

Identification of Ultimate DNA Damaging Oxygen Species

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DNA damage induced by various reactive oxygen species can be characterized using a set of repair endonucleases with defined substrate specificities. DNA damage profiles thus obtained in a cell-free system can be compared with those observed in cellular DNA. Using this approach, we have demonstrated that an illumination of *Salmonella typhimurium* cells with visible light in the presence of methylene blue gives rise to a DNA damage profile very similar to that of singlet oxygen in a cell-free system. Therefore, the genotoxicity observed under these conditions most probably is attributable to the direct action of this species. The damage consists mainly of base modifications that are subject to repair by uvrABC-independent pathways. Revertant frequencies observed in parallel in the strains TA100 and TA2638 indicate a pronounced mutagenicity of the lesions induced. Exposure of *Salmonella typhimurium* to *tert*-butylhydroperoxide gives rise to another form of damage profile that is also different from that produced by hydroxyl radicals in a cell-free system. However, the latter dissimilarity does not exclude hydroxyl radicals as ultimate reactive species, as a very rapid repair of the induced base modifications is observed, which might have distorted the damage profile despite immediate work up.

Introduction

Reactive oxygen species (ROS) such as superoxide, hydroxyl, peroxy and alkoxy radicals, singlet oxygen, and triplet excited carbonyl compounds most probably are generated inside cells as by-products of the normal mitochondrial electron transport. Under certain conditions (oxidative stress) the formation of ROS can be greatly increased (1,2).

Evidence indicating a role for ROS in carcinogenesis has been accumulating continuously. Support not only comes from studies with cell cultures (3) and animal experiments (4), but also from prospective epidemiological studies that reveal an inverse correlation between the individual cancer risk and plasma levels of the antioxidants vitamin C and E and β -carotene (5).

Basically, two different mechanisms have been suggested to explain the effects of ROS. First, ROS might act as promoters, i.e., modulate the expression of genes without damaging the genome (6). Second, ROS might modify the DNA either by direct chemical reaction or via an activation of cellular nucleases, giving rise to mutations. The relevance of this latter initiating capacity of ROS is far from being clear, although the DNA damaging potential of ROS under cell-free conditions is well established. One important question is which of the various ROS can ultimately modify DNA inside cells

and which kind and extent of mutagenicity is associated with that damage. The cellular situation, however, is difficult to analyze because the various species can be generated from one another by numerous enzymatic and nonenzymatic reactions (Fig. 1). High reactivity of one species can be more than compensated by a longer life-time or a more target-directed generation of another.

One way to identify the ultimate DNA damaging species that does not require any detailed information about the metabolic pathways shown in Figure 1 is to use the DNA damage itself as a fingerprint of the modifying species. This is possible because different ROS are known to induce different DNA modifications or at least very different ratios of common modifications. For example, the 5,6-double bond of thymine residues is a

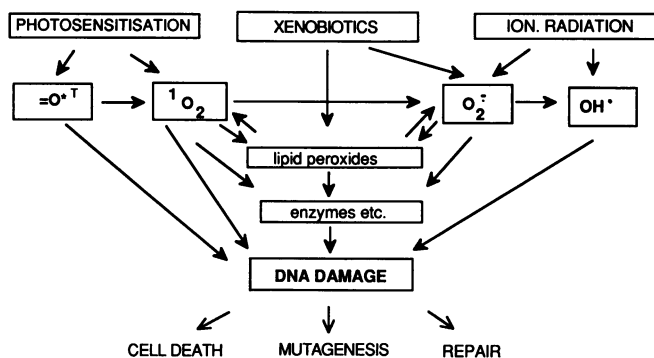


FIGURE 1. Possible pathways for the induction of DNA damage by various reactive oxygen species.

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primary target for hydroxyl radicals (7), while singlet oxygen is known to modify guanines predominantly (8,9). If cellular nucleases were ultimately responsible for DNA damage, strand breaks would be expected to be the only modifications.

To follow up this idea, we have developed a method in which we use repair endonucleases as probes to characterize a given kind of DNA damage: the relative number of modified sites detected by a set of enzymes and the relative number of DNA strand breaks are determined and combined to obtain DNA damage profiles. Damage profiles observed in cellular DNA can be compared to those generated in a cell-free system under conditions for which the ultimate reactive species is already known or can be investigated much more easily.

Assay System

Supercoiled DNA is a suitable target for damage analysis because it is converted by either a single-strand break or by the incision of repair endonucleases into a relaxed form that migrates separately from the supercoiled one in agarose gel electrophoresis (Fig. 2). The relative amounts of supercoiled and relaxed circular DNA are determined by fluorescence scanning after staining with ethidium bromide and are used to calculate the number of strand breaks and endonuclease-sensitive lesions in the DNA [Eq. (1)].

$$\text{ssb} + \text{ess} = -\ln(1.4 \cdot I / (1.4 \cdot I + II)) \quad (1)$$

In Eq. (1), ssb represents the number of single-strand breaks per DNA molecule, and ess is the number of sites recognized and incised by a repair endonuclease if an incubation with the enzyme precedes the gel electrophoresis. I and II are the fluorescence intensities of the supercoiled and relaxed form of the DNA, respectively. The factor 1.4 accounts for the relatively lower fluorescence of ethidium bromide in form I compared to form II (10).

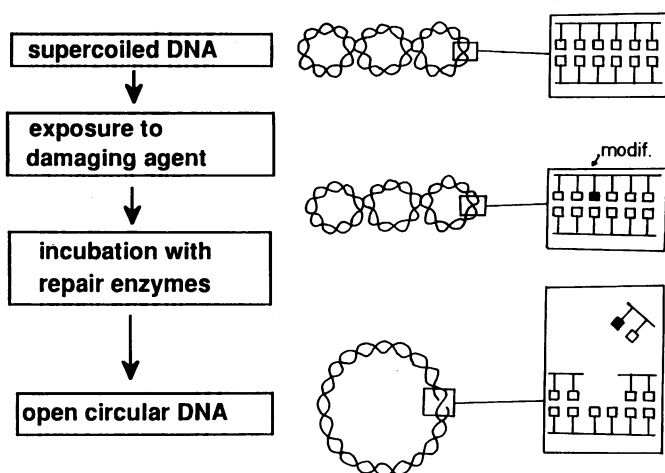


FIGURE 2. Principle of the damage analysis by means of repair endonucleases in supercoiled DNA.

To obtain damage profiles, the conditions of exposure to the damaging agents have to be chosen to generate approximately 0.1 to 2 endonuclease-sensitive sites and/or strand breaks per supercoiled molecule. For a plasmid of 10,000 base pairs the sensitivity therefore is approximately 1 modification per 10^5 base pairs. Experimental details are described in Epe et al. (11,12).

Results

DNA Damage Profiles Observed under Cell-Free Conditions

To analyze DNA damage generated under cell-free conditions, supercoiled DNA from bacteriophage PM2 was exposed to a damaging agent in phosphate buffer. Subsequently, the number of single-strand breaks and the number of modified sites recognized by three different enzyme preparations was determined as described above. Exonuclease III is known to incise sites of base loss (AP sites) exclusively (13). The other enzyme preparations are crude extracts from *Micrococcus luteus* and *Salmonella typhimurium*, respectively. They both contain several different repair endonucleases that have been characterized only in part.

We tested several established sources of ROS with this system (11). In Figure 3, damage profiles generated by NDPO_2 (disodium salt of 1,4-etheno-2,3-benzodioxin-1, 4-dipropanoic acid), methylene blue (MB), or rose bengal (RB) under illumination with visible light, and xanthine/xanthine oxidase/ Fe^{3+} -EDTA are depicted. NDPO_2 is a chemical source of singlet oxygen (14,15), while oxidation of xanthine by xanthine oxidase in the presence of Fe^{3+} is a system generating hydroxyl radicals (16). The ultimate DNA damaging species under all four reaction conditions have been investigated using superoxide dismutase, catalase, or D_2O as solvent (11). According to these studies, the very similar damage profiles induced by NDPO_2 and by the photosensitizers MB and RB can all be assigned to singlet oxygen as the predominant ultimate reactive species, while hydroxyl radicals are responsible for the damage profile induced by xanthine/xanthine oxidase/ Fe^{3+} -EDTA, which is characterized by a much higher relative number of strand breaks compared to endonuclease-sensitive sites.

DNA Damage Profiles Observed in *Salmonella typhimurium*

The cell-free generated damage profiles shown in Figure 3 and assigned to singlet oxygen and hydroxyl radicals serve as a reference for DNA damage produced in cellular DNA. In Figure 4, damage profiles are shown that are observed in plasmid pAQ1 of *Salmonella typhimurium* strains TA1535/pAQ1 and TA1978/pAQ1 after illumination of the bacteria in the presence of MB and after incubation with *tert*-butylhydroperoxide (*t*-BuOOH). The two strains are obtained from the Ames tester strains TA1535 (*rfa*, *uvrB*) and TA1978 (*rfa*) by

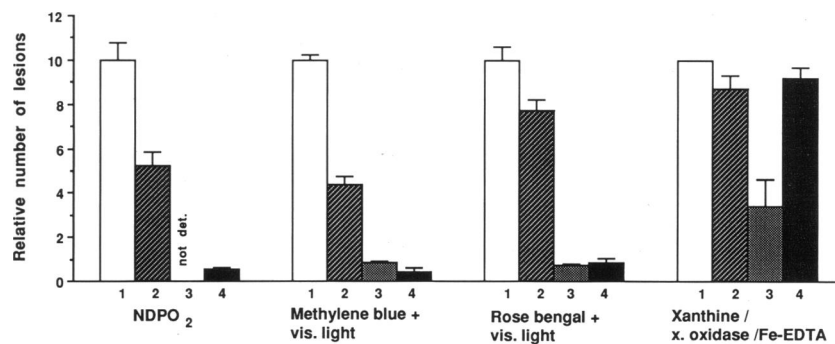


FIGURE 3. DNA damage profiles induced by various agents in phosphate buffer. For each type of damage, columns 1 and 2 indicate sites sensitive to endonucleases from *Micrococcus luteus* (normalized to 10 units) and *Salmonella typhimurium*, respectively, column 3 indicates sites sensitive to exonuclease III from *E. coli* (AP sites), and column 4 represents direct single-strand breaks. Reaction conditions were as follows: (A) 0–100 mM NDPO₂, 2 hr, 37°C; (B) 0–2 min visible light (halogen lamp 1000 W at 95 cm distance) in the presence of methylene blue (10 µg/mL); (C) 0–30 min visible light in the presence of rose bengal (20 µg/mL); (D) 0–4 µM xanthine/xanthine oxidase/10 µM Fe³⁺–EDTA.

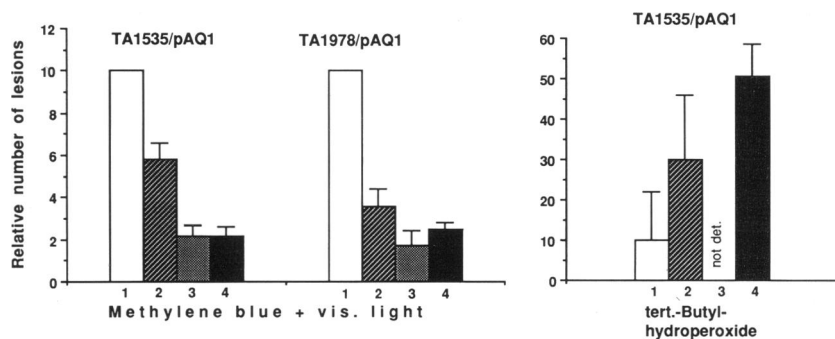


FIGURE 4. DNA damage profiles in *Salmonella typhimurium* TA1535/pAQ1 and TA1978/pAQ1 after exposure to methylene blue and light (0–4 min) (left panel) and *tert*-butylhydroperoxide (0–42 mM; 30 min, 37°C) (right panel). The relative numbers of endonuclease-sensitive sites (columns 1–3) and single-strand breaks (column 4) were determined in the plasmid pAQ1 exactly as for the cell-free damage profiles (Fig. 3).

introduction of the plasmid pAQ1 (6662 base pairs). Plasmids are isolated from the bacteria after exposure to the damaging agents by a procedure avoiding alkaline conditions and assayed for strand breaks and endonuclease-sensitive sites as described above for PM2 DNA.

The DNA damage profiles observed after treatment of the two strains with MB and visible light are similar to those assigned to singlet oxygen in the cell-free system. Therefore, singlet oxygen most probably acts as the ultimate reactive species in the cellular system too. Indirect pathways, e.g., an activation of cellular nucleases or a reduction of singlet oxygen to superoxide and, ultimately, hydroxyl radicals, do not seem to contribute significantly.

After treatment of the bacteria with *t*-BuOOH, significant DNA damage is observed only at very high concentrations (>10 mM). Damage in TA1978/pAQ1 is found to be approximately one-quarter of that in TA1535/pAQ1; therefore, a damage profile can only be calculated for the latter (repair-deficient) strain. It consists predominantly of strand breaks and therefore dif-

fers substantially not only from the profiles caused by singlet oxygen but also from those generated by hydroxyl radicals (xanthine/xanthine oxidase/Fe³⁺–EDTA) (Fig. 3).

Effects of Complexing Agents on Damage Production by *t*-BuOOH

t-BuOOH (50 mM) does not induce detectable DNA damage when incubated with PM2 DNA in phosphate buffer (data not shown); therefore, the DNA damage observed in the cells is not the result of a direct reaction. Evidence that damage is mediated by radicals generated in a Fenton-type reaction is obtained by the inhibitory effect of *o*-phenanthroline (17). As shown in Table 1, formation of single strand breaks is completely prevented by the Fe²⁺-complexing reagent. Ethylenediaminetetraacetic acid (EDTA), the Fe(II) complex of which can catalyze the Fenton reaction, increases the extent of damage, while the addition of Fe(III) as EDTA complex has little influence.

Table 1. Influence of complexing agents on the induction of DNA single-strand breaks in *Salmonella typhimurium* TA1535/pAQ1 by *tert*-butylhydroperoxide.^a

% Damage observed in the presence of ^b			
EDTA 1 mM	<i>o</i> -Phenanthroline		Fe ³⁺ / EDTA 250 μM / 1 mM
	100 μM	500 μM	
175 ± 43	30 ± 18	0 ± 2	127 ± 13

^a *tert*-Butylhydroperoxide, 42 mM, 30 min.

^b The reference damage (100 ± 5%) was determined in the absence of a complexing agent.

Table 2. Repair of DNA damage induced by methylene blue and light and by *tert*-butylhydroperoxide in *Salmonella typhimurium*.

Treatment	Strain	Strand breaks	% Residual damage after 20 min incubation at 37°C ^a	
			Endonuclease-sensitive sites ^b	
			<i>M. luteus</i>	<i>S. typhimurium</i>
MB and light	TA1535/pAQ1	62 ± 5	48 ± 15	0
	TA1978/pAQ1	90 ± 25	46 ± 12	0
<i>t</i> -BuOOH	TA1535/pAQ1	30 ± 15	9 ± 25	0

^a Reference damage (100%) determined after immediate workup.

^b Sites sensitive to enzymes from *Micrococcus luteus* and *Salmonella typhimurium*, respectively.

DNA Repair, Cytotoxicity, and Mutagenicity

The use of *Salmonella typhimurium* as target cells offers the opportunity to measure DNA repair, cytotoxicity, and mutagenicity in parallel with the DNA damage. Thereby, the consequences of the characterized DNA damage can be observed in the same system.

The disappearance of the modified sites generated by *t*-BuOOH or by illumination in the presence of MB during a 20-min repair incubation is shown in Table 2. For both types of damaging agents, data indicate that the repair is not exclusively dependent on the uvrABC excision repair, which is defective in TA1535/pAQ1. Repair after treatment with *t*-BuOOH is very rapid and conceivably has removed most of the lesions even when plasmids are isolated directly after exposure.

Treatment with MB and light is moderately toxic under the conditions necessary to measure damage profiles, i.e., which yield approximately 1 modification in 50,000 base pairs (~ 10% survival). Neither treatment with MB in the dark nor illumination in the absence of MB is toxic, nor does it give rise to measurable DNA damage. The concentration of 10 mM *t*-BuOOH necessary to generate 1 single-strand break in 50,000 base pairs is associated with less than 0.1% survival.

The assay for his⁺ revertants after the exposure of the tester strains TA100 and TA2638 to the same conditions as used for damage analysis indicates a high mutagenicity of the DNA damage caused by MB and light (Table 3). As expected, treatment with MB in the dark or illumination without MB does not induce revertants (data not shown). In contrast, *t*-BuOOH exhibits only moderate mutagenicity with noncytotoxic concentrations (Table 3).

Table 3. Mutagenicity of DNA damage induced by methylene blue and light and by *tert*-butylhydroperoxide in *Salmonella typhimurium*.

Treatment	Linear dose range	Revertant frequency in strain	
		TA100	TA2638
MB and light	0–20 sec	1170 ± 110 rev/min	588 ± 56 rev/min
<i>t</i> -BuOOH, 30 min, 37°C	0–0.5 mM	167 ± 20 rev/mM	223 ± 14 rev/mM

Discussion

The results demonstrate that an analysis of DNA damage using repair endonucleases as probes allows us to obtain damage profiles that can serve as characteristic fingerprints of the ultimate DNA damaging oxygen species. By comparing cellular with cell-free damage profiles, singlet oxygen has been shown to be directly responsible for DNA damage in *Salmonella typhimurium* bacteria illuminated in the presence of MB. Therefore, it may be concluded that singlet oxygen is also responsible for the observed mutagenicity.

Our finding is unexpected in view of the facile one-electron reduction of singlet oxygen to superoxide by NADH and other cellular components (18). As shown in Figure 1, superoxide would be expected to generate hydroxyl radicals that are at least 750-fold more reactive with DNA than hydroxyl radicals (11). Most probably, the result is a consequence of a target-directed generation of singlet oxygen, as MB has a considerably high affinity for DNA (19,20). The importance of this site-specific formation is supported by observations that extracellularly generated singlet oxygen is highly cytotoxic due to membrane damage and therefore lacks mutagenicity (21,22).

The existence of an uvrABC-independent repair mechanism for the singlet oxygen-induced DNA modifications can be regarded as an indication that these modifications are also formed under natural conditions. This is because uvrABC-independent repair of base modification most probably is initiated by repair glycosylases with high substrate specificity; those enzymes known to recognize hydroxyl radical-induced modifications, e.g., endonuclease III from *E. coli* (23), are unlikely to be responsible for the repair observed as singlet

oxygen modifies guanine residues rather than thymines (8,9).

The DNA damage by *t*-BuOOH is most probably mediated by a Fe(II)-catalyzed generation of oxygen radicals as indicated by the inhibitory effect of *o*-phenanthroline. The mechanistic pathways involved appear to be similar to those with H₂O₂ (24) because oxyR mutants of *S. typhimurium* exhibit the same sensitivity to both agents (25). In eukaryotic cells, free radicals have been detected by ESR spectroscopy after exposure of mouse keratinocytes to *t*-BuOOH (26).

The absence of detectable base modifications in DNA damage induced by *t*-BuOOH suggests that it might be ultimately generated by cellular nucleases activated in consequence of the oxygen radical formation. This type of mechanism has been postulated for the clastogenic activity of hydroperoxides derived from arachidonic acid (27) and of benzoylperoxide (28) in mammalian cells. However, in these cases, *o*-phenanthroline is not inhibitory. The very rapid repair of DNA damage induced by *t*-BuOOH, which in the repair-proficient strain is practically complete even without explicit repair incubation, indicates that another explanation for the unexpected damage profile produced by *t*-BuOOH cannot be ruled out: The profile observed might be modified by repair and differ from the original profile. Rapid repair therefore can be an important obstacle for the use of DNA damage profiles to identify ultimately reactive oxygen species.

The results presented here support the notion that the ability of ROS to act as initiators in carcinogenesis is largely governed by the site of their generation and the effectiveness of repair of the resulting lesions. When formed in the vicinity of DNA, singlet oxygen might represent a considerable risk, especially if the removal of the lesions induced is less effective than in the case of hydroxyl radicals, as shown here for *Salmonella* cells.

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