Free Radical Biology & Medicine 47 (2009) 401–409

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Thymine hydroperoxide as a potential source of singlet molecular oxygen in DNA

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ARTICLE INFO

Article history:
Received 26 December 2008
Revised 28 April 2009
Accepted 1 May 2009
Available online 6 May 2009

Keywords:
Singlet molecular oxygen
5-(Hydroperoxymethyl)uracil
Thymine hydroperoxide
Near-infrared emission
HPLC/MS/MS
Chemiluminescence
Chemical trapping
Free radicals

ABSTRACT

The decomposition of organic hydroperoxides into peroxyl radicals is a potential source of singlet molecular oxygen [O_2 ($^1\Delta_g$)] in biological systems. This study shows that 5-(hydroperoxymethyl)uracil (5-HPMU), a thymine hydroperoxide within DNA, reacts with metal ions or HOCl, generating O_2 ($^1\Delta_g$). Spectroscopic evidence for generation of O_2 ($^1\Delta_g$) was obtained by measuring (i) the bimolecular decay, (ii) the monomolecular decay, and (iii) the observation of D_2O enhancement of O_2 ($^1\Delta_g$) production and the quenching effect of NaN_3. Moreover, the presence of O_2 ($^1\Delta_g$) was unequivocally demonstrated by the direct characterization of the near-infrared light emission. For the sake of comparison, O_2 ($^1\Delta_g$) derived from the H_2O_2/HOCl system and from the thermolysis of the *N,N′*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide endoperoxide was also monitored. More evidence of O_2 ($^1\Delta_g$) generation was obtained by chemical trapping of O_2 ($^1\Delta_g$) with anthracene-9,10-divinylsulfonate (AVS) and detection of the specific AVS endoperoxide by HPLC/MS/MS. The detection by HPLC/MS of 5-(hydroxymethyl)uracil and 5-formyluracil, two thymine oxidation products generated from the reaction of 5-HPMU and Ce^{4+} ions, supports the Russell mechanism. These photoemission properties and chemical trapping clearly demonstrate that the decomposition of 5-HPMU generates O_2 ($^1\Delta_g$) by the Russell mechanism and point to the involvement of O_2 ($^1\Delta_g$) in thymidine hydroperoxide cytotoxicity.

Formation of oxidative base lesions within DNA has been associated with mutagenesis and lethality [1]. Thymine has been considered a major target of oxidative base damage, yielding thymine hydroperoxides as primary products [2]. For example, hydroxyl radicals react with this base moiety by addition to the 5,6 double bond, generating eight diastereomers of thymidine, 5(6)-hydroxy-6(5)-hydroperoxy-5,6-dihydrothymidine [2,3]. Additionally, the abstraction of a hydrogen atom from the methyl group of the thymine base leads to formation of 5-(hydroperoxymethyl)-2′-deoxyuridine (5-HPMdU) [3]. Thymidine hydroperoxides can also be formed by the so-called type I mechanism (one-electron oxidation) upon exposure of DNA to UVA light in the presence of a photosensitizer [4,5]. Moreover, 5-HPMdU has been shown to be formed upon reaction of thymidine with peroxyl radicals [6].

Thymidine hydroperoxides are interesting compounds owing to their relative stability compared to other DNA hydroperoxides [3]. 5-HPMdU is of special interest, because its half-life is about 40-fold greater than that of 5(6)-hydroxy-6(5)-hydroperoxy-5,6-dihydrothymidine [3]. However, in the presence of redox-active compounds, such as transition metal ions or hypochlorous acid (HOCl), these stable hydroperoxides can decompose to the reactive peroxyl and alkoxyl radicals. Metal ions as Fe^{2+} and Cu^{2+} are often bound to the phosphate groups of the DNA backbone and to certain amino acids of some proteins, including chromatin [7,8]. Therefore, the reaction of thymine hydroperoxides with Fe^{2+} or Cu^{2+} ions associated with the DNA backbone or the chromatin scaffold could be a source of alkoxyl and peroxyl radicals in DNA [6].

It has been shown that organic hydroperoxides containing α-hydrogen can produce singlet molecular oxygen [O_2 ($^1\Delta_g$)] by the Russell mechanism [9]. In this mechanism, the self-reaction of primary or secondary peroxyl radicals generates a linear tetraoxide intermediate that decomposes to an alcohol, a ketone, and molecular oxygen. Howard and Ingold found that this reaction may generate either an electronically excited ketone or O_2 ($^1\Delta_g$) [10]. In fact, it was shown that the generation of O_2 ($^1\Delta_g$) is favored over that of electronically excited ketone [11].

Abbreviations: O_2 ($^1\Delta_g$), singlet molecular oxygen; 5-HPMU, 5-(hydroperoxymethyl)uracil; 5-HPMdU, 5-(hydroperoxymethyl)-2′-deoxyuridine; 5-HMU, 5-(hydroxymethyl)uracil; 5-HMdU, 5-(hydroxymethyl)-2′-deoxyuridine; 5-FoU, 5-formyluracil; AVS, anthracene-9,10-divinylsulfonate; $AVSO_2$, AVS endoperoxide; $DHPNO_2$, endoperoxide of *N,N′*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide; dGuo, 2′-deoxyguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; LAOOH, linoleic acid hydroperoxide; LAOOCl, chlorinated peroxide intermediate; $LAO^{\bullet}$, linoleic acid alkoxyl radical; $LAOO^{\bullet}$, linoleic acid peroxyl radical; $^{\bullet}OCl$, hypochlorite radical; $Cl^{\bullet}$, chlorine radical.

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doi:10.1016/j.freeradbiomed.2009.05.001

$$O_2\,(^1\Delta_g) \rightarrow O_2\,(^3\Sigma_g^-) + h\upsilon \quad (\lambda = 1270\ \text{nm}) \quad \text{(eq. 1)}$$

$$O_2\,(^1\Delta_g) + O_2\,(^1\Delta_g) \rightarrow 2O_2\,(^3\Sigma_g^-) + h\upsilon \quad (\lambda = 634 \text{ and } 703\ \text{nm}) \quad \text{(eq. 2)}$$

Scheme 1. O_2 ($^1\Delta_g$) monomolecular decay in the near-infrared region (λ 1270 nm, Eq. (1)) and bimolecular decay in the visible region (λ 634 and 703 nm, Eq. (2)).

Recently, Miyamoto et al. showed that the metal ion- [12,13] or HOCl- [14] catalyzed decomposition of linoleic acid hydroperoxides (LAOOH) generates O_2 ($^1\Delta_g$) by the Russell mechanism. Linoleic acid is one of the major fatty acids present in membranes, and the decomposition of its hydroperoxides could be a source of O_2 ($^1\Delta_g$) in biological systems. Other biological sources of O_2 ($^1\Delta_g$) are the enzymatic process of peroxidases [15,16], phagocytosis [17], and the exposure of endogenous photosensitizers (porphyrins, flavins, quinones) to UVA light [18]. Singlet molecular oxygen is a reactive form of oxygen, which reacts with electron-rich biomolecules such as lipids [19], proteins [20–23], and DNA [24,25]. In the last case, O_2 ($^1\Delta_g$) has been shown to oxidize 2′-deoxyguanosine (dGuo) yielding 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), which is a recognized mutagenic lesion [26–28].

Also, O_2 ($^1\Delta_g$) is an excited and short-lived species, which decays to ground state emitting light. Relaxation of O_2 ($^1\Delta_g$) to the ground state produces light emission at 1268 nm in a process called monomol light emission [29]. Additionally, two molecules of O_2 ($^1\Delta_g$) can collide, resulting in the simultaneous relaxation of both molecules, with light emission at 634 and 703 nm. This process is called dimol light emission [30] (Scheme 1). Taking advantage of O_2 ($^1\Delta_g$) light emission, common approaches used for its detection are the light measurements at near-infrared (λ 1270 nm) and visible (λ>570 nm) regions. Another approach used is a chemical trapping methodology. This technique is based on the formation of a specific product upon reaction of O_2 ($^1\Delta_g$) with a chemical trap [31]. The specific product can be detected and identified using appropriate techniques, such as HPLC coupled to mass spectrometry in tandem. In addition, other less specific methods, such as the quencher effect of azide [32] and the increase in O_2 ($^1\Delta_g$) lifetime in the presence of deuterated water [33], are also employed and can be valuable tools if coupled with more specific methodologies.

Because O_2 ($^1\Delta_g$) is potentially mutagenic, the aim of this study was to investigate whether 5-HPMU, a primary hydroperoxide containing α-hydrogen, could be a source of O_2 ($^1\Delta_g$) in DNA. With this purpose, the generation of O_2 ($^1\Delta_g$) from 5-HPMU using Ce^{4+} or HOCl was assessed through the direct spectroscopic detection and characterization of O_2 ($^1\Delta_g$) by dimol and monomol light emission in the near-infrared region, as well as by the observation of deuterium oxide enhancement of O_2 ($^1\Delta_g$) lifetime and the quenching effect of sodium azide. In addition, the reaction mechanism was investigated by chemically trapping the O_2 ($^1\Delta_g$) produced from the reaction of 5-HPMU and Cu^{2+}, Fe^{2+}, or HOCl using anthracene-9,10-divinylsulfonate (AVS). The chemical trapping method was coupled to the detection of the corresponding endoperoxide $AVSO_2$ by HPLC coupled to mass spectrometry in tandem. In addition to O_2 ($^1\Delta_g$), 5-(hydroxymethyl)uracil (5-HMU) and 5-formyluracil (5-FoU), other products characteristic of the Russell mechanism, were detected in the reaction of 5-HPMU and Ce^{4+} ions.

Materials and methods

Materials

Sodium azide (NaN_3), uracil, paraformaldehyde, Dowex-50W, and ammonium cerium(IV) nitrate were obtained from Sigma (St. Louis, MO, USA). Deuterium oxide (D_2O) was from Cambridge Isotope Laboratories (Andover, MA, USA). Hydrogen peroxide (H_2O_2) and potassium hydroxide were purchased from Peróxidos do Brasil (Paraná, Brazil) and Merck (Rio de Janeiro, Brazil), respectively. All of the other solvents were acquired from Merck (Hunterdon, NJ, USA) and were of HPLC grade. The water used in the experiments was treated with the Nanopure water system (Barnsted, Dubuque, IA, USA).

Synthesis and analysis of 5-HMU and 5-HPMU

5-HMU was synthesized following the method proposed by Cline et al. with minor modifications [34]. 5-HPMU was produced by oxidation of 5-HMU using H_2O_2 and 37% HCl [35]. The complete descriptions of the 5-HMU and 5-HPMU syntheses are reported in the supplementary material. Purification, HPLC/MS, and NMR spectroscopic analysis are also described (Supplementary Figs. 1 and 2).

Hydroperoxide measurements

After purification, 5-HPMU was dissolved in water or D_2O, and the hydroperoxide concentration was determined by the ferric–xylenol orange [36] and iodometric methods [37].

Dimol light emission of singlet molecular oxygen

Dimol light emission of O_2 ($^1\Delta_g$) in the visible region was measured with a photon counter device, following the method described previously by Miyamoto et al. [13]. Selective light emission was obtained with a cut-off filter (λ>570 nm) (03FS006, Melles Griot visible filters) placed between the cuvette and the photomultiplier tube. The dimol light emission was monitored in the reactions of 5-HPMU with Ce^{4+} ions or HOCl.

In the first experiment, 900 μl of 2 mM 5-HPMU (final concentration 1.8 mM, prepared in D_2O) was placed in a cuvette inside the equipment and the baseline was monitored. After a short period of time, 100 μl of 50 mM Ce^{4+} ions prepared in D_2O (final concentration 5 mM) was injected into the cuvette, and light emission was recorded. For the second experiment, 125 μl of 40 mM HOCl (final concentration 5 mM) previously prepared in NaOH (10 mM in D_2O) was injected into a cuvette containing 5-HPMU (final concentration 1.8 mM in 15 mM phosphate buffer, pH 7.4, prepared in D_2O). Reactions of 5-HPMU and Ce^{4+} ions or HOCl were also carried out in the presence of NaN_3 (1 or 10 mM) and with replacement of D_2O by a mixture of D_2O:H_2O (35:65, v/v). All light emission experiments were acquired in triplicate and performed with stirring. Solutions of Ce^{4+} ions and HOCl were injected manually into the cuvette.

Monomol light emission of singlet molecular oxygen in the near-infrared region

Monomol light emission of O_2 ($^1\Delta_g$) at 1270 nm was monitored with a special photon counting apparatus developed in our laboratory and equipped with a monochromator capable of selecting emission in the near-infrared region (800–1400 nm) as described by Miyamoto et al. [13]. The monomol light emission was monitored during the reaction of 5-HPMU (final concentration 1.8 mM) with increasing concentrations of Ce^{4+} ions or HOCl (final concentration 0.1 to 10 mM). For the reactions with Ce^{4+} ions, 5-HPMU (final concentration 1.8 mM, in D_2O) was placed into a cuvette inside the equipment and the baseline was monitored for a short period of time. After that, 200 μl of each Ce^{4+} solution dissolved in D_2O (final concentrations 0.1, 0.5, 1, 2, 5, and 10 mM) was injected into the cuvette and light emission was recorded. For the reactions with HOCl, 5-HPMU (final concentration 1.8 mM, in D_2O) was placed into a cuvette inside the equipment and the baseline was monitored for a short period of time. Then, 200 μl of each HOCl solution prepared in NaOH (10 mM, in D_2O) (final concentrations 0.1, 0.5, 1, 2, 5, and 10 mM) was injected into the cuvette. The reactions of 5-HPMU and Ce^{4+} or HOCl were carried out under stirring and performed at least in triplicate. The monitoring of monomol light emission in the reactions of 1.8 mm 5-HPMU and 5 mM Ce^{4+} ions or HOCl was also carried out in the presence of NaN_3 (1 or