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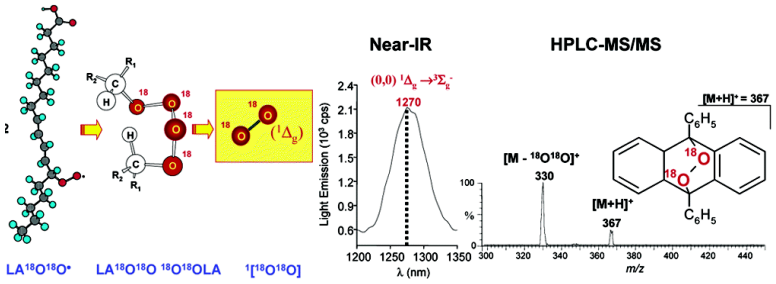

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Singlet Molecular Oxygen Generated from Lipid Hydroperoxides by the Russell Mechanism: Studies Using ^{18}O-Labeled Linoleic Acid Hydroperoxide and Monomol Light Emission Measurements

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Abstract: The decomposition of lipid hydroperoxides into peroxyl radicals is a potential source of singlet oxygen ($^1O_2$) in biological systems. We report herein on evidence of the generation of $^1O_2$ from lipid hydroperoxides involving a cyclic mechanism from a linear tetraoxide intermediate proposed by Russell. Using ^{18}O-labeled linoleic acid hydroperoxide (LA^{18}O^{18}OH) in the presence of Ce^{4+} or Fe^{2+}, we observed the formation of ^{18}O-labeled $^1O_2$ ($^{18}[^1O_2]$) by chemical trapping of $^1O_2$ with 9,10-diphenylanthracene (DPA) and detected the corresponding ^{18}O-labeled DPA endoperoxide (DPA^{18}O^{18}O) by high-performance liquid chromatography coupled to tandem mass spectrometry. Spectroscopic evidence for the generation of $^1O_2$ was obtained by measuring (i) the dimol light emission in the red spectral region ($\lambda > 570$ nm); (ii) the monomol light emission in the near-infrared (IR) region ($\lambda = 1270$ nm); and (iii) the quenching effect of sodium azide. Moreover, the presence of $^1O_2$ was unequivocally demonstrated by the direct spectral characterization of the near-IR light emission. For the sake of comparison, $^1O_2$ deriving from the H_2O_2/OCl^- and H_2O_2/MoO_4^{2-} systems or from the thermolysis of the endoperoxide of 1,4-dimethylnaphthalene was also monitored. These chemical trapping and photoemission properties clearly demonstrate that the decomposition of LA^{18}O^{18}OH generates $^{18}[^1O_2]$, consistent with the Russell mechanism and pointing to the involvement of $^1O_2$ in lipid hydroperoxide mediated cytotoxicity.

Introduction

Lipid peroxidation is considered a key chemical event of the oxidative stress associated with several diseases.[1−6] Lipid hydroperoxides are the primary products of lipid peroxidation, and it is assumed that their decomposition is involved in the generation of toxic products and in the induction of tissue injury.[7−9] Lipid hydroperoxides are also generated in singlet molecular oxygen [$^1O_2$, O_2 ($^1\Delta_g$)] mediated oxidations[10,11] and by the action of enzymes such as lipoxygenases[12] and cyclooxygenases.[13] Despite their relative stability in organic solutions, lipid hydroperoxides are easily decomposed by metal ions, generating peroxyl and/or alkoxyl radicals, which are responsible for the propagation of lipid peroxidation and the generation of toxic compounds.[14]

Following the suggestion of Russell,[15] Howard and Ingold[16] found that the self-reaction of peroxyl radicals generates $^1O_2$. Russell proposed the formation of a cyclic mechanism from a linear tetraoxide intermediate that decomposes to give an alcohol, ketone, and molecular oxygen (Scheme 1). It has been postulated that this reaction may generate either an electronically excited oxygen molecule (Scheme 1A) or an electronically excited ketone (Scheme 1B). Indeed, $^1O_2$ and triplet carbonyls have been identified as the chemiluminescence (CL) emitters in the ultraweak CL associated with lipid peroxidation in a biological system.[17−19] The generation of $^1O_2$ during the metal-catalyzed decomposition of alkylhydroperoxides has been studied by several authors.[20−24] Niu and Mendenhall reported

(1) Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G. *Free Radic. Biol. Med.* **1992**, *13*, 341−390.
(2) Berliner, J. A.; Heinecke, J. W. *Free Radic. Biol. Med.* **1996**, *20*, 707−727.
(3) Markesbery, W. R. *Free Radic. Biol. Med.* **1997**, *23*, 134−147.
(4) Rowley, D.; Gutteridge, J. M. C.; Blake, D.; Farr, M.; Halliwell, B. *Clin. Sci.* **1984**, *66*, 691−695.
(5) Baynes, J. W. *Diabetes* **1991**, *40*, 405−412.
(6) Otamiri, T.; Sjodahl, R. *Cancer* **1989**, *64*, 422−425.
(7) Esterbauer, H. *Am. J. Clin. Nutr.* **1993**, *57*, 779S−786S.
(8) Girotti, A. W. *J. Lipid Res.* **1998**, *39*, 1529−1542.
(9) Spiteller, G. *Chem. Phys. Lipids* **1998**, *95*, 105−162.
(10) Terao, J.; Matsushita, S. *J. Am. Oil Chem. Soc.* **1977**, *54*, 234−238.
(11) Frankel, E. N.; Neff, W. E.; Bressler, T. R. *Lipids* **1979**, *14*, 961−967.
(12) Brash, A. R. *J. Biol. Chem.* **1999**, *274*, 23679−23682.
(13) Hamberg, M.; Samuelsson, B. *Biochim. Biophys. Acta* **1980**, *617*, 545−547.
(14) Gardner, H. W. *Free Radic. Biol. Med.* **1989**, *7*, 65−86.
(15) Russell, G. A. *J. Am. Chem. Soc.* **1957**, *79*, 3871−3877.
(16) Howard, J. A.; Ingold, K. U. *J. Am. Chem. Soc.* **1968**, *90*, 1057−1058.
(17) King, M. M.; Lai, E. K.; McCay, P. B. *J. Biol. Chem.* **1975**, *250*, 6496.
(18) Sugioka, K.; Nakano, M. *Biochim. Biophys. Acta* **1976**, *423*, 203−216.
(19) Sies, H. *Arch. Toxicol.* **1987**, *60*, 138−143.
(20) Hawco, F. J.; O'Brien, P. J. *Biochem. Biophys. Res. Commun.* **1976**, *76*, 354−361.
(21) Kanofsky, J. R. *J. Org. Chem.* **1986**, *51*, 3386−3388.
(22) Koga, S.; Nakano, M.; Uehara, K. *Arch. Biochem. Biophys.* **1991**, *289*, 223−229.
(23) Niu, Q.; Mendenhall, G. D. *J. Am. Chem. Soc.* **1990**, *112*, 1656−1657.
(24) Mendenhall, G. D.; Sheng, X. C. *J. Am. Chem. Soc.* **1991**, *113*, 8976−8977.

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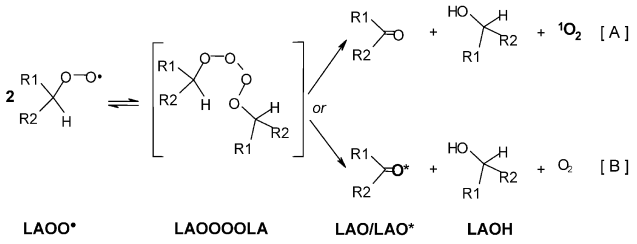
Scheme 1. Proposed Russell Mechanism for the Self-Reaction of Peroxyl Radicals (LAOO•) Involving a Cyclic Mechanism from a Linear Tetraoxide Intermediate (LAOOOOLA) and the Corresponding Products: [A] Alcohol (LAOH), Ketone (LAO), and $^1O_2$; or [B] Alcohol (LAOH), Excited Ketone (LAO*), and O_2



that the yields of $^1O_2$, in the case of simple alkylhydroperoxides, ranged from 3.9% to 14.0%.[23] In contrast, the yields of excited carbonyls were 10^3-10^4 lower, suggesting that the self-reaction of peroxyl radical deriving from fatty acids generates predominantly $^1O_2$.[24]

Singlet oxygen exhibits substantial reactivity toward electron-rich organic molecules including, among others, the guanine moiety of DNA.[25,26] Evidence has accumulated indicating that $^1O_2$ is implicated in the genotoxic effect of the UVA component of solar radiation and likely plays an important role in the cell signaling cascade[27] and in the induction of gene expression.[28]

We report here on studies we pursued to clearly demonstrate the generation of $^1O_2$ from lipid hydroperoxides by the mechanism proposed by Russell. For this purpose, we used ^{18}O-labeled linoleic acid hydroperoxide ($LA^{18}O^{18}OH$) and ceric ion (Ce^{4+}) or ferrous ion (Fe^{2+}) as a mechanistic tool. Our study was based on the specific detection and quantification of ^{18}O-labeled singlet oxygen ($^{18}[^1O_2]$) generated by the combination of two ^{18}O-labeled peroxyl radicals, using 9,10-diphenylanthracene (DPA) as the chemical trap. The corresponding DPA endoperoxide containing labeled oxygen ($DPA^{18}O^{18}O$) was detected and quantified by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Additional evidence for the generation of $^1O_2$ was obtained by direct measurement of (i) dimol light emission in the red spectral region ($\lambda > 570$ nm) using a red-sensitive photomultiplier (PMT); (ii) monomol light emission in the near-infrared (IR) region ($\lambda = 1270$ nm) with a liquid nitrogen cooled germanium diode detector; and (iii) the $^1O_2$ spectrum in the near-IR region using a specific IR-PMT coupled to a monochromator.

Experimental Section

Materials. Linoleic acid (LA) and sodium azide were obtained from Sigma (St. Louis, MO). Ceric sulfate was supplied by BDH Limited (Poole, U.K.). Methylene blue and iron(II) sulfate heptahydrate came from Merck (Rio de Janeiro, Brazil and Darmstadt, Germany, respectively). Silica gel 60 (230−400 mesh), deuterium oxide, 9,10-diphenylanthracene, and 9,10-dibromoanthracene were from Aldrich (Steinheim, Germany), and hydrogen peroxide was purchased from Peróxidos do Brasil (Paraná, Brazil). All of the other solvents were of HPLC grade and were acquired from Merck (Rio de Janeiro, Brazil). The water used in the experiments was treated with the Nanopure Water System (Barnstead, Dubuque, IA).

Synthesis of ^{18}O-Labeled Linoleic Acid Hydroperoxide ($LA^{18}O^{18}OH$). $LA^{18}O^{18}OH$ was synthesized by photooxidation of linoleic acid under an $^{18}O_2$-saturated atmosphere using methylene blue as the photosensitizer. Briefly, 1 g of linoleic acid was dissolved in 50 mL of chloroform previously purged with nitrogen gas for 20 min. Next, 100 μL of methylene blue solution (100 mM in methanol) was added, and the oxygen contained in the system was removed by successive freezing and thawing under vacuum. This procedure was repeated at least five times to ensure complete removal of $^{16}O_2$. Thereafter, the whole system was connected to an $^{18}O_2$ gas cylinder (Isotec-Sigma, St. Louis, MO) under 0.5 atm. The mixture was irradiated with two tungsten lamps (500 W) for 5 h and stirred continuously. The $LA^{18}O^{18}OH$ was purified by column chromatography, using silica gel 60 (230−400 mesh) according to the method described by Kühn.[29] After purification, the $LA^{18}O^{18}OH$ was dissolved in chloroform and stored at −20 °C. The $LA^{18}O^{18}OH$ concentration was determined by absorbance at 234 nm ($\epsilon_{234} = 25\ 000\ M^{-1}\ cm^{-1}$),[30] considering that 60% of the hydroperoxides contain conjugated diene.[10,11]

Synthesis of the $LA^{18}O^{18}OH$ was confirmed by mass spectrometry, using a Quattro II mass spectrometer (Micromass, Manchester, U.K.) in the negative electrospray ionization mode (ESI^-). The source temperature of the mass spectrometer was kept at 100 °C, and the flow rates of drying and nebulizing gas were optimized at 300 and 15 L/h, respectively. The cone voltage was set to 20 V, and the collision energy was set at 30 eV. The capillary potential and high electrode potential were set to 3.0 and 0.5 kV, respectively. Full scan data were acquired over a mass range from 50 to 400 *m*/*z*. For the analysis, 20 μL of $LA^{18}O^{18}OH$ solution (300 pg/μL) was injected through an LC-18 column (Supelco, 150 × 4.6 mm, 5 μm) and eluted with a linear gradient from 75% to 100% acetonitrile for 10 min at a flow rate of 1 mL/min. The data were processed and transformed into molecular mass values on a mass scale by means of the Mass Lynx NT data system, version 3.20 (Micromass, Altricham, U.K.).

Chemical Detection of Singlet Molecular Oxygen. The generation of $^1O_2$ was monitored by chemical trapping with 9,10-diphenylanthracene (DPA). This method is based on the rapid and specific reaction of $^1O_2$ with DPA ($k_r = 1.3 \times 10^6\ M^{-1}\ s^{-1}$), which forms a stable DPA endoperoxide ($DPAO_2$).[31,32] The $DPAO_2$ was quantified using 9,10-dibromoanthracene (DBA) as an internal standard. $DPAO_2$ and DBA were analyzed by an HPLC system (SPD 10 AV*VP*, Shimadzu, Kyoto, Japan) connected to an automatic SIL-10AD*VP* autoinjector and to a UV−visible detector (SPD 10 AV*VP*, Shimadzu, Kyoto, Japan) and a Quattro II Micromass mass spectrometer (Manchester, U.K.). The compounds were diluted 1:125 in acetonitrile, and 50 μL was injected. The reaction products were separated by a reversed-phase Luna LC-18 column (150 × 4.6 mm, 5 μm particle, Phenomenex, CA), using a gradient system of acetonitrile (solvent B) and water (solvent A). Elution was started with a increasing linear gradient of B from 75% to 100% over the course of 10 min, followed by an isocratic elution for 7 min, ending with a decreasing gradient back to 75% within 3 min at a flow rate of 0.8 mL/min. The eluent was monitored with a UV detector at 210 nm and with the mass spectrometer utilizing atmospheric pressure chemical ionization (APCI) in the positive ion mode. The probe and source temperature of the mass spectrometer were held at 300 and 150 °C, respectively. $DPAO_2$ was monitored at a cone voltage of 15 V and collision energy of 15 eV. In the same run, DBA was monitored at a cone voltage of 20 V and collision energy of 40 eV. Full-scan data were acquired over a mass range of 100−500 Da. The quantification was performed in the multiple reaction monitoring mode (MRM), by selecting a specific mass transition of the target compound. The data

(25) Cadet, J.; Douki, T.; Pouget, J. P.; Ravanat, J. L. *Methods Enzymol.* **2000**, *319*, 143−153.
(26) Kang, P.; Foote, C. S. *J. Am. Chem. Soc.* **2002**, *124*, 4865−4873.
(27) Klotz, L. O.; Briviba, K.; Sies, H. *Methods Enzymol.* **2000**, *319*, 130−143.
(28) Ryter, S. W.; Tyrrell, R. M. *Free Radic. Biol. Med.* **1998**, *24*, 1520−1534.
(29) Kühn, H.; Wiesner, R.; Lankin, V. Z.; Nekrasov, A.; Alder, L.; Schewe, T. *Anal. Biochem.* **1987**, *160*, 24−34.
(30) Mulliez, E.; Leblanc, J. P.; Girerd, J. J.; Rigaud, M.; Chottard, J. C. *Biochim. Biophys. Acta* **1987**, *916*, 13−23.
(31) Corey, E. J.; Taylor, W. C. *J. Am. Chem. Soc.* **1964**, *86*, 2909−2914.
(32) Turro, N. J.; Chow, M. F.; Rigaudy, J. *J. Am. Chem. Soc.* **1981**, *103*, 7218−7224.

were processed and transformed into molecular mass values on mass scale using the aforementioned software.

Incubations of $LA^{18}O^{18}OH$ with Ceric Ion. $LA^{18}O^{18}OH$ was incubated with Ce^{4+} in D_2O:chloroform (1:1, v/v) and DMSO:chloroform (1:1, v/v) systems. A Ce^{4+} solution was prepared in D_2O or DMSO, and the DPA and $LA^{18}O^{18}OH$ solutions were prepared in chloroform. In the case of the reaction conducted under deaerated conditions, Ce^{4+} and DPA/$LA^{18}O^{18}OH$ solutions were purged with nitrogen gas for 20 min. For the reaction, an aliquot of 100 μL of Ce^{4+} solution (final concentration: 25 mM) and 100 μL of DPA/ $LA^{18}O^{18}OH$ solution (final concentration: 60 and 25 mM, respectively) was poured into a small glass vial and incubated under vigorous mixing at 37 °C for 1 h. Similarly, deaerated incubation was conducted in a sealed flask purged with nitrogen. Following incubation, an aliquot of the samples was dried under a nitrogen stream and diluted three times in acetone. An aliquot of 50 μL of this solution was mixed with 50 μL of sodium azide (final concentration: 20 mM) and 25 μL of 1 mM DBA solution in acetonitrile.

Incubations of $LA^{16}O^{16}OH$ with Ceric or Ferrous Ion. $LA^{16}O^{16}OH$ was incubated with Ce^{4+} or Fe^{2+} in D_2O:chloroform (1:1, v/v). A Ce^{4+} or Fe^{2+} solution was prepared in D_2O, and the DPA and $LA^{16}O^{16}OH$ solutions were prepared in chloroform. For the reaction, an aliquot of 100 μL of Ce^{4+} or Fe^{2+} solution (final concentration: 50 mM) and 100 μL of DPA/$LA^{16}O^{16}OH$ solution (final concentration: 60 and 50 mM, respectively) was poured into a small glass vial and incubated under vigorous mixing at 37 °C for 1 h. Following incubation, an aliquot of the samples was dried under a nitrogen stream and diluted three times in acetone. An aliquot of 50 μL of this solution was mixed with 50 μL of sodium azide (final concentration: 20 mM) solution in acetonitrile.

Dimol Light Emission of Singlet Oxygen (λ = 634 and 703 nm). Light emission of $^1O_2$ in the visible region was measured with a photoncounter, following the method described previously by Cadenas and Sies.[33,34] The photoncounting device consisted of a red-sensitive photomultiplier tube (9203BM Thor EMI Electron Tubes, U.K.) cooled to −20 °C by a thermoelectric cooler (FACT 50 MKIII, EMI Gencom, Plainview, NY). The potential applied to the photomultiplier was −1.200 V. The phototube output was connected to an amplifier discriminator (model 1121, Princeton Instruments, NJ), which transmitted the signal to a computer. Selective light emission at wavelengths λ > 570 nm was obtained with a cutoff filter (03IFS006, Melles Griot visible filters) placed between the cuvette and the photomultiplier tube. Sample solutions were poured into a thermostated glass cuvette (35 mm × 7 mm × 55 mm) with mirrored walls, and the reactant was injected through a small polyethylene tube under continuous stirring. The CL signal was monitored during the injection of 0.1 mL of 100 mM Ce^{4+} solution in methanol (final concentration: 2 mM) into 5 mL of 0.2 mM LAOOH solution in methanol. For comparison, the CL signal of the reaction of H_2O_2 by hypochlorite was also monitored. The reaction was performed by injecting 0.4 mL of 0.28 M hypochlorite solution (final concentration: 22.4 mM) into 5 mL of 2.2 mM hydrogen peroxide solution.

Monomol Light Emission of Singlet Oxygen (λ = 1270 nm). Infrared light emission of $^1O_2$ at 1270 nm was monitored with a liquid nitrogen cooled photodiode germanium detector (Ge-Diode, model EI-L, Edinburgh Instruments Ltd, Livingston, U.K.), sensitive in the spectral region from 800 to 1700 nm, as described elsewhere.[34] A silicon filter and a band-pass filter at 1270 nm with a 10 nm half-bandwidth were used (Spectrogon U.K. Ltd., Glenrothes, UK). The Ge-Diode system was equipped with a bias power supply (model PS-1) set to provide 160 V to the detector, a muon filter (model MF-1) to filter cosmic and ionizing radiation, an optical chopper (Bentham 218, Bentham Instruments, U.K.) set at a frequency of 125 Hz, and a lock-in amplifier (Bentham 225, Bentham Instruments, U.K.) to process the signal from the detector. The detector's signal was registered by the F-900 version 6.22 software program (Edinburgh Analytical Instruments, Livingston, U.K.). The optimal condition to detect the $^1O_2$ monomol light emission was adjusted with the endoperoxide of 1,4-dimethylnaphthalene ($DMNO_2$, 15 mM in chloroform) heated to 40 °C. For the experiments, the photodiode detector was filled with liquid nitrogen and left to equilibrate for 2 h before use. The assay was conducted at 37 °C in a thermostated quartz cuvette (10 mm × 10 mm × 30 mm) under continuous stirring with a small magnetic bar (Cuv-o-stir, model 333, Hellma, Mülheim, Germany). After the baseline was recorded with the assay solvent, the reactant was injected into the cuvette with a syringe injection pump (Syringe Pump Model 22, Harvard Apparatus, MA). For the assay, 0.5 mL of LAOOH (100 mM in methanol, final concentration: 20 mM) was infused through a small polyethylene tube into 2.0 mL of 200 mM Ce^{4+} solution in methanol at a constant flux at a flow rate of 0.7 mL/min. The effect of NaN_3 was tested by injecting a 10 mM solution of NaN_3 by another tube immediately following the appearance of an intense CL signal in the LAOOH and Ce^{4+} reaction.

Singlet Oxygen Spectrum in the Infrared Region. The $^1O_2$ monomol light emission spectrum was measured with a special photocounting apparatus developed in our laboratory and equipped with a monochromator capable of selecting emissions in the near-infrared region (800−1400 nm). The apparatus consisted of a photomultiplier tube (R5509 PMT, Hamamatsu Photoniks KK, Shizuoka, Japan) cooled to −80 °C with liquid nitrogen (S600 PHOTOCOOL, PC176TSCE005 cooler, Products for Research Inc., MA) to reduce the dark current. The power was provided by a high voltage DC power supply (Model C3360, Hamamatsu Photoniks KK, Shizuoka, Japan), and the applied potential was set to −1.5 kV. The light emitted from the sample was processed through a monochromator (M300, Bentham Instruments, U.K.) equipped with a diffraction grating (Type G306R1u0, Bentham Instruments, U.K.) capable of selecting wavelengths in the infrared region. The control of the monochromator and the acquision of data were done by means of the F900 software described earlier. Experiments were conducted in quartz cuvettes under continuous stirring, as described above. Typically, from three to five scans in the range of 1200−1350 nm were recorded and averaged to yield the spectrum. The $^1O_2$ spectrum generated in the LAOOH/Ce^{4+} reaction was recorded during the injection of 200 mM Ce^{4+} (final concentration: 40 mM) into 1.8 mL of 100 mM LAOOH solution in methanol at a flow rate of 0.7 mL/min. The $^1O_2$ spectra generated in the H_2O_2/OCl^-, H_2O_2/MoO_4^{2-} reaction and during the thermodissociation of $DMNO_2$ were measured similarly. For the H_2O_2/OCl^-, 2 mL of hypochlorite (0.28 M) was injected into 1 mL of 3 M H_2O_2 (final concentration: 1 M) at a flux rate of 1.4 mL/min, and, for the H_2O_2/ MoO_4^{2-}, 0.1 mL of 0.5 M MoO_4^{2-} (final concentration: 25 mM) was added to 1.9 mL of 35% H_2O_2, with the reaction proceeding autocatalytically. In the case of the endoperoxide, a 15 mM solution of $DMNO_2$ in chloroform was incubated at 37 °C.

Results and Discussion

Synthesis and Characterization of ^{18}O-Labeled Linoleic Acid Hydroperoxide ($LA^{18}O^{18}OH$). $LA^{18}O^{18}OH$ was synthesized by photooxidation, using methylene blue as a sensitizer in an atmosphere saturated with ^{18}O-isotopically labeled molecular oxygen ($^{18}O_2$). The incorporation of two atoms of ^{18}O into the hydroperoxyl moiety of linoleic acid (LA) is consistent with data from electrospray ionization mass spectrometry in the negative mode (ESI^-/MS) (Figure 1).

Mass spectra of both LAOOH (Figure 1A) and $LA^{18}O^{18}OH$ (Figure 1B) show two intense peaks, one corresponding to the molecular ion and the other corresponding to the dehydrated product. The mass spectrum of LAOOH displays a molecular

(33) Cadenas, E.; Sies, H. *Methods Enzymol.* **1984**, *105*, 221−231.
(34) Di Mascio, P.; Sies, H. *J. Am. Chem. Soc.* **1989**, *111*, 2909−2914.

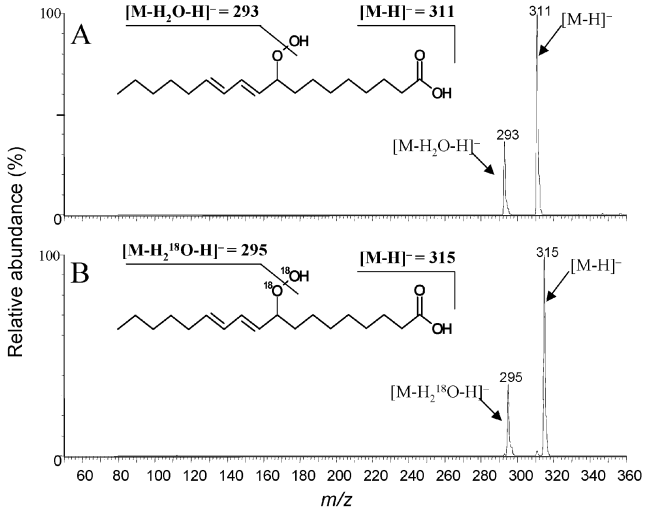
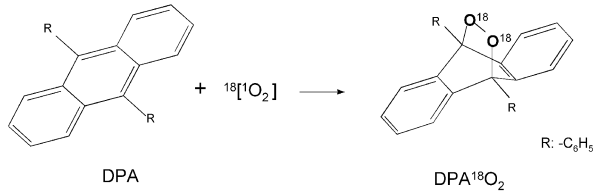


Figure 1. Electrospray ionization mass spectra of (A) unlabeled (LAOOH) and (B) ^{18}O-labeled (LA^{18}O^{18}OH) linoleic acid hydroperoxide obtained in the negative ion mode. The structure of 9-hydroperoxyoctadienoic acid, one of the four LAOOH isomers, is represented in the figure.

Scheme 2. Chemical Trapping of $^{18}[^1O_2]$ with 9,10-Diphenylanthracene (DPA) Yielding the Corresponding ^{18}O-Labeled DPA Endoperoxide (DPA^{18}O^{18}O)



ion $[M - H]^-$ at *m*/*z* 311 and a dehydrated product $[M - H_2O - H]^-$ at *m*/*z* 293. The mass spectrum of LA^{18}O^{18}OH exhibits an intense ion at *m*/*z* 315, corresponding to the labeled hydroperoxide molecular ion $[M - H]^-$, and another peak at *m*/*z* 295, corresponding to the labeled dehydrated product $[M - H_2{}^{18}O - H]^-$. The increase by 4 atomic mass units (amu) for the molecular ion and 2 amu for the dehydrated product in the mass spectrum of LA^{18}O^{18}OH indicates that two atoms of ^{18}O were successfully incorporated into the hydroperoxyl group. The detection of only trace amounts of the ion peak at *m*/*z* 311 (LA-^{16}O^{16}OH) demonstrates that LA^{18}O^{18}OH is almost 100% pure.

Characterization of Singlet Oxygen Generated in the Reaction of LA^{18}O^{18}OH and Ceric Ion, Using 9,10-Diphenylanthracene. Labeled $^1O_2$ ($^{18}[^1O_2]$) generated through the reaction of LA^{18}O^{18}OH and Ce^{4+} was chemically trapped with 9,10-diphenylanthracene (DPA), generating the corresponding labeled endoperoxide (DPA^{18}O^{18}O) (Scheme 2).

The analysis of $DPAO_2$ generated in the reaction of LA^{18}O-^{18}OH with Ce^{4+}, performed by HPLC-MS in the full scan mode, revealed that besides the DPA^{18}O^{18}O, two other DPA adduct species were detected: the unlabeled endoperoxide (DPA^{16}O^{16}O), and endoperoxide with one labeled oxygen (DPA^{18}O^{16}O). The tandem mass spectra (MS2 full scan) of all endoperoxides were recorded, by selecting in the first analyzer (MS1) the molecular ions of DPA^{16}O^{16}O, DPA^{18}O^{16}O, and DPA^{18}O^{18}O, at *m*/*z* 363 (Figure 2A), at *m*/*z* 365 (Figure 2B), and at *m*/*z* 367 (Figure 2C), respectively. As expected, all of the endoperoxides showed an intense fragment ion at *m*/*z* 330 corresponding to the loss of one oxygen molecule.

Figure 3 shows typical chromatograms of $DPAO_2$ quantification. The reaction products were separated by HPLC, and the peaks eluting from the column were monitored by the UV at 210 nm (Figure 3A) and by recording the specific mass transitions in the multiple reaction monitoring mode (MRM) of the mass spectrometer. The following mass transitions were monitored for DPA endoperoxides: *m*/*z* 367 to 330 for DPA^{18}O^{18}O (Figure 3B), *m*/*z* 365 to 330 for DPA^{18}O^{16}O (Figure 3C), and *m*/*z* 363 to 330 for DPA^{16}O^{16}O (Figure 3D). For the internal standard, 9,10-dibromoanthracene (DBA) (Figure 3E), the mass transition from *m*/*z* 336 to 176, corresponding to the loss of two Br ($[(M - 2Br) + H]^+ = m/z$ 176) from the molecular ion ($[M + H]^+ = m/z$ 336), was monitored.

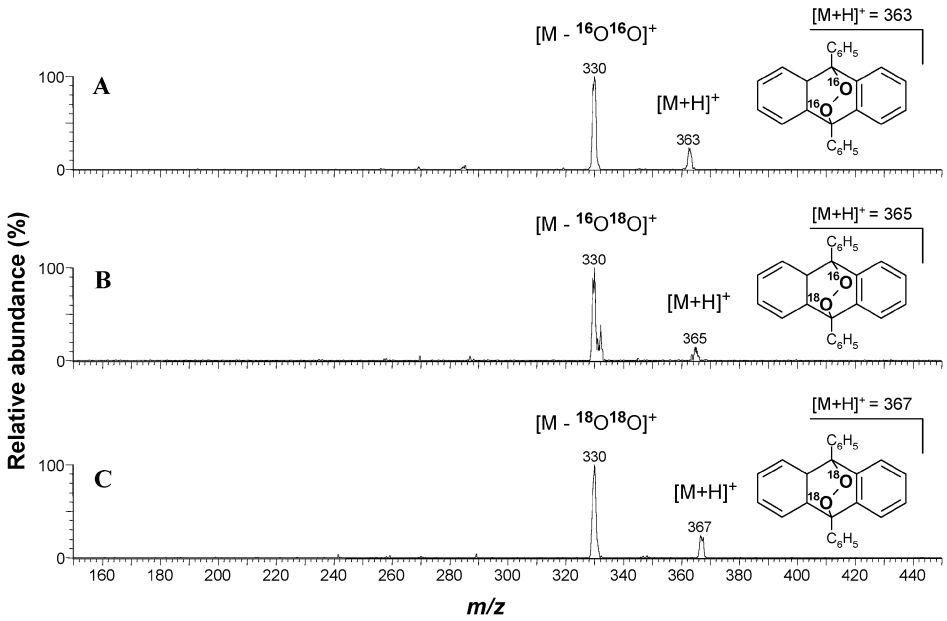


Figure 2. Positive APCI-mass spectra in the MS/MS mode of the endoperoxides of 9,10-diphenylanthracene formed in the incubation of 25 mM LA^{18}O-^{18}OH with 25 mM Ce^{4+} and 60 mM DPA in D_2O:chloroform (1:1, v/v) under vigorous mixing at 37 °C for 1 h. The MS2 full scan was acquired by selecting in the first analyzer the ions with $m/z = 363$, 365, or 367. (A) Spectrum of DPA^{16}O^{16}O. (B) Spectrum of DPA^{16}O^{18}O. (C) Spectrum of DPA^{18}O^{18}O.

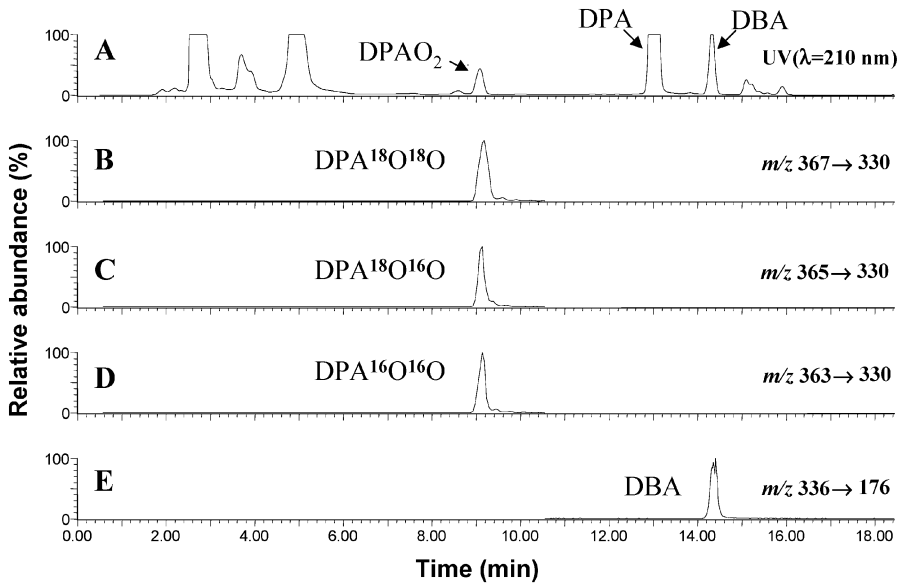


Figure 3. Typical chromatograms for the quantification of the corresponding ^{18}O-labeled DPA endoperoxide ($DPAO_2$) by HPLC coupled to tandem mass spectrometry in the MRM mode. (A) UV chromatogram at 210 nm. Specific mass transitions for (B) $DPA^{18}O^{18}O$, *m/z* 367 → 330; (C) $DPA^{16}O^{18}O$, *m/z* 365 → 330; (D) $DPA^{16}O^{16}O$, *m/z* 363 → 330; and (E) internal standard 9,10-dibromoanthracene (DBA), *m/z* 336 → 176. $LA^{18}O^{18}OH$ (25 mM) was incubated with 25 mM Ce^{4+} and 60 mM DPA in D_2O:chloroform (1:1, v/v) under vigorous mixing at 37 °C for 1 h.

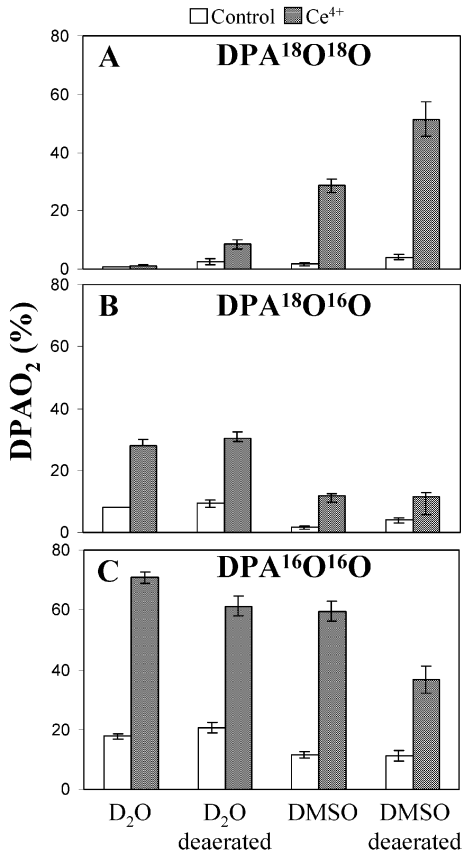


Figure 4. Relative percentage of the 9,10-diphenylanthracene endoperoxides (A) $DPA^{18}O^{18}O$, (B) $DPA^{18}O^{16}O$, and (C) $DPA^{16}O^{16}O$. The control, 25 mM $LA^{18}O^{18}OH$ (□) or 25 mM $LA^{18}O^{18}OH$ with 25 mM Ce^{4+} (■), reactions were carried out in D_2O, deaerated D_2O, DMSO, and deaerated DMSO, all containing chloroform (1:1, v/v), and 60 mM DPA under vigorous mixing at 37 °C for 1 h. Data are mean values ± SD ($n = 4$) for different experiments.

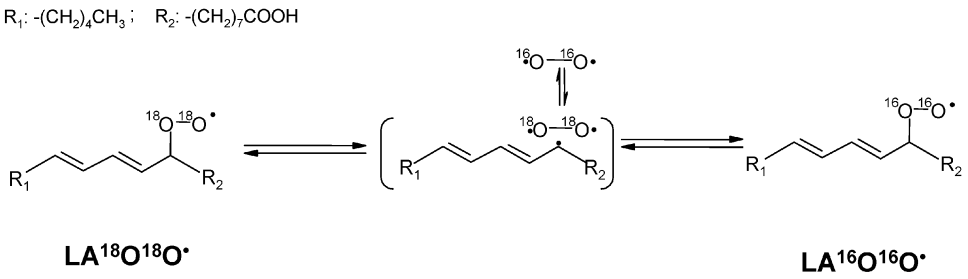
Figure 4A shows the generation of $DPA^{18}O^{18}O$ in the incubation of $LA^{18}O^{18}OH$ with Ce^{4+}, clearly demonstrating the formation of $^{18}[^1O_2]$ that probably occurs via the combination of ^{18}O-labeled peroxyl radical of linoleic acid ($LA^{18}O^{18}O^{\bullet}$). On the other hand, $DPA^{18}O^{16}O$ (Figure 4B) and $DPA^{16}O^{16}O$ (Figure 4C) were also detected, indicating that the generation of $^1O_2$ consisted of $^{18}O^{16}O$ and $^{16}O^{16}O$.

The yields of the total endoperoxides formed in D_2O, deaerated D_2O, DMSO, and deaerated DMSO were 5.0%, 1.5%, 1.5%, and 0.9%, respectively. The ratios of these three differently labeled endoperoxides ($DPA^{18}O^{18}O$:$DPA^{18}O^{16}O$:$DPA^{16}O^{16}O$) in the same experimental conditions were (1.1:28.1:70.8), (8.5:30.3:61.2), (28.6:11.9:59.5), and (51.5:11.8:36.7), respectively. These data suggest that besides labeled peroxyl radicals ($LA^{18}O^{18}O^{\bullet}$), two other peroxyl radicals containing unlabeled oxygen ($LA^{16}O^{16}O^{\bullet}$) and one labeled oxygen ($LA^{18}O^{16}O^{\bullet}$) are involved in the generation of $^1O_2$.

According to Russell's mechanism, the bimolecular reaction of $LA^{18}O^{18}O^{\bullet}$ with $LA^{16}O^{16}O^{\bullet}$ or $LA^{18}O^{16}O^{\bullet}$ would generate $^1O_2$ containing ^{18}O and ^{16}O, while the self-reaction of $LA^{16}O^{16}O^{\bullet}$ or $LA^{18}O^{16}O^{\bullet}$ would generate $^1O_2$ containing unlabeled oxygen.

Our results show that, when $LA^{18}O^{18}OH$ and Ce^{4+} were incubated in a deaerated solution of D_2O or DMSO, the amount of $DPA^{18}O^{18}O$ (Figure 4A) increased, while the amount of $DPA^{16}O^{16}O$ (Figure 4C) decreased, suggesting the involvement of an oxygen exchange mechanism during the incubation of $LA^{18}O^{18}OH$ and Ce^{4+}. Data from the literature suggest that $^{16}O_2$ dissolved in the reaction system can easily replace the $^{18}O_2$ of the peroxyl radical moiety.[35] On the basis of this fact, we hypothesized that $LA^{18}O^{18}O^{\bullet}$, which is formed by the one-electron oxidation of $LA^{18}O^{18}OH$ mediated by Ce^{4+}, has its oxygen rapidly replaced by the residual $^{16}O_2$ dissolved in the reaction system, thereby generating $LA^{16}O^{16}O^{\bullet}$ (Scheme 3). Another mechanism for the generation of $DPA^{16}O^{16}O$ may occur by the energy transfer from $^{18}[^1O_2]$ to $^{16}O_2$ in the fundamental ground state ($^3\Sigma_g^-$), yielding $^{16}[^1O_2]$. In fact, Jones and Baves showed that 50% of the $^{16}[^1O_2]$ was converted to $^{18}[^1O_2]$ in the gas phase in the presence of $^{18}O_2$ ($^3\Sigma_g^-$).[36] The triplet carbonyls

Scheme 3. ^{16}O-Exchange Reaction of ^{18}O-Labeled Linoleic Acid Hydroperoxide ($LA^{18}O^{18}OH$) with $^{16}O_2$



produced during the Russell mechanism could also be involved in such exchange mechanism by quenching $^{16}O_2$ ($^3\Sigma_g^-$), yielding $^{16}[^1O_2]$.[37]

The amount of $DPA^{18}O^{18}O$ (Figure 4A) increased 26 times and $DPA^{18}O^{16}O$ decreased by more than 60% (Figure 4B) when the incubation was done in DMSO as compared to that done in D_2O, while using DMSO led to a 16% decrease in the amount of $DPA^{16}O^{16}O$. From this result, we can speculate that the interaction of $LA^{18}O^{18}OH$ with water could form $LA^{18}O^{16}OH$, thus lowering the yields of $^{18}[^1O_2]$ when the reaction is conducted in D_2O. A similar exchange of water with the peroxyl group has also been proposed by Loidl-Stahlhofen.[38] It is known that, under acidic conditions, hydroperoxides are prone to heterolytic cleavage (Hock/Criegee rearrangement). During this process, water could exchange with the protonated hydroxyl group, forming $LA^{18}O^{16}OH$ (Supporting Information Figure 1).

Our results show variations in the amount of $DPA^{18}O^{18}O$ according to the level of oxygen dissolved in the system and the presence of water. Nevertheless, they serve as evidence for the generation of $^{18}[^1O_2]$ in the $LA^{18}O^{18}OH$ and Ce^{4+} reaction. While the purity of the labeled $LA^{18}O^{18}OH$ used in this study was near 100%, nonlabeled products such as $DPA^{18}O^{16}O$ and $DPA^{16}O^{16}O$ were detected.

When Fe^{2+} was used instead of Ce^{4+}, $DPA^{18}O^{18}O$ was also detected in the same incubation conditions (Supporting Information Figure 2). Figure 5A shows the amount of $DPAO_2$ generated in the reaction of $LA^{16}O^{16}OH$ in the presence of Fe^{2+} as compared to that with Ce^{4+}. In this experiment, after 1 h of incubation, 50% of $LA^{16}O^{16}OH$ was consumed, and the yields of $DPAO_2$ for Ce^{4+} and Fe^{2+} were 6.6 ± 1.1% and 1.0 ± 0.4%, respectively. Figure 5B shows the time course of $DPAO_2$ formation without metal (a) and in the presence of Fe^{2+} (b) or Ce^{4+} (c).

Besides the oxygen production, decomposition products arising from the Russell mechanism, ketone and alcohol, were also detected by HPLC mass spectrometry as described in Supporting Information Figures 3 and 4, respectively.

In oxygen-18 tracer studies with mass spectrometry, Bartlett and Traylor, using cumylperoxy radicals which do not have the α-hydrogen, demonstrated that evolution of oxygen occurs during the interaction between the radicals via a head-to-head mechanism.[39,40] Using similar labeling experiments coupled to electron spin resonance spectroscopy, Bennett and Howard also

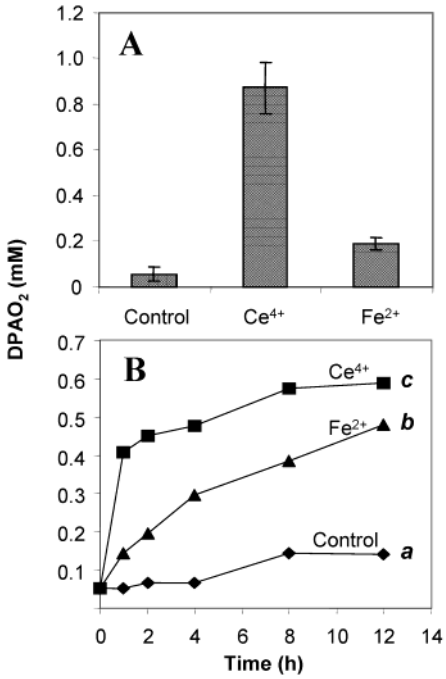


Figure 5. Quantification of $DPAO_2$ formed in the reaction of LAOOH with Ce^{4+} or Fe^{2+}. (A) $DPAO_2$ concentration in the reaction of 50 mM LAOOH with 50 mM Ce^{4+} or Fe^{2+} and 60 mM DPA in D_2O:chloroform (1:1, v/v) at 37 °C for 1 h. Data are mean values ± SD ($n = 4$) for different experiments. (B) Time-course of $DPAO_2$ generation in the same experimental condition as described for (A). (a) Control: DPA alone; (b) with Fe^{2+}; and (c) with Ce^{4+}.

obtained evidence supporting the generation of molecular oxygen by the Russell mechanism, using a mixture of equimolar amounts of various ^{18}O-labeled and unlabeled peroxyl radicals.[41] Furthermore, the oxygen evolved from the self-reaction of primary, secondary, and tertiary alkylperoxy radicals involves a head-to-head interaction between two radicals instead of a head-to-tail reaction. However, they have not found whether the oxygen generated in this reaction was in the singlet state.

Considering the products detected here ($^1O_2$, ketone, and alcohol), the interaction of two linoleic acid peroxyl radicals is most likely to occur by a head-to-head mechanism, as proposed by Russell.

These results described here provide new evidence of the generation of $^1O_2$ during the decomposition of lipid hydroperoxides catalyzed by metal ions (Ce^{4+} and Fe^{2+}), involving the bimolecular interaction of $LA^{18}O^{18}O^{\bullet}$ via a cyclic mechanism from a linear tetraoxide intermediate. Moreover, the detection of $DPA^{18}O^{16}O$ and $DPA^{16}O^{16}O$ showed additional mechanistic features involved in the hydroperoxide decomposition.

(35) Chan, H. W.-S.; Levett, G.; Matthew, J. A. *Chem. Phys. Lipids* **1979**, *24*, 245−256.
(36) Jones, I. T. N.; Bayes, K. D. *J. Chem. Phys.* **1972**, *2*, 1003−1004.
(37) Darmanyan, A. P.; Foote, C. S. *J. Phys. Chem.* **1993**, *97*, 4573−4576.
(38) Loidl-Stahlhofen, A.; Hannemann, K.; Spiteller, G. *Biochim. Biophys. Acta* **1994**, *1213*, 140−148.
(39) Bartlett, P. D.; Traylor, T. G. *J. Am. Chem. Soc.* **1963**, *85*, 2407−2410.
(40) Traylor, T. G. *J. Am. Chem. Soc.* **1963**, *85*, 2411−2413.
(41) Bennett, J. E.; Howard, J. A. *J. Am. Chem. Soc.* **1973**, *95*, 4008−4010.

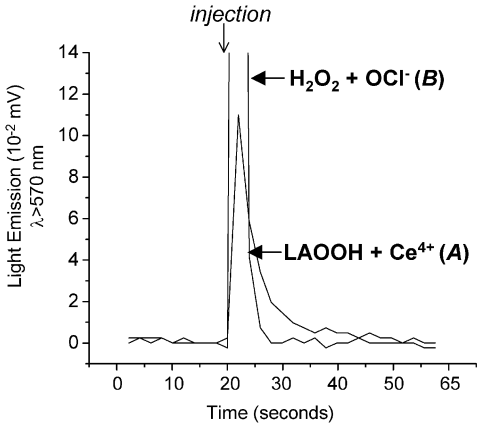


Figure 6. Dimol light emission of singlet oxygen (λ > 570 nm) generated in the reaction of (A) LAOOH/Ce^{4+} and (B) H_2O_2/hypochlorite. The light emission signal was monitored during the injection of 100 mM Ce^{4+} solution in methanol (final concentration: 2 mM) into 5 mL of 0.2 mM LAOOH methanol solution under continuous stirring. The reaction H_2O_2/hypochlorite was performed by injecting 0.4 mL of 0.28 M hypochlorite solution (final concentration: 22.4 mM) into 5 mL of 2.2 mM hydrogen peroxide solution.

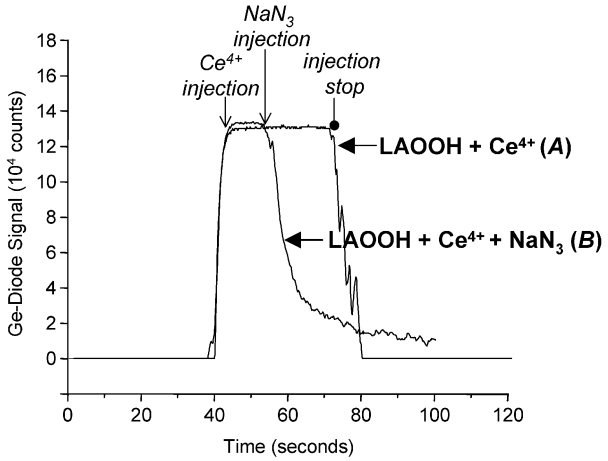


Figure 7. Monomol light emission at λ = 1270 nm of singlet oxygen generated by the reaction of LAOOH/Ce^{4+} (A), and the quenching effect of $^1O_2$ by NaN_3 (B). For the assay, 0.5 mL of 100 mM LAOOH methanol solution (final concentration: 20 mM) was infused into 2.0 mL of 200 mM Ce^{4+} methanol solution at a constant flow rate of 0.7 mL/min under continuous stirring. NaN_3 (final concentration: 10 mM) was injected by another polyethylene tube.

Characterization of Singlet Oxygen Generated in the Reaction of LAOOH and Ceric Ion by Chemiluminescence Measurements. The chemiluminescence (CL) produced by a chemical reaction provides important information about the excited species being generated. The measurement of CL originating from the radiactive transition of $^1O_2$ to its ground state ($^1\Delta_g \rightarrow {}^3\Sigma_g^-$) is an important method for the detection and characterization of $^1O_2$.[42−44] Two types of CL arise from $^1O_2$: dimol light emission (eq 1), which corresponds to the simultaneous deactivation of two molecules of $^1O_2$ to the ground state with photoemission in the visible red region, and monomol light emission (eq 2), which corresponds to the deactivation of one molecule of $^1O_2$ to the ground state with photoemission in the near-IR at 1270 nm.

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

$$O_2(^1\Delta_g) \rightarrow O_2(^3\Sigma_g^-) + h\nu \quad (\lambda = 1270 \text{ nm}) \quad (2)$$

The CL arising from the reaction of LAOOH and Ce^{4+} was recorded in the visible red region (λ > 570 nm) (Figure 6) and in the near-IR region (λ = 1270 nm) (Figure 7). As shown in Figure 6 (trace A), an injection of Ce^{4+} into a solution of LAOOH produced an intense light in the red spectral region. For comparative purposes, the reaction of H_2O_2 and hypochlorite (eq 3) was used here as an authentic source of $^1O_2$.[45]

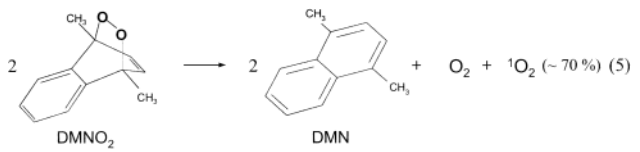
$$H_2O_2 + OCl^- \rightarrow H_2O + Cl^- + {}^1O_2 \quad (\sim 100\%) \quad (3)$$

Figure 7 shows the CL signal recorded in the near-IR region using the Ge-Diode detector. An intense emission signal was observed upon injection of Ce^{4+} into the LAOOH solution, indicating that $^1O_2$ was generated (Figure 7, trace A). For further characterization of $^1O_2$, the effect of azide, a known physical quencher of $^1O_2$, was tested. As expected, the injection of azide into the LAOOH and Ce^{4+} mixture effectively quenched the germanium diode signal (Figure 7, trace B).

In addition to the direct kinetic detection of the monomol emission of $^1O_2$ at 1270 nm, further direct evidence of $^1O_2$ generation was obtained by measuring the infrared light emission spectrum produced in the LAOOH and Ce^{4+} reaction. Figure 8 shows the characteristic monomol light emission spectra of $^1O_2$ ($^1\Delta_g \rightarrow {}^3\Sigma_g^-$), with maximum intensity at 1270 nm, obtained for the LAOOH and Ce^{4+} reaction (Figure 8A). For comparison, we recorded the infrared light emission spectrum of $^1O_2$ generated by H_2O_2/hypochlorite (Figure 8B), H_2O_2/molybdate (Figure 8C), and 1,4-dimethylnaphthalene endoperoxide (DMNO_2) (Figure 8D). As mentioned earlier, the reaction of H_2O_2/hypochlorite (eq 3) is a well-known source of $^1O_2$, which yields $^1O_2$ in almost stoichiometric amounts. Disproportionation of H_2O_2 catalyzed by molybdate ions is known to generate a huge flux of $^1O_2$ in aqueous solutions at room temperature (eq 4).[46]

$$2H_2O_2 \xrightarrow{MoO_4^{2-}} 2H_2O + {}^1O_2 \quad (\sim 50\%) \quad (4)$$

Thermolysis of DMNO_2 (eq 5), generating the dimethylnaphthalene (DMN) and the $^1O_2$, is temperature-dependent.[47−49] At a fixed temperature of 37 °C, DMNO_2 releases 88 μM/min of $^1O_2$ in a constant flux. As expected, an emission maximum at 1270 nm was observed in all of the systems tested, which is an unambiguous proof of $^1O_2$ generated in the LAOOH and Ce^{4+} reaction.



(42) Khan, A. U.; Kasha, M. *J. Am. Chem. Soc.* **1970**, *92*, 3293−3300.
(43) Krasnovsky, A. A. *Photochem. Photobiol.* **1979**, *29*, 29−36.
(44) Kasha, M.; Khan, A. U. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6047.
(45) Khan, A. U.; Kasha, M. *J. Chem. Phys.* **1963**, *39*, 2105.
(46) Aubry, J.-M.; Cazin B. *Inorg. Chem.* **1988**, *27*, 2013.
(47) Di Mascio, P.; Bechara, E. J. H.; Rubim, J. C. *Appl. Spectrosc.* **1992**, *46*, 236−239.
(48) Briviba, K.; Saha-Möller, C. R.; Adam, W.; Sies, H. *Biochem. Mol. Biol. Int.* **1996**, *38*, 647−651.

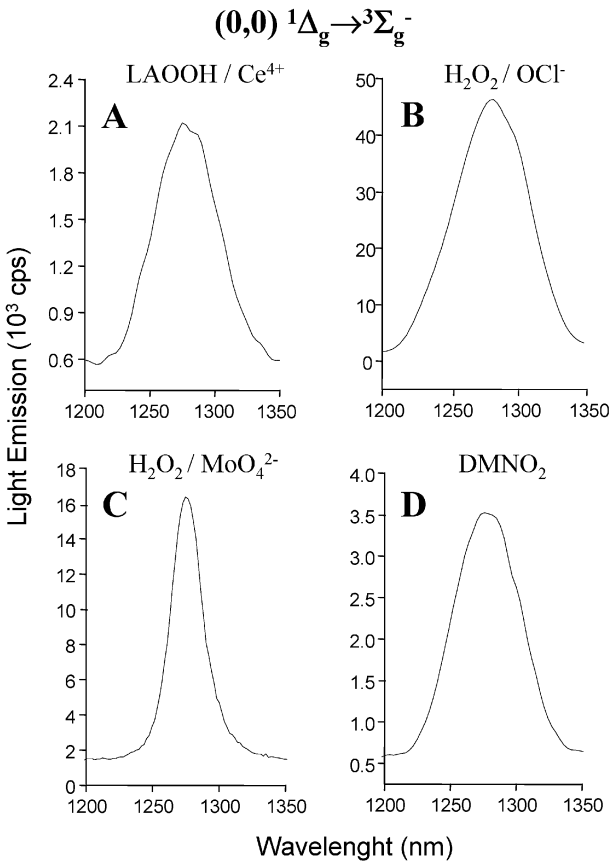


Figure 8. Monomol light emission spectra of singlet oxygen ($^1\Delta_g \rightarrow {}^3\Sigma_g^-$) obtained in the reaction of (A) LAOOH/Ce^{4+}; (B) H_2O_2/OCl^-; (C) H_2O_2/MoO_4^{2-}; and (D) $DMNO_2$. LAOOH/Ce^{4+} reaction was recorded during the injection of 200 mM of Ce^{4+} (final concentration: 40 mM) into 1.8 mL of 100 mM LAOOH methanol solution at a flow rate of 0.7 mL/min. H_2O_2/OCl^- reaction: 2 mL of 0.28 M hypochlorite was injected into 1 mL of 3 M H_2O_2 (final concentration: 1 M) at a flux rate of 1.4 mL/min. H_2O_2/MoO_4^{2-} reaction: 0.1 mL of 0.5 M MoO_4^{2-} (final concentration: 25 mM) was added to 1.9 mL of 10 M H_2O_2 with the reaction proceeding autocatalytically. $DMNO_2$: 15 mM in chloroform was incubated at 37 °C. For equipment conditions, see the Experimental Section.

Conclusions

Taken together, the results discussed herein provide direct evidence of the generation of $^1O_2$ during the decomposition of LAOOH catalyzed by metal ions (Ce^{4+} and Fe^{2+}). Detection of ^{18}[$^1O_2$] in the LA^{18}O^{18}OH and Ce^{4+} reaction serves as strong supporting evidence for Russell's mechanism, in which two peroxyl radicals combine via an intermediate tetraoxide, generating an alcohol, a ketone, and singlet oxygen. The emission of dimol light in the red spectral region ($\lambda > 570$ nm) and the emission of monomol light in the near-infrared region ($\lambda = 1270$ nm), accompanied by the quenching effect of sodium azide, are indications of molecular oxygen in the excited singlet state. Moreover, the emission of $^1O_2$ dimol and monomol light with a characteristic intense band at 1270 nm unequivocally demonstrates the generation of oxygen in the excited singlet state.

Considering that lipid hydroperoxides generate easily in cell membranes, these results point to a potential role of $^1O_2$ in the cytotoxicity mechanism of LAOOH. Taking into account the longer half-lifetime of peroxyl radical (7 s) as compared to those of other reactive oxygen species (LAO$^\bullet$, LA$^\bullet$, $O_2^{\bullet-}$, $^1O_2$, HO$^\bullet$ are 1×10^{-7}, 1×10^{-9}, 1×10^{-6}, 1×10^{-7}, 1×10^{-10} s, respectively),[50] this allows for the migration of fatty acid peroxyl radicals to other sites, where it can combine with another peroxyl radical and generate $^1O_2$.

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Supporting Information Available: Electrospray ionization mass spectra of LA^{18}O^{16}OH, the exchange product of LA^{18}O^{18}OH with water; detection of DPA endoperoxide ($DPAO_2$) formed in the incubation of LA^{18}O^{18}OH with Fe^{2+}; analysis of the labeled ketone (LA^{18}O) and the alcohol (LA^{18}OH) after the reaction of LA^{18}O^{18}OH with Ce^{4+} by HPLC-APCI-MS (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(49) Pierlot, C.; Aubry, J. M.; Briviba, K.; Sies, H.; Di Mascio, P. *Methods Enzymol.* **2000**, *319*, 3−20.
(50) Pryor, W. A. In *Free Radical in Molecular Biology, Aging, and Disease*; Armstrong, D., Sohal, R. S., Cutler, R. G., Slater, T. F., Eds.; Raven Press: New York, 1984; pp 13−41.