - proB*)$_{XIII}$ [F' *lacI*⁻ *Z*⁻.*proB*⁺]	G:C→C:G
CC104	*ara*⁻. Δ(*lac - proB*)$_{XIII}$ [F' *lacI*⁻ *Z*⁻.*proB*⁺]	G:C→T:A
CC105	*ara*⁻. Δ(*lac - proB*)$_{XIII}$ [F' *lacI*⁻ *Z*⁻.*proB*⁺]	A:T→T:A
CC106	*ara*⁻. Δ(*lac - proB*)$_{XIII}$ [F' *lacI*⁻ *Z*⁻.*proB*⁺]	A:T→G:C
AB1157	*F*⁻ *thr-1, leuB6, thi-1, argE3, his-4, proA2, lacY1, galK2, xyl-5, ara-14, rspL13, tsx-33, supE44*	
BW375	As AB 1157, but *nth*	
BH20	As AB 1157, but *fpg*	
BH160	As AB 1157, but *nth fpg*	

*The CC strains were a gift from Prof. J.H. Miller (University of California, Los Angeles, CA, USA). The BW strain was a gift from Dr. Bernard Weiss (University of Michigan Medical School, Ann Arbor, MI, USA). The BH strains were a gift from Dr. S. Boiteux (DRR, CEA Fontenay aux Roses, France). The AB strain was from our laboratory stock.

Growth conditions

Bacterial cultures were grown overnight in M9 minimal medium (13) containing glucose (4 g/L) supplemented with 2.5 mg/mL casamino acids and 1 µg/mL thiamine at 37°C under shaking. The supplemented medium was designated M9S. A starting inoculum (0.25 mL, ≈10^8 cells) was taken from these cultures and the cells were grown in 10 mL fresh M9S medium until the mid-exponential phase (2 x 10^8 cells/mL).

Survival experiments

Cultures in the mid-exponential phase of growth (pretreated or not with 1 mM dipyridyl) were challenged with 20 mM H_2O_2 (30% perhydrol; Merck, 7722-84-1, Brazil) for 20 min (CC strains) or 5 min (AB1157 and base excision repair mutant strains) in M9S medium at 37°C under shaking. Residual H_2O_2 was inactivated by the addition of excess catalase (5 µg/mL; EC 1.11.1.6 Sigma 9001-05-2, USA). Samples were collected at the end of the incubation time, diluted in M9 salt solution, and spread on lysogeny broth (LB) medium (13) solidified with 1.5% agar (Difco). The colony forming units (CFU) were scored after overnight incubation at 37°C.

Pretreatment with dipyridyl

Cultures in the mid-exponential growth phase were incubated with the iron chelator dipyridyl (1 mM; 2,2'- bipyridine, Sigma, 366-18-7) for 20 min in M9S medium at 37°C, with shaking. Treatment with the metal ion chelator alone did not affect cell viability (data not shown). Cultures treated with 1 mM dipyridyl are referred to as iron-depleted culture.

Analysis of the Lac⁻⟶Lac⁺ mutagenesis induced by H_2O_2

Mutagenesis assays for transitions and transversions are based upon the Lac⁻→Lac⁺ reversion of specific mutations in the *lacZ* gene located on an F'-plasmid described by Coulondre and Miller (14) and Cupples and Miller (11). Reversion is examined in a set of strains (CC101 to 106) in which the Lac⁺ phenotype is recovered after specific base substitution restores codon 461 of the *lacZ* gene to Gln. We have screened base changes using the CC strains after treatment with 15 and 20 mM H_2O_2 for 20 min with or without dipyridyl (1 mM) pretreatment. Cultures in the mid-exponential growth phase were challenged as indicated in the section Survival experiments. Aliquots (0.8 mL) were taken for survival and mutagenesis experiments, centrifuged for 4 min at 9500 *g* and resuspended in 0.4 mL. An aliquot of 0.2 mL was added to 2 mL LB and incubated for 20 h at 37°C with shaking (150 rpm). Next, a 0.1-mL portion was spread on minimal medium containing 0.4% lactose as a carbon source (11) and incubated for 72 h at 37°C to allow growth of Lac⁺ revertants. Viable cell numbers in the 20-h culture were counted on LB plates after 24 h at 37°C. The mutation frequency is reported as number of mutant cells per 10^8 viable cells. Data are reported as means ± SEM of three experiments, as indicated in the tables. Fold increase in mutation frequency (base substitution) was compared between strains by ANOVA and the Tukey-Kramer multiple comparison test (GraphPad InsTat, GraphPad Software Inc., USA). The level of significance was set at $P < 0.01$ in all analyses.

As a control we measured the sensitivity of CC strains to challenge with 20 mM H_2O_2 and observed that the survival was about 10% and for dipyridyl-pretreated cells the survival was 2 to 5% (data not shown).

Analysis of the Rif^S⟶Rif^R mutagenesis induced by H_2O_2

Mutagenesis experiments were performed as described by Sedgwick and Goodwin (12). Cells in the mid-exponential

phase of growth, pretreated with 1 mM dipyridyl or not (20 min), were treated with 20 mM H_2O_2 for 5 min. All strains showed at least 60% survival after the H_2O_2 challenge. Aliquots were taken for survival experiments, and 0.1-mL samples were added to 3 mL of melted LB containing 0.75% agar, which was then layered on 15-mL LB plates. An additional 3-mL layer was added to the plates, which were then incubated at 37°C for 5 h to allow cell division and mutation fixation. After this period, a final 3-mL layer of melted LB containing 800 µg/mL rifampicin (Sigma 13292-46-1) was added and plates were then incubated for 16 h. Diffusion of the antibiotic through the medium led to a final concentration of 100 µg/mL rifampicin. Rifampicin-resistant CFU represented cells with a mutated *rpoB* gene and mutation frequency was expressed as the ratio between the number of rifampicin-resistant CFU and the number of viable cells after treatment detected in the corresponding survival experiment.

Results and Discussion

To analyze the nature of the lesions produced when bacterial cultures are pretreated with 2-2'dipyridyl and challenged with H_2O_2 at high concentrations, we conducted mutagenesis assays for transitions and transversions based upon the $Lac^- \rightarrow Lac^+$ reversion of specific mutations in the *lacZ* gene located on an F'-plasmid described by Coulondre and Miller (14) and Cupples and Miller (11).

A clearly predominant A:T→T:A (CC105) transversion was seen when strains were treated with H_2O_2 concentrations ≥15 mM (Table 2). On the other hand, these concentrations induced a completely different pattern of base changes in cultures pretreated with 2-2'dipyridyl. In this case there was a clear preference for G:C→A:T (CC102) transition instead of the massive A:T→T:A (CC105) transversion induced by H_2O_2 alone (Tables 2 and 3). It should be noted that A:T→T:A (CC105) base substitution appears as the second highest mutation frequency of base substitution when H_2O_2 treatment is performed under iron deprivation (Table 3).

By our characterization of DNA base substitution induced by treatment with high concentrations of H_2O_2 upon iron deprivation, we obtained a clear difference in the profile of DNA lesions induced in the two situations. A:T→T:A transversion induced by H_2O_2 can be related to translesion synthesis at AP sites that is strongly dependent on SOS

Table 2. Mutagenesis of CC culture strains exposed to H_2O_2.

Strain	Substitution	0 mM H_2O_2	15 mM H_2O_2		20 mM H_2O_2	
		Mut/10^8 cell	Mut/10^8 cell	Fold increase	Mut/10^8 cell	Fold increase
CC101	A:T→C:G	1.7 ± 0.3	12.5 ± 1.5	7.4	55.3 ± 30.9	32.5
CC102	G:C→A:T	0.5 ± 0.3	0.0	0.0	0.0	0.0
CC103	G:C→C:G	0.6 ± 0.2	6.0 ± 4.2	9.8	18.4 ± 10.8	30.2
CC104	G:C→T:A	3.3 ± 1.4	33.7 ± 5.7	10.2	50.0 ± 11.5	15.2
CC105	A:T→T:A	0.5 ± 0.2	978.8 ± 242.0*	1856.9	162.9 ± 6.9*	309.0
CC106	A:T→G:C	0.3 ± 0.1	0.5 ± 0.1	1.7	0.1 ± 0.0	0.2

Cultures in the mid-exponential phase of growth in M9S medium at 37°C under shaking were exposed to H_2O_2 treatment for 20 min. The data represent the mean of at least 3 experiments and are used in Table 3 and Figure 1. *P < 0.01 compared to all other strains (Tukey-Kramer test).

Table 3. Mutagenesis of CC culture strains exposed to dipyridyl and H_2O_2.

Strain	Substitution	Dipyridyl + 0 mM H_2O_2	Dipyridil + 15 mM H_2O_2		Dipyridyl + 20 mM H_2O_2	
		Mut/10^8 cell	Mut/10^8 cell	Fold increase	Mut/10^8 cell	Fold increase
CC101	A:T→C:G	2.4 ± 0.5	4.8 ± 0.1	2.0	8.6 ± 2.0	3.5
CC102	G:C→A:T	0.5 ± 0.1	68.7 ± 4.7*	135.8	138.9 ± 40.8*	275.0
CC103	G:C→C:G	0.8 ± 0.1	5.9 ± 3.4	7.2	2.2 ± 1.3	2.7
CC104	G:C→T:A	16.7 ± 6.9	25.0 ± 7.0	1.5	14.1 ± 1.3	0.8
CC105	A:T→T:A	0.7 ± 0.1	15.1 ± 8.8	20.2	56.9 ± 21.0	76.0
CC106	A:T→G:C	1.4 ± 0.2	0.6 ± 0.1	0.4	0.9 ± 0.0	0.6

Cultures in the mid-exponential phase of growth in M9S medium at 37°C under shaking were pretreated with 1 mM dipyridyl for 20 min and then exposed to H_2O_2 treatment for 20 min. The data represent the mean of at least 3 experiments. *P < 0.01 compared to all other strains (Tukey-Kramer test).

response (15).

The appearance of A:T→T:A is consistent with a preference for adenine (A rule) insertion in the bypass at AP sites (16). Therefore, we assume that our observation is consistent with the production of AP sites, a well-known type of oxidative DNA damage (17), after exposure to high H_2O_2 concentrations. However, we cannot exclude the possibility of another oxidative base damage, such as 2-hydroxyadenine, that can also induce A:T→T:A transversion by mispairing with adenine. The accumulation of 2-hydroxyadenine is reported to occur after H_2O_2 treatment in human cells (18,19).

The clear preferential induction of G:C→A:T transition by high H_2O_2 upon iron deprivation indicates that the nature of DNA base damage induced by this challenge may be different from that induced by H_2O_2 alone. The occurrence of 8oxo-G as well as AP sites due to damaged guanine in DNA does not support the appearance of this transition. Both lesions would produce G:C→T:A as a result of mispairing between 8oxo-G with A or by preferential insertion of adenine at an AP site (20). This led us to focus on cytosine as the main target for DNA lesion induced by H_2O_2 upon iron ion deprivation.

Hydroxyl ion reacts with cytosine by adding to the C5-C6 double bond leading to the formation of cytosine glycol, which is unstable and can break down further to 5-hydroxy-2'deoxycytosine (5-OHdC), 5-hydroxy-2'deoxyuracil (5-OHdU) and uracil glycol (21,22). The major stable oxidation product of cytosine is 5-OHdU that preferentially pairs with adenine (13). 5-Hydroxy-2'deoxyuracil shares this same property and it would also result in the G:C→A:T transition (19,23). Whenever 5-OHdC is present in DNA, it is removed by the action of endonuclease III (Nth) via the N-glycosylase/β elimination reaction, by the formamidopyrimidine-DNA glycosylase (Fpg) via the N-glycosylase/β,δ elimination reaction (24,25) in *E. coli* or by their homologs in eukaryotes (26,27). Nth and Fpg can remove 5-OHdU from DNA through the cited mechanisms and additionally by uracil DNA N-glycosylase (Ung) generating AP sites (25).

Mutagenesis induced by 20 mM H_2O_2 upon iron deprivation in the *rpoB* gene, performed as described by Sedgwick and Goodwin (12), was evaluated using either *nth* and *fpg* single mutants or double mutants. A higher mutation frequency was detected in the double mutant (Figure 1) for both conditions. We detected a 110-fold increase in mutation frequency in dipyridyl-pretreated cells and a 20-fold increase in cultures not treated with the metal chelator, suggesting the appearance of additional and/or different lesions in cells challenged with H_2O_2 upon iron deprivation. In both cases the sum of the mutation frequency observed in the single mutants did not correspond to that observed in the double mutant. This behavior may indicate that both *nth* and *fpg* share a role in preventing the appearance of mutagenic lesions. We suggest that 5-OHdC and 5-OHdU are the premutagenic DNA lesions produced by the challenge of H_2O_2 in iron-depleted cultures. We assume that the G:C→A:T transition would be due to the mispairing of damaged cytosine, in this case 5-OHdC, with adenine. In the case of 5-OHdU we can speculate that it would be converted to an AP site, since Ung is active in *nth fpg* double mutants, leading to base substitution of C to T as a consequence of the preference for adenine insertion during translesion synthesis at an AP site. The nature of base substitution in the *nth, fpg* and *nth fpg* background is presently under investigation by our group.

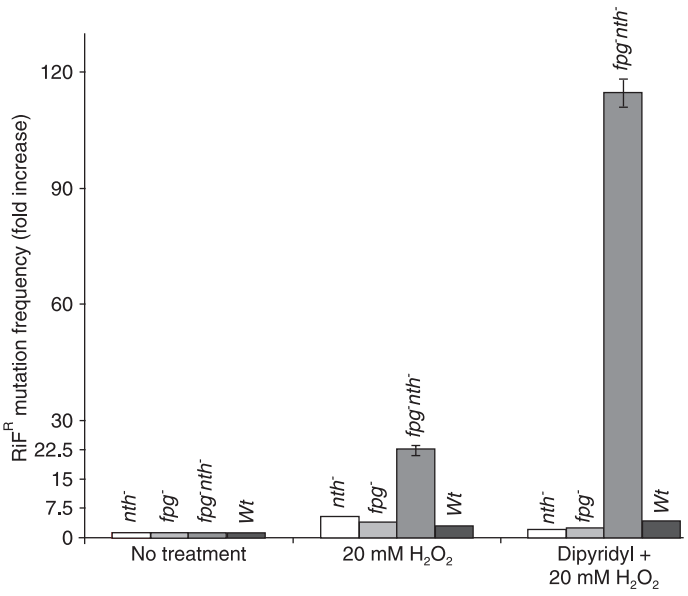

Figure 1. Mutagenesis of *Escherichia coli* cultures exposed to dipyridyl and H_2O_2. Cultures in the mid-exponential phase of growth in M9S medium at 37°C under shaking were pretreated or not with 1 mM dipyridyl for 20 min and then submitted to H_2O_2 treatment for 5 min.

We suggest that lethal and mutagenic pathways produced by high concentrations (more than 15 mM) of H_2O_2 in *E. coli* under iron deprivation might be a result of cytosine oxidation, yielding their mutagenic products 5-OHdC and/or 5-OHdU. Taken together, our data support the hypothesis of two different pathways for H_2O_2-induced lesions upon iron deprivation, which depend on H_2O_2 concentration.

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