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DNA DAMAGE BY CHEMICALLY GENERATED SINGLET OXYGEN

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A naphthalenic endoperoxide was used as a non-photochemical source of singlet oxygen ($^1O_2$) to examine some interactions between this reactive oxygen species and DNA. High molecular weight DNA (ca. 10^8 daltons) was exposed to 120 mol m^{-3} $^1O_2$ (cumulative concentration) and analyzed for interstrand crosslinkage by hydroxyl apatite chromatography following formamide denaturation. No evidence for $^1O_2$-induced interstrand crosslinking was obtained. The capacity of $^1O_2$ to generate strand breaks in single-stranded (ss) and double-stranded (ds) DNA was investigated by sucrose gradient centrifugation analysis of bacteriophage øX174 DNA. No direct strand breaks could be detected at neutral pH, whereas extensive strand breakage was observed after treatment with alkali. Possible biological consequences of $^1O_2$-exposure were assessed by examining the plaque-forming capacity of ss and ds øX174 DNA molecules using wildtype *Escherichia coli* spheroplasts as recipients. Without any further treatment with heat or alkali, exposure to the endoperoxide resulted in a time- and dose-dependent inactivation, ss DNA being considerably more sensitive than ds DNA. From the present results and those reported earlier (Nieuwint *et al.*,[20]) we infer that $^1O_2$-induced inactivation of øX174 DNA is not due to DNA backbone breakage nor to interstrand crosslinking, but rather to some form of damage to the base or sugar moiety of the DNA, the exact nature of which remains to be elucidated.

KEY WORDS: DNA crosslinking, DNA damage, DNA inactivation, singlet oxygen.

INTRODUCTION

Singlet oxygen ($^1O_2$) can be generated by a class of dyes known as "photodynamic sensitizers". These compounds are capable of capturing photons and transferring the energy to ground-state O_2, which then becomes activated to the excited singlet state, $^1\Delta g$. $^1O_2$, which is a chemically aggressive species, has a life-time of several μsec in aqueous solutions (4.4 μsec in H_2O and 55 μsec in D_2O)[1–3] and is thus capable of traveling several μm before it is quenched by a solvent molecule.[4,5]

$^1O_2$ is a common product in living cells, since many sensitizers are of biological origin, while light and oxygen are usually also present. Recent work has demonstrated that certain enzymatically controlled reactions occur in living cells that generate $^1O_2$ in the absence of light.[6–9] The potential harm from endogenous $^1O_2$ depends upon a number of factors, such as rate and site(s) of $^1O_2$ production, presence of quenchers, and vulnerability of critical targets.

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In view of the possible involvement of $^1O_2$ in carcinogenesis[10] it is of interest to determine its reactivity towards DNA. Much work has been carried out to characterize the action of photodynamically generated $^1O_2$ on DNA, nucleotides, nucleosides and bases. Such studies have shown that guanine is specifically attacked in such a way that in DNA alkali-labile sites are produced.[11–17] Fiel *et al.*[18] have reported DNA backbone breakage by photodynamic porphyrins *without* alkaline treatment. However, in photodynamic systems it is often uncertain to what extent an effect can be ascribed to $^1O_2$, since (1) other activated oxygen species (e.g. $O_2^{-\cdot}$) may be generated in addition to $^1O_2$, and (2) activated oxygen species may react with the sensitizer generating secondary reactive products.[17]

Thus, Kornhauser *et al.*[19] found different breakdown products from guanosine after exposure to photodynamically generated as opposed to radiofrequency-discharge-generated singlet oxygen. Nieuwint *et al.*[20] recently reported DNA backbone breakage induced by a photodynamic system (rose bengal plus light), which effect, however, could not be mimicked by a relative excess of $^1O_2$ generated *chemically* using the thermodissociable endoperoxide of 3,3′-(1,4-naphthylidene)diproprionate ($NDPO_2$). Here we extend our observations on $NDPO_2$-induced effects on DNA. First, a search was made for the possible induction of interstrand crosslinks in high molecular weight DNA. Second, some results on the biological inactivation of bacteriophage øX174 DNA by $NDPO_2$ are described.

MATERIALS AND METHODS

Materials

Tritium-labeled high molecular weight mammalian DNA (ca. 10^8 daltons) was isolated from Chinese hamster ovary cells according to the procedure described by Poll *et al.*[21]

Single-stranded (ss) and double-stranded (ds) DNA molecules from bacteriophage øX174 were obtained as described by Blok *et al.*[22] and Baas *et al.*[23] Preparations of ds circular DNA contained ca. 70% RFI DNA (supercoiled replicative form) and ca. 30% RFII DNA (open relaxed form), as indicated by sucrose gradient centrifugation. Plaque-forming activity of the DNA was determined on *Escherichia coli* K12 (AB1157) spheroplasts, as described by Blok *et al.*[22]

The water-soluble endoperoxide of disodium 3,3′-(1,4-naphthylidene)diproprionate ($NDPO_2$) was obtained as described before[20] and stored at $-70°C$ until use.

Exposure to $^1O_2$

At 37°C the $NDPO_2$ dissociates with first order kinetics ($t_{1/2} = 23$ min) yielding NDP and molecular O_2, half of which is in the triplet ground state, the other half being in the excited singlet state. No other reactive oxygen species are formed in this reaction.[20,24,25] For $^1O_2$ exposure $NDPO_2$ was dissolved in 50 mol m^{-3} Tris HCl pH 7.8, at 0°C. Various concentrations of $NDPO_2$ were added to DNA solutions and incubated at 37°C for 3 h. These exposure conditions allowed more than 99.5% of the available $^1O_2$ to be released. The cumulative $^1O_2$ concentration was calculated from the increase in absorbance at 288 nm, at which NDP (but not $NDPO_2$) has an absorbance peak ($\varepsilon_{288} = 7780$).

DNA interstrand crosslinking

A solution of ^{3}H-thymidine labeled DNA, isolated from Chinese hamster ovary cells, in 5 mol m^{-3} Tris HCl pH 7.8, 1 mol m^{-3} ethylene diaminetetraacetate (EDTA), was incubated at 37°C for 3 h with $NDPO_2$ (85 mg/ml). Untreated DNA and DNA treated with preincubated (3 h, 37°C) $NDPO_2$ were used as controls. Interstrand crosslinking was determined by denaturation (dialysis for 3 h against formamide at 50°C and subsequent dialysis against the above Tris EDTA buffer) followed by hydroxyl apatite chromatography to separate ss from ds DNA. With this technique crosslinked DNA as well as non-cross-linked palindromic DNA (5–10%) is recovered in the ds DNA peak.[21]

Exposure of øX174 DNA

Fifty μl $NDPO_2$ solution in 50 mol m^{-3} Tris HCl pH 7.8 were added to 50 μl of a solution containing ss or ds 0X174 DNA (20 μg/ml) in 50 mol m^{-3} Tris HCl pH 7.8 and incubated at 37°C. To stop the reaction at various time intervals 10 μl aliquots were diluted in 1 ml 50 mol m^{-3} Tris HCl pH 7.8, on ice, followed by freezing at −20°C. Alternatively, for analysis on sucrose gradients, reactions were stopped by diluting 1:1 with ice-cold 50 mol m^{-3} Tris buffer followed by storage on ice until the analysis. Biological activity of the DNA was assayed as described.[22] The highest concentration $NDPO_2$ preincubated at 37°C for 3 h (and thus being converted into NDP) had no detectable effect on the plaque-forming capacity of the DNA; incubation with the starting material NDP was also without effect.

Alkali treatment of DNA

DNA was tested for alkali-labile sites by addition of 1/10 of a volume 1 mol dm^{-3} NaOH (final pH about 12.5). After 30 min incubation at 37°C the solution was neutralized by addition of 1 mol dm^{-3} HCl in 50 mol m^{-3} Tris buffer to give a pH of about 7.5, and stored on ice until further analysis.

Sucrose gradient centrifugation of øX174 DNA

Linear sucrose gradients (for ss DNA: 5 to 40% (w/v) in 10 mol m^{-3} NaCl, 0.1 mol m^{-3} EDTA, 1 mol m^{-3} phosphate buffer pH 7.2; for ds DNA: 10 to 30% (w/v) in 0.3 mol dm^{-3} NaCl, 0.1 mol m^{-3} EDTA, 10 mol m^{-3} Tris HCl pH 7.3) were prepared in cellulose nitrate tubes. About 1.2 μg of DNA in 0.25 ml was layered on top of the gradients. Centrifugation was for 19 h at 20°C, 36000 r.p.m. for ss DNA, and 25000 r.p.m. for ds DNA, in a SW41 rotor of a Spinco ultracentrifuge. After centrifugation the gradients were sucked out from the bottom of the tubes. The absorbance at 260 nm was monitored with a Zeiss PMQ-2 spectrophotometer and recorded with a Kipp BD8 flatbed recorder. The percentages of (un)broken molecules were derived from the sedimentation patterns obtained (e.g. see Figure 1). The number of breaks in ss DNA are calculated from changes in these percentages. For ds DNA this can be done by the method described by Van der Schans *et al.*[26]

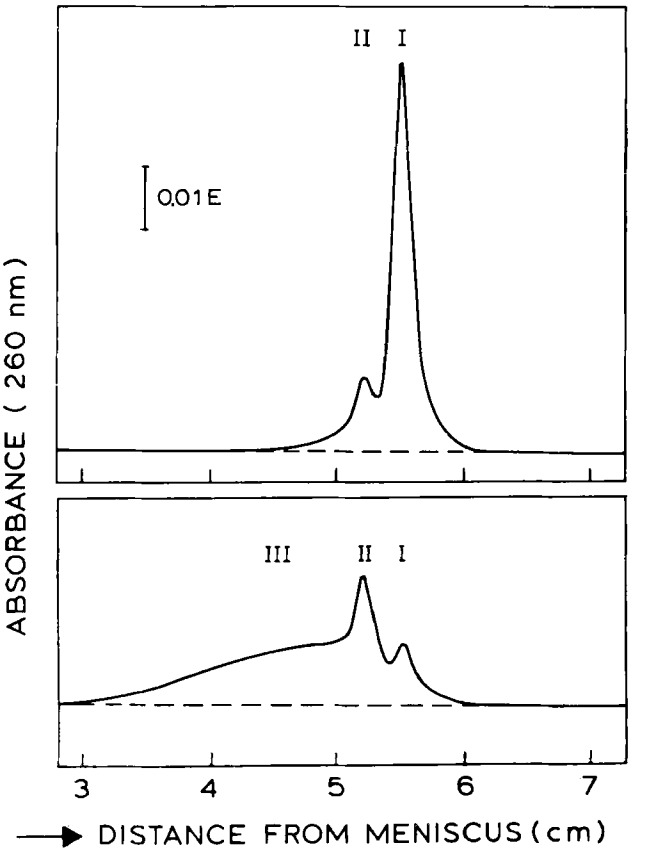

FIGURE 1 Sucrose gradient sedimentation patterns of ss øX174 DNA molecules subjected to about 1.3 mol m^{-3} (cumulative concentration) of $^1O_2$ generated from $NDPO_2$ during 3 h at 37°C. Centrifugation was carried out either without further treatment (upper panel) or following alkali treatment (lower panel) as described in Materials and Methods. $^1O_2$-exposed DNA without alkali treatment was indistinguishable from untreated control DNA. I: circular DNA; II: molecules containing one break (i.e. ss full-length linear molecules); III: molecules containing two or more breaks.

D_2O solvent effect

The D_2O solvent effect was determined by using reaction mixtures of which the components had been made up with D_2O (99.75% deuterium oxide, Merck A.G., Germany) instead of H_2O. Because the DNA stock solutions were in H_2O-containing buffers, the final D_2O concentrations in the experimental solutions were 97.8% for ss and 98.6% for ds øX174 DNA.

RESULTS

Search for $^1O_2$-dependent DNA interstrand crosslinking

Cells from patients with the chromosome breakage syndrome Fanconi anaemia are hypersensitive to the clastogenic (chromosome breaking) effect of crosslinking

agents[27] and to that of D_2O.[28] The critical involvement of $^1O_2$ in the origin of spontaneous chromosomal breakage in Fanconi anaemia has recently been suggested.[29] It was thus considered of interest to assess the capacity of $^1O_2$ to produce DNA crosslinks. However, as shown in Table I, exposure to $^1O_2$ at a cumulative concentration as high as 120 mol m^{-3} failed to induce detectable interstrand crosslinking in high molecular weight DNA. In contrast, treatment with 8-methoxypsoralen plus UVA, as determined in a separate set of experiments,[21] resulted in extensive crosslinking of the DNA (Table I).

$^1O_2$-induced formation of DNA strand breaks

In previous studies no direct strand breaks could be detected in ds plasmid DNA after exposure to $^1O_2$, chemically generated by $NDPO_2$, although alkali-labile lesions could readily be observed.[20] In this study we also examined this for ss øX174 DNA. The experiments show (Figure 1, upper panel) that $^1O_2$ failed to induce direct strand breaks in ss DNA, but again, after alkali treatment extensive fragmentation was observed (Figure 1, lower panel). In agreement with earlier results obtained with ds plasmid DNA, ds øX174 DNA molecules were unaffected by $NDPO_2$ exposure without alkali treatment. Control experiments in which ss and ds øX174 DNA preparations were incubated with NDP for at least 3 h at 37°C did not reveal any induction of strand breaks, even after treatment with alkali.

$^1O_2$-induced biological inactivation of øX174 DNA

As shown in Figure 2, exposure of ss øX174 DNA to $NDPO_2$ at 37°C caused loss of biological activity, with a kinetics of inactivation that paralleled the kinetics of $NDPO_2$ dissociation. Moreover, when the incubation with $NDPO_2$ was carried out in D_2O instead of H_2O, inactivation was markedly stimulated, indicating that inactivation was indeed due to $^1O_2$. Ds DNA exhibited similar kinetics of biological inactivation, but was considerably (ca. two orders of magnitude) less sensitive than ss DNA (results not shown).

DISCUSSION

In this study we have made use of the thermodissociable endoperoxide $NDPO_2$ as a chemical source of $^1O_2$ to investigate the reactivity of this oxygen species towards

TABLE I
Search for $^1O_2$-induced interstrand crosslinking[a]

Treatment	% ds-DNA[b]
None	6.6; 6.6
NDP (85 mg/ml)[c]	7.4; 7.7; 8.8; 9.8
$NDPO_2$ (85 mg/ml)[d]	8.6; 8.9; 9.2; 11.9
8-methoxy-psoralen (2 μg/ml) + UVA[e]	61; 63; 66; 67; 68; 69

[a]^{3}H-labeled DNA from Chinese hamster ovary cells was exposed to $NDPO_2$ at 37°C for 3 h and analyzed for DNA interstrand crosslinkage as described in Materials & Methods.
[b]ds DNA: DNA that resisted denaturation due to palindromic sequences and interstrand crosslinks.
[c]$NDPO_2$ preincubated at 37°C for 3 h.
[d]cumulative $^1O_2$ concentration ca. 120 mol m^{-3}.
[e]UVA irradiation was 3.5 mW/cm^2; see [21] for details of the procedure.

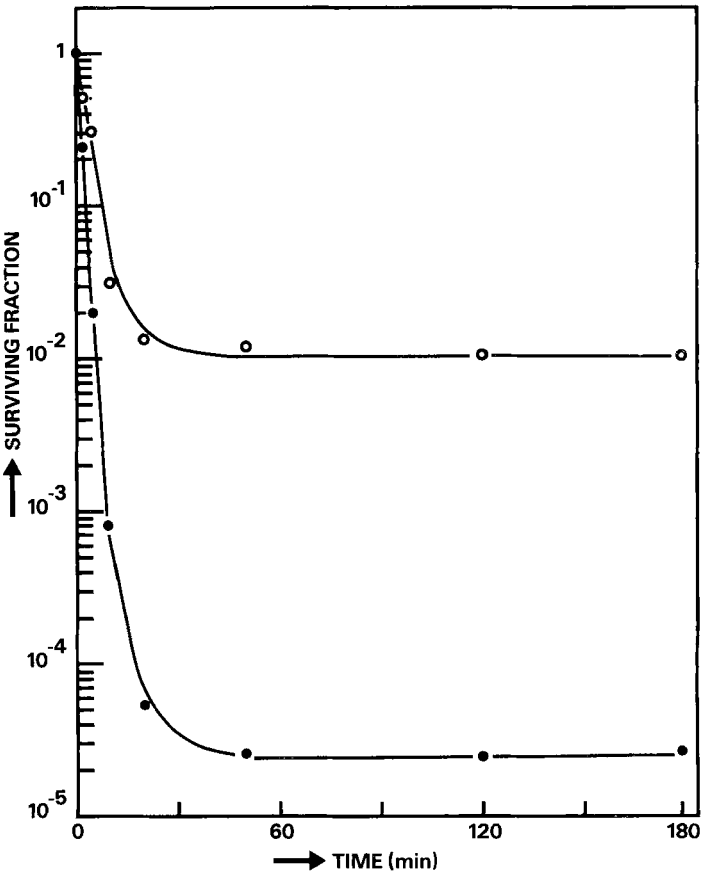

FIGURE 2 Loss of biological activity of ss øX174 DNA molecules as a function of time in the presence of $^1O_2$-generating endoperoxide $NDPO_2$, at 37°C. Cumulative $^1O_2$ concentration was 1.7 mol m^{-3}. o——o, H_2O; ●——●, 97.8% D_2O.

DNA. This system has an important advantage over photochemical sources of $^1O_2$ previously utilized in similar investigations in that "type I" (i.e. sensitizer-mediated) reactions, often complicating the interpretation of photochemical effects, do not occur in this system. In addition, $^1O_2$ is the only type of reactive oxygen species generated,[24,25] which minimizes possible interference from free radical species, such as $O_2^{-\cdot}$ or $OH^{\cdot}$. Furthermore, the only other product formed in the reaction, NDP, appears to be totally inert towards DNA. These features make the present system ideally suited for the study of $^1O_2$ effects on the physico-chemical and biological properties of DNA.

Using relatively large amounts of endoperoxide-generated $^1O_2$ we were unable to detect strand breaks in ss or in ds DNA molecules (cf. reference 20); also, interstrand crosslinks could not be detected (Table I). However, as further discussed below, $^1O_2$ was clearly able to inactivate bacteriophage øX174 DNA, at least part of which could be accounted for by alkali-labile lesions.

Both ss and ds øX174 DNA was inactivated by $NDPO_2$, although ss DNA appeared to be much more susceptible. This difference in sensitivity is typically found

in a variety of damaging processes like in reactions with OH and H radicals[30,31] and the formation of apurinic sites.[32,33] This effect may be related to either the conformation of the DNA in which loss of the double helix structure facilitates the introduction of damage,[32,34] or to enzymatic repair processes.[31]

The observation that the inactivation curves (Figure 2) initially show a steep decline, but then strongly deflect is consistent with the kinetics of thermal dissociation of $NDPO_2$, which yields $^1O_2$ and NDP in a first order reaction. At the end of the process only NDP is present as a possible reactant, which, however, was shown to be inert towards DNA.

The inactivation of DNA is greatly stimulated by using D_2O instead of H_2O as a solvent. Along with the observations of Aubry *et al.*[24] and Chou and Frei[25] who showed that the thermal decomposition of $NDPO_2$ leads to the evolution of O_2 in the $^1\Delta g$ state as the only reactive oxygen species, the D_2O solvent effect further substantiates that the inactivation of 0X174 DNA is indeed to be attributed to $^1O_2$. The presence of a solvent effect also indicates that there is competition between the reaction of $^1O_2$ with DNA and the quenching reaction of $^1O_2$ by the solvent molecules.

Further support for the proposal that $^1O_2$ is indeed responsible for damaging the DNA is derived from the sucrose gradient sedimentation analysis. We were unable to detect any significant direct strand breaks, both with ss and ds DNA. The reaction conditions used in these experiments (3 h, 37°C) are expected to convert labile lesions into breaks in the case of OH and H radical damage, especially in ss, but also partly in ds øX174 DNA.[35,36] Therefore, a contribution from $H^{\cdot}$ and $OH^{\cdot}$ to the observed damage may be excluded. At the same time these data show that the nature of the damage induced by $^1O_2$ must be different from $H^{\cdot}$- and $OH^{\cdot}$-induced damage.

The observation that breaks can be detected after alkali treatment only, indicates that at least part of the damage in øX174 DNA is due to alkali-labile lesions. Such lesions could be apurinic sites, which are known to inactivate both ss and ds øX174 DNA molecules, and which can only be detected as a break after an alkali treatment.[33] However, more work is needed to verify this suggestion. We estimate that the alkali-labile lesions, as detected by the procedure used (30 min, 37°C, pH 12.5), would account for only about one third of the inactivating damage in ss øX174 DNA. The nature of the remaining damage is currently under investigation.

In conclusion we infer that $^1O_2$ is capable of inactivating both ss and ds øX174 DNA. This inactivation is not due to strand breaks nor to interstrand crosslinks, but probably to some form of base or sugar damage. Although at least part of the damage could be detected as alkali-labile lesions, the exact nature of this damage remains to be elucidated.

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