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Singlet Oxygen Induces Oxidation of Cellular DNA*

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Jean-Luc Ravanat, Paolo Di Mascio‡, Glaucia R. Martinez‡, Marisa H. G. Medeiros‡, and Jean Cadet§

From the Laboratoire “Lésions des Acides Nucléiques” Service de Chimie Inorganique et Biologique, UMR CNRS 5046, Département de Recherche Fondamentale sur la Matière Condensée, CEA Grenoble, 17 Avenue des Martyrs, F-38054 Grenoble Cedex 9, France and the ‡Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, CEP 05513–970, São Paulo, SP, Brazil

The aim of the present work was to evaluate the potential for $^1\text{O}_2$ to induce oxidation of cellular DNA. For this purpose cells were incubated in the presence of a water-soluble endoperoxide whose thermal decomposition leads to the formation of singlet oxygen. Thereafter, DNA was extracted and the level of several modified DNA bases was determined by HPLC analysis coupled to a tandem mass spectrometric detection. A significant increase in the level of 8-oxo-7,8-dihydro-2'-deoxyguanosine was observed upon incubation of the cells with the chemical generator of $^1\text{O}_2$, whereas the level of the other DNA bases measured remained unchanged. To demonstrate that singlet oxygen is directly involved in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine, the corresponding ^{18}O -labeled endoperoxide was used. Incubation of the cells with such a generator of ^{18}O -labeled singlet oxygen results in the formation of ^{18}O -labeled 8-oxo-7,8-dihydro-2'-deoxyguanosine in the nuclear DNA. This result clearly demonstrates that singlet oxygen, when released within cells, is able to directly oxidize cellular DNA.

Modification of cellular DNA upon exposure to reactive oxygen and nitrogen species is the likely initial event involved in the induction of the mutagenic and lethal effects of various oxidative stress agents (1–3). As an example, the deleterious effects of UVA radiation are, at least partly, explained in terms of photooxidation of cellular DNA (4, 5). The mechanism of UVA-mediated photooxidative damage to DNA is not completely established. Evidence has been accumulated over the years for the significant implication of singlet oxygen, as the result of UVA activation (4, 6) of endogenous photosensitizers (porphyrins, flavins, . . .) not yet characterized. However, a type I mechanism involving the initial formation of a DNA radical cation, that could be predominantly located at guanine sites due the lowest ionization potential of the latter base and/or to the possibility of charge transfer in DNA (7), could not be excluded. To our knowledge, no clear evidence has been provided to demonstrate that singlet oxygen is able to oxidize cellular DNA. It should be added, however, that $^1\text{O}_2$ is known to be mutagenic and genotoxic (2, 3, 8). In addition, singlet oxygen has been identified as the reactive oxygen species in-

involved in numerous biological processes. Among others we may cite neutrophils phagocytosis (9) and enzymatic processes (10).

Reactions of singlet oxygen with nucleosides and isolated DNA are well documented. Interestingly, it was shown that $^1\text{O}_2$ oxidizes, among the nucleosides, almost exclusively the guanine base. Singlet oxygen reacts with free dGuo¹ and short oligonucleotides to give rise to the overwhelming formation of the 4*R* and 4*S* diastereomers of 4-hydroxy-8-oxo-4,8-dihydro-2'-deoxyguanosine, together with a small amount of 8-oxodGuo (11–15). In contrast, 8-oxodGuo was found to be the major oxidation product formed upon exposure of isolated DNA to $^1\text{O}_2$ (6, 11, 16). However, 8-oxodGuo cannot be considered as a specific biological marker of $^1\text{O}_2$, since this DNA lesion could be formed under various conditions of oxidative stress, including those generated by one-electron process (17), hydroxyl radical (18), and Fenton-type reactions (19). On one hand, this explains why 8-oxodGuo could be used as an ubiquitous biomarker of DNA oxidation (20–22). On the other hand, the formation of 8-oxodGuo in cellular DNA could not be attributed to the initial formation of $^1\text{O}_2$. During the recent past, water-soluble generators of $^1\text{O}_2$, that consist of aromatic hydrocarbon endoperoxides, became available (23–25). Thermal decomposition of the latter compounds is used to efficiently produce singlet oxygen (26). Interestingly, the water-soluble non-ionic endoperoxide DHPNO₂ (23) was recently found to be incorporated into cells upon incubation (27). Using such a chemical generator of singlet oxygen, Klotz *et al.* (27) were able to show the activation of specific genes upon intracellular release of singlet oxygen.

The purpose of the present work was to use DHPNO₂ to determine whether intracellular singlet oxygen is able to oxidize nuclear DNA. Emphasis was placed on the determination of the mechanism of DNA oxidation. For this purpose, the ^{18}O -labeled endoperoxide DHPN¹⁸O₂ was prepared to assess if the formation of oxidative damage results from the direct reaction of $^1\text{O}_2$ within cellular DNA. It was clearly shown that intracellular formation of singlet oxygen is able to efficiently produce 8-oxodGuo.

EXPERIMENTAL PROCEDURES

Cell Culture and Preparation—The THP1 monocyte cell line used for this study was obtained as described previously (28). Before treatment with the endoperoxide, the cells were recovered by centrifugation (250 × *g*, 4 min) and washed twice with PBS buffer.

Preparation of the Endoperoxide—The water-soluble non-ionic endoperoxide DHPNO₂ was prepared by methylene blue-mediated photo-

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§ To whom correspondence should be addressed: Laboratoire “Lésions des Acides Nucléiques,” DRFMC/SCIB, CEA/Grenoble, F-38054 Grenoble Cedex 9, France. Tel.: 33-4-76-88-49-87; Fax: 33-4-76-88-50-90; E-mail: jcadet@cea.fr.

¹ The abbreviations used are: dGuo, 2'-deoxyguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; DHPN, *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide; DHPNO₂, 1,4-endoperoxide of DHPN; DHPN¹⁸O₂, [¹⁸O₂]1,4-endoperoxide of DHPNO₂; HPLC-MS/MS, high performance liquid chromatography coupled to tandem mass spectrometry.

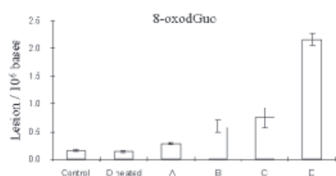


FIG. 1. Measurement of 8-oxodGuo in DNA of cells treated in the presence of varying amounts of DHPNO₂. Cells (30×10^6 per sample) suspended in 400 μ l of PBS buffer were treated for 1 h at 37 °C with either 5 μ l (A), 10 μ l (B), 20 μ l (C), or 50 μ l (D) of DHPNO₂. Control cells were not treated or treated with 50 μ l of heat-deactivated DHPNO₂ (D heated).

sensitization of DHPN as reported previously (29). The corresponding labeled DHPN¹⁸O₂ endoperoxide, whose synthesis is described in detail elsewhere (30), was obtained using the same protocol, in the presence of ¹⁸O-labeled molecular oxygen.

Incubation of the Cells with Either DHPNO₂ or DHPN¹⁸O₂—Cells (30×10^6 per sample) were collected by centrifugation prior to being suspended in either 200 or 400 μ l of PBS buffer, to which different amounts of either 210 mM DHPNO₂ or 130 mM DHPN¹⁸O₂ were added. For control experiment, complete deactivation of DHPNO₂ was obtained by heating the endoperoxide for 40 min at 70 °C. The cell suspension was incubated at either 4 or 37 °C for increasing periods of time. After incubation, the cells were recovered by centrifugation and washed with 5 ml of PBS buffer to remove the excess of endoperoxide prior to DNA extraction.

DNA Extraction—Extraction of cellular DNA was performed according to a previously reported procedure (14, 28) with one major modification. Typically, 10 mM sodium azide, a well known singlet oxygen quencher, was added to the buffered solutions (14, 28) used to isolate DNA (buffer A, 320 mM sucrose, 5 mM MgCl₂, 10 mM Tris-HCl, 0.1 mM deferoxamine, 1% Triton X-100, 10 mM NaN₃, pH 7.5; and buffer B, 10 mM Tris-HCl, 5 mM EDTA-Na₂, 0.15 mM deferoxamine, 10 mM NaN₃, pH 8.0). Sodium azide addition was made to prevent oxidation due to the presence of residual endoperoxide during cell lysis and subsequent DNA isolation (see below).

8-OxodGuo Measurement—The level of 8-oxodGuo in cellular DNA was assessed using the recently available HPLC-MS/MS assay (31, 32). This required a quantitative enzymatic digestion of DNA into nucleosides following a reported optimized procedure (31, 33). Accurate quantification of the level of 8-oxodGuo was obtained by using an isotopically labeled M + 5 internal standard (31, 32). Typically, 1.5 pmol of [M + 5] 8-oxodGuo was added to the DNA sample prior to its enzymatic digestion. The resulting hydrolysate was directly injected onto the HPLC column coupled to the mass spectrometric detector. The output of the column was also connected to a UV detector set up at 260 nm for quantification of the DNA amount through the measurement of dGuo (31, 32). Both 284 to 168 and 289 to 173 transitions were used for the detection of 8-oxodGuo and the corresponding [M + 5]-labeled internal standard, respectively (31, 32). In addition, the 286 to 170 transition was recorded to monitor the formation of [M + 2] ¹⁸O-labeled 8-oxodGuo. The measurement of the other DNA lesions, including thymidine glycols, 5-(hydroxymethyl)-2'-deoxyuridine, 5-formyl-2'-deoxyuridine, and 8-oxo-7,8-dihydro-2'-deoxyadenosine, was performed as described previously (32). Results, expressed as the number of lesion formed per 10⁶ DNA bases, represent the average and S.D. of, at least, three independent determinations.

RESULTS

Incubation of cell suspension for 1 h at 37 °C with DHPNO₂ results in the formation of 8-oxodGuo in cellular DNA (Fig. 1) as determined using the HPLC-MS/MS assay. The background level of 8-oxodGuo in cellular DNA was estimated to be close to 0.2 modification per 10⁶ DNA bases (Fig. 1, Control). Incubation of the cells with increasing amounts of DHPNO₂ (conditions A, B, C, and D) results in a significant increase (up to 10 times) in the level of 8-oxodGuo. No significant increase in the level of 8-oxodGuo was observed when cells were incubated with 50 μ l of deactivated DHPNO₂ (Fig. 1) nor when the incubation was performed at 4 °C (Fig. 2).

In the absence of the endoperoxide the HPLC-MS/MS assay is sensitive enough (Fig. 3) to measure endogenous 8-oxodGuo and to quantify its amount by isotope dilution using ¹⁵N₅-

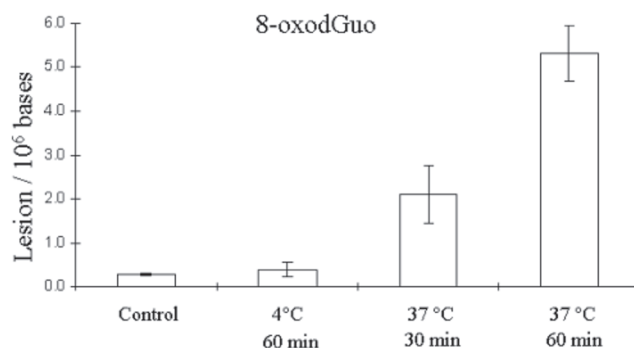


FIG. 2. Measurement of 8-oxodGuo in DNA of cells treated for increasing periods of time in the presence of DHPNO₂. Cells (30×10^6 per sample, four samples per condition) suspended in 200 μ l of PBS buffer were treated by 50 μ l of DHPNO₂ at either 4 °C for 60 min or at 37 °C for 30 and 60 min.

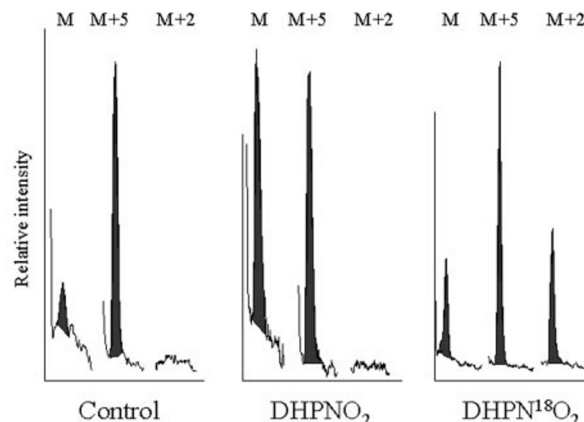


FIG. 3. Typical HPLC-MS/MS chromatograms obtained for the detection of 8-oxodGuo in cellular DNA. Cells were not treated (Control), treated with either 50 μ l of the unlabeled endoperoxide (DHPNO₂) or of the corresponding ¹⁸O-labeled endoperoxide DHPN¹⁸O₂. For each sample, the mass spectrometric detection method was set up to monitor endogenous 8-oxodGuo (M) the internal standard (M+5) as well as ¹⁸O-labeled 8-oxodGuo (M+2).

labeled [M + 5] 8-oxodGuo as the internal standard (Fig. 3, Control). When cells are incubated with DHPNO₂ an increase in the level of 8-oxodGuo is observed (Fig. 3). Furthermore, as expected, no [M + 2] ¹⁸O-labeled 8-oxodGuo could be detected in either DHPNO₂-treated cells or in the control experiment. When cells were incubated with the labeled endoperoxide DHPN¹⁸O₂ a slight increase of 8-oxodGuo is observed, and [M + 2] ¹⁸O-labeled 8-oxodGuo is detected (Fig. 3). The results obtained after treatment of the cells with two different concentrations of the labeled endoperoxide are summarized in Fig. 4.

An attempt was also made to evaluate if the formation of 8-oxodGuo is mainly due to the intracellular production of singlet oxygen. For this purpose, the cells were first incubated with DHPN¹⁸O₂ at 4 °C to allow the penetration of the endoperoxide into cells. Thereafter, the cells were collected by centrifugation to remove the excess of DHPN¹⁸O₂ and resuspended in PBS buffer before incubation at 37 °C. Under these conditions (Fig. 5, column C) the formation of [M + 2] 8-oxodGuo could be detected. However, without removing the excess of endoperoxide (Fig. 5, column D) an about 3-fold higher amount of the lesion is measured.

DISCUSSION

Incubation of the cell suspension for 1 h at 37 °C in the presence of DHPNO₂ results in a significant formation of 8-oxodGuo. No increase is observed if the endoperoxide is deactivated (by heating) confirming the involvement of singlet oxy-

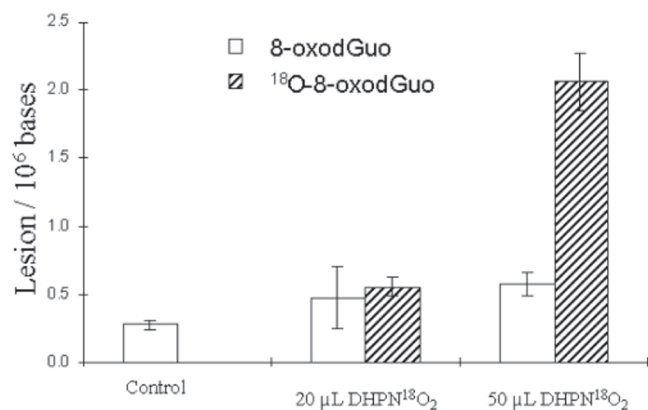


FIG. 4. Levels of 8-oxodGuo and [M + 2] ¹⁸O-labeled 8-oxodGuo measured in cellular DNA upon treatment of cells with either 20 or 50 μl of DHPN¹⁸O₂. Results represent the average and S.D. of four independent determinations.

gen in the formation of the lesion. The background level of 8-oxodGuo was determined to be close to 0.2 lesion per 10⁶ DNA bases. This relatively low value is in agreement with recent data (14, 34) and confirms that former measurements have led to an overestimation of the level of 8-oxodGuo in cellular DNA due to an artifactual oxidation during the work-up (35). No detectable increase in the formation of other DNA lesions, including thymidine glycols, 5-(hydroxymethyl)-2'-deoxyuridine, 5-formyl-2'-deoxyuridine, and 8-oxo-7,8-dihydro-2'-deoxyadenosine (data not shown), was observed upon incubation of the cells in the presence of DHPNO₂ as determined by HPLC-MS/MS measurement (32). The results clearly show that ¹O₂ is able to selectively generate 8-oxodGuo in cellular DNA, in agreement with the known reactivity of ¹O₂ toward the guanine moiety of isolated DNA.

An experiment was designed to demonstrate that 8-oxodGuo is not produced after cell lysis. For this purpose, cells were incubated in the presence of DHPNO₂ at 4 °C. Under these conditions, since the endoperoxide is stable at 4 °C, the production of singlet oxygen during the incubation is very low, and the amount of residual DHPNO₂ left is maximum during cell lysis. Under these conditions, no increase in 8-oxodGuo was observed (Fig. 3). This demonstrates that 8-oxodGuo is not formed during cell lysis and DNA isolation. This could be explained, at least partly, by the fact that the first step of the DNA extraction protocol consists in the purification of the nuclei (14), which is accompanied by the elimination of a large amount of the endoperoxide. In addition, sodium azide, a well known singlet oxygen quencher, was added to the different isolation buffers to scavenge singlet oxygen and thus to prevent DNA oxidation due to the presence of the residual generator of ¹O₂ (DHPNO₂). The results (Figs. 1 and 2) show that the release of ¹O₂ from the thermal decomposition of DHPNO₂ is able to induce oxidation to the guanine base of cellular DNA.

However, such an oxidation could result from either the direct reaction of ¹O₂ within cellular DNA (Scheme 1) or the oxidative stress induced by the intracellular production of singlet oxygen. Therefore, a chemical generator of labeled singlet oxygen DHPN¹⁸O₂ (30) was used to distinguish between the two possible mechanisms. For this purpose, the cell suspension was incubated with either DHPNO₂ or DHPN¹⁸O₂, and the amount of 8-oxodGuo was assessed by using the accurate and highly sensitive HPLC-MS/MS assay. The latter assay allows the simultaneous quantification of endogenous 8-oxodGuo, together with [M + 2]-labeled 8-oxodGuo. The latter compound is formed by the reaction of dGuo with ¹⁸O-labeled ¹O₂ generated by the thermal decomposition of DHPN¹⁸O₂. When cells are incubated with DHPNO₂ an increase in the level of 8-oxodGuo

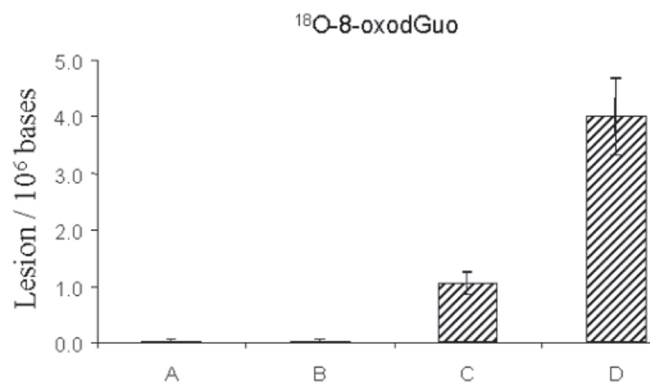
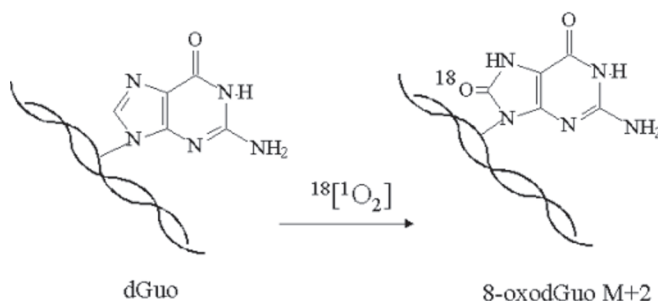


FIG. 5. Formation of [M + 2] ¹⁸O-labeled 8-oxodGuo in cellular DNA upon incubation in the presence of DHPN¹⁸O₂. Condition A, no endoperoxide; conditions B, C, and D, cells were treated with 50 μl of DHPN¹⁸O₂. Samples A, C, D, were incubated at 37 °C for 1 h, whereas sample B was kept at 4 °C. For sample C, prior to the incubation at 37 °C, cells were first incubated 15 min with the endoperoxide at 4 °C. Thereafter, 5 ml of PBS was added, and cells were recovered by centrifugation to remove the excess of endoperoxide which has not penetrated into cells.



SCHEME 1. Reaction of ¹⁸O-labeled singlet oxygen ¹⁸[¹O₂] within the guanine moiety of cellular DNA.

is observed (Fig. 3). Furthermore, as expected, no [M + 2] ¹⁸O-labeled 8-oxodGuo could be detected in either DHPNO₂-treated cells or in the control experiment (Fig. 3). A non-significant variation in the level of unlabeled 8-oxodGuo and an important formation of ¹⁸O-labeled 8-oxodGuo is measured when cells were incubated with the labeled endoperoxide. The results, summarized in Fig. 4, indicate that, as already observed with the unlabeled endoperoxide, the formation of 8-oxodGuo is almost linear with the amount of endoperoxide used. Interestingly, taking into account that the isotopic enrichment of the endoperoxide is about 85% (30), the result indicates that the formation of 8-oxodGuo observed when cells are treated with the labeled endoperoxide could be almost entirely attributed to the direct reaction of singlet oxygen within nuclear DNA, following the reaction described in Scheme 1.

An attempt was also made to evaluate if the formation of 8-oxodGuo is mainly due to the intracellular production of singlet oxygen. For this purpose, the excess of endoperoxide was removed by centrifugation after incubation of the cells with the labeled endoperoxide. However, without removing the excess of endoperoxide (Fig. 5) an about 3-fold higher amount of the lesion is measured. This would indicate that 8-oxodGuo could be formed by intracellular as well as extracellular generated singlet oxygen. However, the difference in the formation of 8-oxodGuo could be probably explained by the fact that an equilibrium exists between the intra- and extracellular localization of the endoperoxide. Removing extracellular DHPN¹⁸O₂ probably results in a decrease of its intracellular concentration that may explain the lower amount of 8-oxodGuo measured. In

addition, penetration of the endoperoxide into cells within a 15-min period at 4 °C is probably not complete. Therefore, it may be concluded that the formation of 8-oxodGuo is due almost exclusively to the intracellular formation of $^1\text{O}_2$. This is in agreement with the fact that the endoperoxide used has been shown to have predominantly an intracellular localization due to its non-ionic chemical character (27).

A rapid estimation could be made to determine the yield of formation of the lesion. From data reported in Fig. 4, about two ^{18}O -labeled 8-oxodGuo per 10^6 DNA bases could be formed when 30×10^6 cells were treated in 200 μl of PBS buffer with 50 μl of 130 mM DHPN $^{18}\text{O}_2$. Two 8-oxodGuo per 10^6 bases represent 2×6000 lesions per cell (cell having 6×10^9 DNA bases) corresponding to $2 \times 6000 \times 30 \times 10^6 = 3.6 \times 10^{11}$ 8-oxodGuo formed. Since the concentration of DHPN $^{18}\text{O}_2$ was 130 mM and the volume used was 50 μl , it may be inferred that $50 \times 10^{-6} \times 130 \times 10^{-3} \times 6.02 \times 10^{23} = 3.9 \times 10^{18}$ molecules of DHPN $^{18}\text{O}_2$ were available. Taking into account that about half of the oxygen release by the thermal decomposition of the endoperoxide is in its singlet state (23, 36), this indicates that, under the conditions used, about 2×10^{18} molecules of $^1\text{O}_2$ were able to give rise to 3.6×10^{11} molecules of 8-oxodGuo. Therefore, 5.4×10^6 molecules of $^1\text{O}_2$ are required for the formation of one 8-oxodGuo. Similar results could be obtained if the estimation is performed using the results presented in the different figures. However, the yield of formation is higher when the final concentration of the endoperoxide is increased. For example, the amount of 8-oxodGuo in the experiment reported in Fig. 2 is two times higher than that obtained in the experiment reported in Fig. 1. In the first experiment 50 μl of DHPNO $_2$ were used to treat 30×10^6 cells in 400 μl of PBS buffer, whereas, in the second one, the same experiment was carried out but in using only 200 μl of PBS buffer.

The low yield of formation of the lesion highlights the low efficiency for singlet oxygen to oxidize cellular DNA. This might be due, at least partly, to the powerful protection afforded by the cellular defenses to prevent the formation of mutagenic DNA damage. In this respect the scavenging or deactivating properties of carotenoids and polyamines toward singlet oxygen have already been demonstrated (37).

The present results have demonstrated that singlet oxygen is able to induce oxidation of cellular DNA. Such an oxidation reaction results in the selective formation of 8-oxodGuo. The use of a pure chemical generator of ^{18}O -labeled singlet oxygen allows us to demonstrate that the formation of the lesion is due to the direct reaction of $^1\text{O}_2$ within cellular DNA. These experiments, however, do not define the origin of the 8-oxodGuo present in cellular DNA before treatment with DHPNO $_2$, which is likely to arise from oxygen metabolism.

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