Direct Evidence of Singlet Molecular Oxygen [O₂ (¹Δg)] Production in the Reaction of Linoleic Acid Hydroperoxide with Peroxynitrite

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Abstract: Peroxynitrite (ONOO⁻), a biologically active species, can induce lipid peroxidation in biological membranes, thereby leading to the formation of various hydroperoxides. We report herein on the formation of singlet molecular oxygen [O₂ (¹Δg)] in the reaction of peroxynitrite with linoleic acid hydroperoxide (LAOOH) or ¹⁸O-labeled LAOOH. The formation of O₂ (¹Δg) was characterized by (i) dimol light emission in the red spectral region ($\lambda > 570$ nm) using a red-sensitive photomultiplier; (ii) monomol light emission in the near-infrared region ($\lambda = 1270$ nm) with a liquid nitrogen-cooled germanium diode or a photomultiplier coupled to a monochromator; (iii) the enhacing effect of deuterium oxide on chemiluminescence intensity, as well as the quenching effect of sodium azide; and (iv) chemical trapping of O₂ (¹Δg) or ¹⁸O-labeled O₂ (¹Δg) with the 9,10-diphenylanthracene (DPA) and detection of the corresponding DPAO₂ or ¹⁸O-labeled DPA endoperoxide by HPLC coupled to tandem mass spectrometry. Moreover, the presence of O₂ (¹Δg) was unequivocally demonstrated by a direct spectral characterization of the near-infrared light emission attributed to the transition of O₂ (¹Δg) to the triplet ground state. For the sake of comparison, O₂ (¹Δg) deriving from the thermolysis of the endoperoxide of 1,4-dimethylnaphthalene or from the H₂O₂/hypochlorite and H₂O₂/molybdate systems were also monitored. These novel observations identified the generation of O₂ (¹Δg) in the reaction of LAOOH with peroxynitrite, suggesting a potential O₂ (¹Δg)-dependent mechanism that contributes to cytotoxicity mediated by lipid hydroperoxides and peroxynitrite reactions in biological systems.

Introduction

Lipid peroxidation of polyunsaturated fatty acids leads to a complex mixture of hydroperoxides.[1,2] Attention has focused on this process because of its role in destructive biological processes. The oxidation of lipids has been implicated in modifications of membrane structure, DNA damage and protein modification.[3–6] It is also increasingly recognized that lipid peroxidation is associated with the mechanism of tumor initiation,[7–10] deposition of arterial plaque,[11,12] radiation damage,[13,14] and aging.[15,16]

Chemiluminescence (CL) arising from lipid peroxidation has been used as a sensitive detector of oxidative stress both in vitro and in vivo.[17,18] Several authors have attributed ultraweak CL associated with lipid peroxidation to the radiative deactivation of singlet oxygen [O₂ (¹Δg)] and to triplet-excited carbonyls.[19–21] It has been proposed that the latter emitters arise from the thermolysis of dioxetane intermediates[22] and annihilation of alkoxyl,[23] as well as peroxyl radicals.[24–27] Following the suggestion of Russell,[24] Howard and Ingold[25] found that the self-reaction of peroxyl radicals generates O₂ (¹Δg). Russell

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Scheme 1. Proposed Russell Mechanism for the Self-Reaction of Linoleic Acid (LA) Peroxyl Radicals (LAOO•) Generating the Tetraoxide Intermediate (LAOOOOLA) and the Corresponding Products: (**A**) Alcohol (LAOH), Ketone (LAO) and O_2 ($^1\Delta_g$); or (**B**) Alcohol (LAOH), Excited Ketone (LAO*) and O_2 ($^3\Sigma_g^-$)
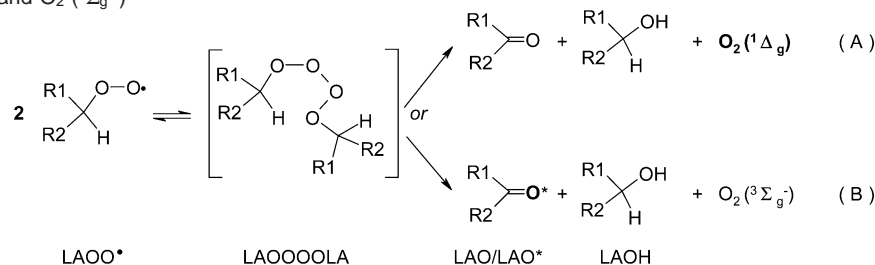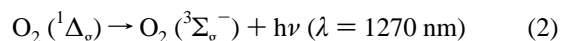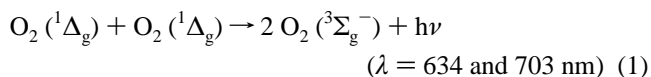

proposed the formation of a cyclic mechanism from a linear tetraoxide intermediate that decomposes to give an alcohol, ketone and molecular oxygen (Scheme 1). It has been postulated that this reaction may generate either an electronically excited oxygen molecule (Scheme 1A) or an electronically excited ketone (Scheme 1B). Indeed, O_2 ($^1\Delta_g$) and triplet carbonyls have been identified as the CL emitters in the ultra-weak CL associated with lipid peroxidation in biological system.[28,29] Niu and Mendenhall reported that the yields of $^1O_2$, in the case of simple alkylhydroperoxides, ranged from 3.9 to 14.0%.[30] By 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 O_2 ($^1\Delta_g$).[31]

Singlet oxygen displays considerable reactivity toward electron-rich organic molecules including, among others, the guanine moiety of DNA.[32,33] Evidence has accumulated indicating that O_2 ($^1\Delta_g$) is implicated in the genotoxic effect of the UVA component of solar radiation and is likely to play an important role in the cell signaling cascade[34] and induction of gene expression.[35] Various authors have investigated the generation of O_2 ($^1\Delta_g$) in biological systems, which has been proposed to occur by type II photosensitization mechanism[36] and enzymatic processes of peroxidases and oxidases.[37,38] Other chemical reactions are also able to produce O_2 ($^1\Delta_g$), such as the reaction of hydrogen peroxide with hypochlorite[39] and the reaction of ONOO$^-$ with hydrogen peroxide[40] or *tert*-butyl hydroperoxide.[41]

Peroxynitrite (oxoperoxonitrate (1$^-$), ONOO$^-$) and its conjugated acid, peroxynitrous acid (ONOOH, pK_a = 6.8), are strong oxidants of biological importance produced by the reaction of the superoxide anion radical and nitrogen monoxide.[42,43] Peroxynitrite reacts rapidly ($k = 10^3$–10^6 M^{-1} s^{-1}) with a number of biological targets, including lipids, thiols, amino

acid residues, and DNA bases.[44–46] Among biomolecules, lipids containing polyunsaturated fatty acids are key targets of peroxynitrite oxidation.[47–49] Studies in vitro have shown that the reaction of peroxynitrite with pure lipids generates nitrated oxidized derivatives[50] as well as several lipid oxidation products, including lipid peroxides such as linoleic acid hydroperoxide.[47] It is known that one-electron oxidation of lipid hydroperoxides mediated by strong oxidants generates peroxyl radicals. We postulate, now, that the reaction of lipid hydroperoxides with peroxynitrite can also promote the generation of peroxyl radicals, thus generating O_2 ($^1\Delta_g$) as an intermediate oxidant by following the mechanism proposed by Russell.

For this purpose, we study here the chemiluminescence-generating reaction of linoleic acid hydroperoxides (LAOOH) or ^{18}O-labeled LAOOH (LA$^{18}O^{18}$OH) with ONOO$^-$, using (i) CL measurement of the dimol and monomol light emission in the visible ($\lambda > 570$ nm) and infrared ($\lambda = 1270$ nm) spectral region (eq 1 and 2, respectively); (ii) the enhanced effect of D_2O and sodium azide quenching on the CL; (iii) the near-infrared emission spectrum characteristic of O_2 ($^1\Delta_g$); and (iv) the chemical trap of O_2 ($^1\Delta_g$) by the 9,10-diphenylanthracene (DPA) and quantification and detection of the corresponding DPAO$_2$ or ^{18}O-labeled DPA endoperoxide (DPA$^{18}O^{18}O$) respectively (eq 3 and 7), to provide additional evidence for the formation of O_2 ($^1\Delta_g$) in this system

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

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

$$DPA + O_2 \, (^1\Delta_g) \rightarrow DPAO_2 \quad (3)$$

Materials and Methods

Chemicals. Peroxynitrite was synthesized from sodium nitrite (0.6 M) and hydrogen peroxide (0.65 M) in a quenched-flow reactor;[46,51]

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excess hydrogen peroxide was used to minimize nitrite contamination.[52] To eliminate excess hydrogen peroxide, the ONOO$^-$ was treated with manganese dioxide. The concentration of the ONOO$^-$ stock solutions was determined spectrophotometrically at 302 nm with an extinction coefficient of 1670 M^{-1} cm^{-1}.[53] For the experiments, a solution of 100 mM ONOO$^-$ was prepared in 0.1 M NaOH or D$_2$O immediately prior to use. The endoperoxide of 1,4-dimethylnaphthalene (DMNO$_2$) was synthesized and used according to a method described previously.[54] The endoperoxide of 9,10-diphenylanthracene (DPAO$_2$) was prepared by photosensitized oxidation.[55] The 9,10-diphenylanthracene (DPA) was from Aldrich (Steinheim, Germany). Linoleic acid (LA) and all the other chemicals were from Sigma (St.Louis, MO) and the solvents were from Merck (Darmstadt, Germany). Sodium hypochlorite was analyzed spectrophotometrically in an alkaline solution (0.1 M NaOH) at 292 nm with an extinction coefficient of 350 M^{-1} cm^{-1}.[56] All the solutions were prepared with distilled water purified with a Millipore Milli-Q system (Bedford, MA).

Linoleic Acid Hydroperoxide Synthesis and HPLC/Mass Spectrometry Analysis. Linoleic acid hydroperoxides (LAOOH) were synthesized by photooxidation using methylene blue as a sensitizer. Typically, 1 g of linoleic acid was dissolved in 50 mL of chloroform containing 0.2 mM of methylene blue and irradiated with a tungsten lamp (500 W) for 5 h. Irradiation was conducted in an ice-bath under a continuous flux of oxygen. The methylene blue was removed and LAOOH separated by silica gel column chromatography, following the procedure described by Kühn.[57] Briefly, the products were placed in the column and separated by a discontinuous gradient of *n*-hexane: diethyl ether from 9:1 to 5:5 (v/v). The concentration of LAOOH was determined spectrophotometrically at 234 nm ($\epsilon = 25 \times 10^3$ M^{-1} cm^{-1}),[58] considering that 60% of the hydroperoxides contain conjugated diene.[1,2]

LAOOH was analyzed by HPLC electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) in the negative ion mode. LAOOH was separated in an LC-18 column (Supelco, 150 × 4.6 mm, 5 μm) and eluted for 10 min with a linear gradient of 75% to 100% acetonitrile. An ESI/APCI-MS analysis was made using a Platform II mass spectrometer (Micromass, Altricham, U.K.). 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 l/h and 15 l/h, respectively. The cone voltage was set to 20 V. Capillary potential and high electrode potential were set to 3.0 kV and 0.5 kV, respectively. For APCI-MS analysis the probe temperature was set to 250 °C. Full scan data were acquired over a mass range of 100−400 *m/z*. 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.).

Synthesis of ^{18}O-Labeled Linoleic Acid Hydroperoxide (LA^{18}O^{18}OH). LA^{18}O^{18}OH was prepared 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. Then, 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 5 times to ensure complete removal of ^{16}O$_2$. Thereafter, the whole system

was connected to an ^{18}O$_2$ gas cylinder under 0.5 atm. The mixture was irradiated with two tungsten lamps (500 W) for 5 h and stirred continuously. Characterization and purity of LA^{18}O^{18}OH was performed by MS as described above.

Dimol Light Emission of Singlet Oxygen. Low-level CL was measured with a single-photon counting system, described elsewhere,[59] equipped with a red-sensitive photomultiplier cooled to −20 °C by a thermoelectric cooler. The potential applied to the photomultiplier was −1.2 kV. The phototube output was connected to an amplifier discriminator (Model 1121, Princeton Instruments, NJ) and to the computer for data acquisition. Selective light emission at wavelengths of $\lambda > 570$ nm was obtained using a cutoff filter (Melles Griot visible filters, 03IFS006) placed between the cuvette and the photomultiplier tube. Sample solutions were poured into a thermostat-equipped glass cuvette (35 mm × 6 mm × 55 mm) with mirrored walls at 37 °C. Typically, the reactant was injected into the solution contained in the cuvette using a syringe injection pump (Syringe Pump Model 22, Harvard Apparatus, MA) at a controlled flow rate.

Monomol Light Emission of Singlet Oxygen. The infrared photoemission of O$_2$ ($^1\Delta_g$) at $\lambda = 1270$ nm was measured with a liquid nitrogen-cooled germanium photodetector (Ge-Diode, Model EI-L, Edinburgh Analytical Instruments, Livingston, UK) or a special photocounting apparatus, which is described in the next method. The Ge-Diode detector, which is sensitive in the spectral region from 800 to 1800 nm, has a detector area of 0.25 cm^2 and a sapphire window, as described elsewhere.[60] A silicon filter and a band-pass filter at $\lambda = 1270$ nm with a 10 nm half-bandwidth were used (Spectrogon U.K. Ltd., Glenrothes, UK). A muon filter (Model MF-1) was used to filter cosmic and ionizing radiations. The power was provided by a bias power supply (Model PS-1) set to 160 V. The signal was processed through an optical chopper (Bentham 218, Bentham Instruments, UK) with a frequency of 125 Hz and a Lock-in Amplifier (Bentham 225, Bentham Instruments, UK). Emission signal data were acquired using the F-900 ver. 6.22 software program (Edinburgh Analytical Instruments, Livingston, UK). For the experiments, the photodiode detector was filled with liquid nitrogen and left to equilibrate for 2 h before use. The assay was conducted in a thermostated quartz cuvette (10 × 10 × 30 mm) under continuous stirring (Cuv-o-stir, model 333, Hellma. Mülheim, Germany) at 37 °C. After recording the baseline with the assay solvent, the reactant was injected into the cuvette by means of a syringe injection pump, as describe earlier herein. Monomol light emissions were also measured using a monochromator fixed at $\lambda = 1270$ nm, with the equipment described in the next paragraph.

Spectral Measurements of Singlet Oxygen in the Near-Infrared Region. The singlet oxygen monomol light emission spectrum was measured with a special photocounting apparatus developed in our laboratory, equipped with a monochromator capable of selecting emissions in the near-infrared region (800−1400 nm). The apparatus consists 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, UK) equipped with a diffraction grating (Type G306R1u0, Bentham Instruments, UK) capable of selecting wavelengths in the infrared region. The phototube output was connected to the computer and the signal acquired. The monochromator was controlled and the data acquired using the F-900 ver. 6.22 software program (Edinburgh Analytical Instruments, Livingston, U. K.). Experiments were conducted in quartz cuvetts, as described above. Typically, 3−5 scans in the range of 1200−1350 nm were recorded and averaged to yield the spectrum.

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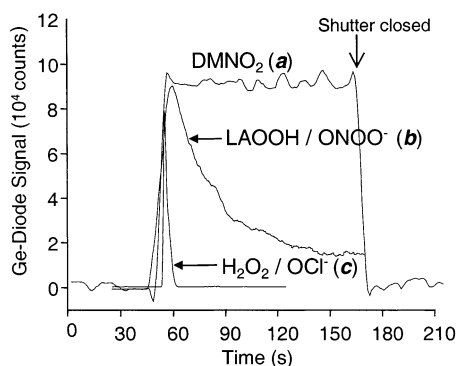(58) Mulliez, E.; Leblanc, J. P.; Girerd, J. J.; Rigaud, M.; Chottard, J. C. *Biochim. Biophys. Acta* **1987**, *916*, 13−23.

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Chemical Trapping of Singlet Oxygen and HPLC/Mass Spectrometry Analysis. To chemically detect O_2 ($^1\Delta_g$), we used 9,10-diphenylanthracene (DPA). This method is based on the rapid and specific reaction of O_2 ($^1\Delta_g$) with DPA ($kr = 1.3 \times 10^6$ M^{-1} s^{-1}) (eq 7), which forms a stable DPA endoperoxide ($DPAO_2$).[61] LAOOH (final concentration, 50 mM) was incubated with $ONOO^-$ (final concentration, 50 mM) in a biphasic system composed of chloroform and D_2O in the presence of 60 mM DPA and protected from light. For purposes of comparison, 60 mM DPA was incubated with 5 mM $DMNO_2$, a known generator of O_2 ($^1\Delta_g$). $DPAO_2$ was separated from DPA by HPLC, using a system consisting of LC 10ADVP pumps connected to an automatic SIL-10ADVP injector (Shimadzu, Tokyo, Japan). For analytical purposes, the system was equipped with a 150×4.6 ID mm (particle size 5 μm) Supelco C18 reverse-phase column and a 20×2.1 mm (particle size 5 μm) Supelco C18 guard precolumn (Supelco, Bellefonte, PA), connected to a Shimadzu SPD-M10AVVP diode-array. Calibration curves for DPA and $DPAO_2$ were drawn in the range of 0.04–0.75 mM and 0.001–0.04 mM, respectively. After incubation, the samples were diluted 1:125 and 30 μL was injected into the HPLC. Solvent A and B were water and acetonitrile, respectively. The compounds were eluted using an increasing linear gradient of B from 65% to 100% during the first 30 min, keeping it constant for 10 min and returning to 65% in 5 min. The flow-rate was 0.6 mL/min. The data were acquired using the Shimadzu CLASS–VP, version 5.3 software program. Atmospheric pressure chemical ionization mass spectrometry analyses in the positive ion mode were made with a Platform II mass spectrometer (Micromass, Altricham, U. K.). The probe and source temperature of the mass spectrometer were maintained at 300 °C and 150 °C, respectively. $DPAO_2$ was monitored at a cone voltage of 15 V and the collision energy set to 15 eV. In the same run, the internal standard 9,10-dibromoanthracene was monitored at a cone voltage of 20 V and a collision energy of 45 eV. Full-scan data were acquired over a mass range of 100–500 m/z. Quantification was done in the multiple reaction monitoring mode (MRM) and the data were processed and transformed into molecular mass values on a mass scale using the aforementioned software program.

Results and Discussion

Light Emission Detection of Singlet Oxygen. The measurement of chemiluminescence (CL) originated by the radiative transition of O_2 ($^1\Delta_g$) to its ground state is an important method for the detection and characterization of O_2 ($^1\Delta_g$). Two types of CL derive from O_2 ($^1\Delta_g$): dimol emission and monomol emission. In this experiment, the mixture of LAOOH and $ONOO^-$ produced a rapid increase in light emission in the wavelength corresponding to monomol and dimol emission of O_2 ($^1\Delta_g$) (see eq 1 and 2). As illustrated in Figure 1, trace b, the injection of $ONOO^-$ (5.2 mM concentration at CL maximum intensity) into a solution of 10 mM LAOOH in methanol produced a strong light emission in the red spectral region ($\lambda > 570$ nm). For comparative purposes, O_2 ($^1\Delta_g$) chemically generated in the thermodissociation of 1,4-dimethylnaphthalene endoperoxide ($DMNO_2$) (Figure 1, trace a) or in the oxidation of H_2O_2 by hypochlorite (Figure 1, trace c) were monitored.

Thermolysis of $DMNO_2$ into the parent hydrocarbon (DMN) and molecular oxygen (eq 4) is temperature-dependent.[61] At a fixed temperature, $DMNO_2$ liberates O_2 ($^1\Delta_g$) at a constant flux, as depicted in Figure 1. In contrast, the yield of O_2 ($^1\Delta_g$) in the H_2O_2/hypochlorite system (eq 5) is almost quantitative but very fast compared with the thermodecomposition of $DMNO_2$ and the reaction of LAOOH and $ONOO^-$. During injection of

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Figure 1. Dimol light emission of O_2 ($^1\Delta_g$) generated in the reaction of LAOOH and peroxynitrite. Lane *a*, thermodissociation of 6 mM $DMNO_2$ in chloroform; Lane *b*, injection of 100 mM $ONOO^-$ (5.2 mM after 18 s of injection) into 4 mL of 10 mM LAOOH in methanol, final pH 9; and Lane *c*, injection of 328 mM hypochlorite (9.2 mM after 10 s of injection) into 4 mL of 20 mM H_2O_2. Injection flux was set to 0.7 mL/min. All solutions were maintained under mixing at 37 °C.

peroxynitrite or LAOOH solution the pH changed from 6 to 9 or 13 to 11, respectively.

(4)

$$H_2O_2 + OCl^- \rightarrow Cl^- + H_2O + O_2\ (^1\Delta_g) \qquad (5)$$
$$(\sim 100\ \%)$$

Using the Ge-Diode detector, the monomol light emission at $\lambda = 1270$ nm was observed in the reaction of LAOOH/peroxynitrite, as shown in Figure 2, trace b. Likewise, the production of O_2 ($^1\Delta_g$) from H_2O_2/hypochlorite and from $DMNO_2$ are shown in traces c and a, respectively. Neither $ONOO^-$ nor LAOOH nor hypochlorite elicited CL when present alone.

The CL's intensity increased with the $ONOO^-$ concentration tested from 10 to 100 mM (data not shown). Singlet oxygen production was calibrated based on the rate of DMN generated from $DMNO_2$, considering that 70% of the oxygen released is in the excited state.[61] At 37 °C, thermodissociation of $DMNO_2$ yielded 88 μM O_2 ($^1\Delta_g$) min^{-1}, so that we roughly estimated a flash of O_2 ($^1\Delta_g$) production of 318 μM O_2 ($^1\Delta_g$) for the reaction LAOOH/peroxynitrite shown in trace b of Figure 2. This corresponds to the generation of about 1% of O_2 ($^1\Delta_g$) from the reaction of equimolar amounts of LAOOH and $ONOO^-$.

To further characterize the generation of O_2 ($^1\Delta_g$) in the reaction of LAOOH and peroxynitrite, the effects of deuterated water and azide on CL intensity were examined using both the Ge-Diode detector (Figure 3A) and the special monochromator

Figure 2. Monomol light emission of O_2 ($^1\Delta_g$) generated in the reaction of LAOOH and peroxynitrite. Lane *a*, thermodissociation of 13 mM DMNO$_2$ in methanol; Lane *b*, injection of 100 mM LAOOH methanol solution (22.5 mM after 50 s of injection) into 2 mL of 100 mM ONOO$^-$, final pH 11; and Lane *c*, injection of 328 mM hypochlorite (8 mM after 4 s of injection) into 2 mL of 20 mM H_2O_2. Injection flux was set to 0.7 mL/min. All solutions were maintained under mixing at 37 °C.
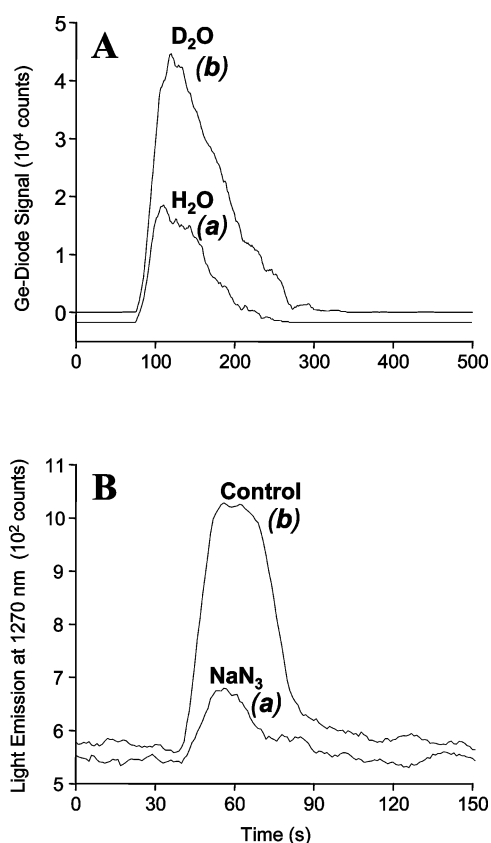
Figure 3. Effect of D_2O and NaN$_3$ on the monomol emission of O_2 ($^1\Delta_g$) generated in the reaction of LAOOH and ONOO$^-$. A, time course of light emission monitored by Ge-Diode detector during injection of 100 mM LAOOH methanol solution (5.5 mM after 10 s of injection) into 2 mL of 100 mM ONOO$^-$ in H_2O (*a*), and 67% D_2O (*b*), final pH 11; and B, time interval of light emission monitored by photomultiplier with a monochromator set to $\lambda = 1270$ nm during injection of 100 mM peroxynitrite in 67% D_2O (5.5 mM after 10 s of injection) into 2 mL of 10 mM LAOOH, final pH 9 (*a*) without NaN$_3$ and (*b*) with 1 mM NaN$_3$. The LAOOH solution was injected at a flow rate of 0.7 mL/min. All solutions were maintained under mixing at 37 °C.

coupled to a highly sensitive photomultiplier (Figure 3B). Upon replacement of H_2O by 67% D_2O, the monomol emission at $\lambda = 1270$ nm was increased (Figure 3A). This is consistent with the fact that the lifetime of O_2 ($^1\Delta_g$) increases in D_2O.[62] In

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addition, sodium azide effectively quenched the CL signal's intensity (Figure 3B, line *b* compared with Figure 3B line *a*), which is a strong indication that O_2 ($^1\Delta_g$) is the emitter.

Considering the fact that peroxynitrite decomposition generates hydroxyl radical (•OH) or "•OH-like species",[63−65] we assessed the possible involvement of these species in the process of O_2 ($^1\Delta_g$) generation. For this purpose, we tested the effect of known •OH radical scavengers, dimethyl sulfoxide (DMSO, 0.2−10% v/v) and mannitol (1 and 5 mM) in the CL signal. In both cases, CL was strongly inhibited. CL decreased by 50% with the addition of 1.5 mM mannitol and 0.5% DMSO. The addition of desferrioxamine, a known metal chelator and scavenger of hydroxyl radicals,[66] also decreased the CL intensity. It should be noted that DMSO and mannitol concentration used here, did not affect the O_2 ($^1\Delta_g$) CL signal's intensity generated from the thermodissociation of DMNO$_2$. Therefore, it is strongly suggested that •OH or "•OH-like" species are involved in the O_2 ($^1\Delta_g$) generating process.

Singlet Oxygen Spectrum. Besides the direct kinetic detection of the monomol emission of O_2 ($^1\Delta_g$) at $\lambda = 1270$ nm, we also recorded the infrared light emission spectrum of O_2 ($^1\Delta_g$) produced by the reaction of LAOOH with peroxynitrite (Figure 4 A). The emission spectra of O_2 ($^1\Delta_g$) generated in the oxidation of H_2O_2 by hypochlorite[39] (eq 5) (Figure 4B), the thermodissociation of DMNO$_2$[54] (eq 4) (Figure 4C), and the disproportionation of H_2O_2 catalyzed by molybdate ions[67] (eq 6) (Figure 4D) were also recorded for purposes of comparison. As expected, an emission maximum at $\lambda = 1270$ nm, characteristic of O_2 ($^1\Delta_g$) monomol emission, was observed in all the systems tested, confirming the generation of O_2 ($^1\Delta_g$) in the reaction of LAOOH and peroxynitrite

$$2\,H_2O_2 \xrightarrow{MoO_4^{2-}} 2\,H_2O + O_2\,(^1\Delta_g) \qquad (6)$$
$$(\sim 50\,\%)$$
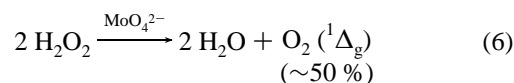
Chemical Trapping of Singlet Oxygen. The O_2 ($^1\Delta_g$) in the reaction of LAOOH and ONOO$^-$ was chemically detected using the O_2 ($^1\Delta_g$) chemical probe DPA (eq 7), which is suitable for reactions in organic phases.[68] The water soluble disodium salt anthracene derivative, anthracene-9,10-diyldiethyl sulfate was recently used to chemically trap O_2 ($^1\Delta_g$).[69] Detection of the anthracene endoperoxide (DPAO$_2$) provided further evidence of the formation of O_2 ($^1\Delta_g$) in this system (Figure 5B*d*). DPA
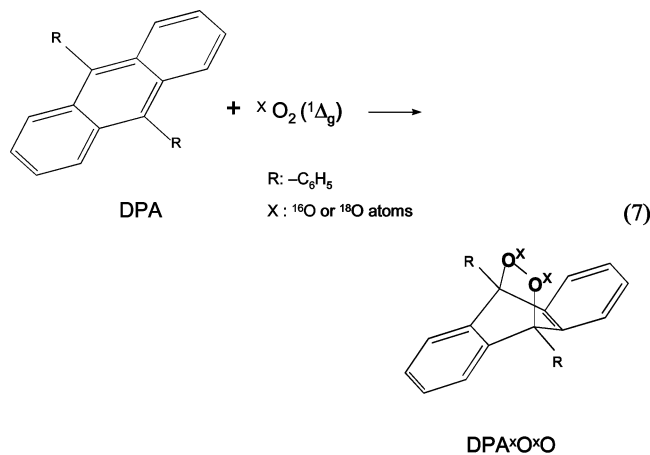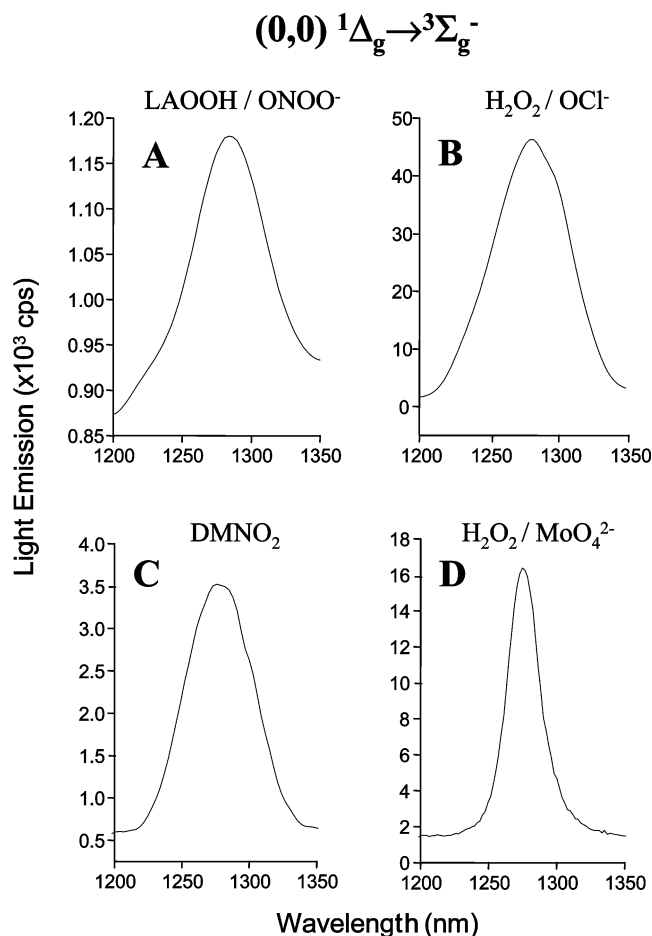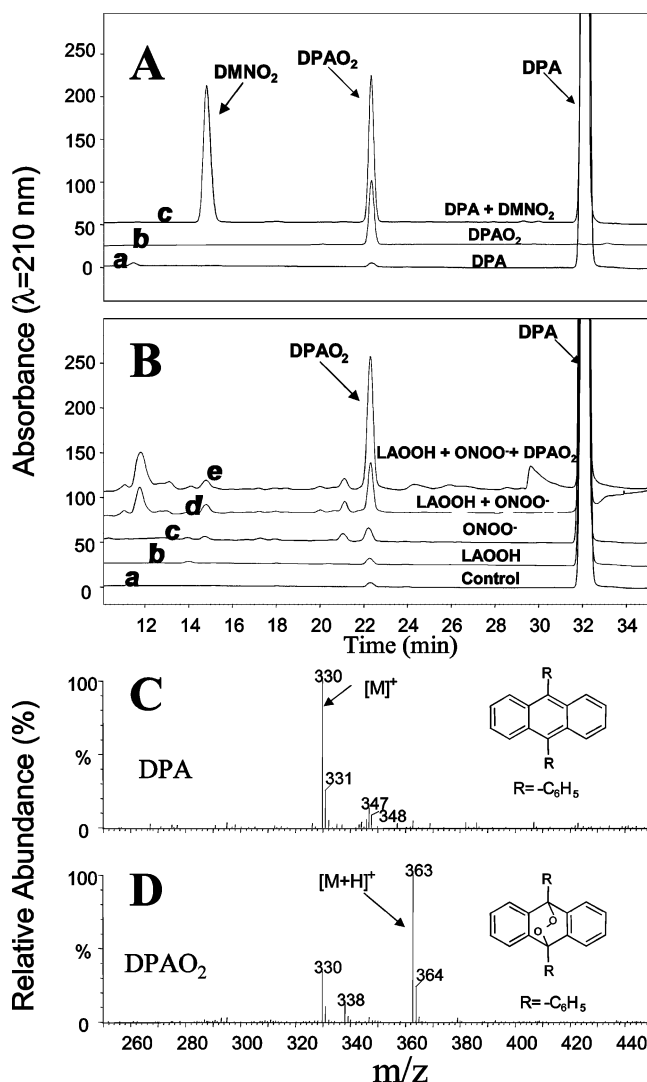
Figure 4. Monomol light emission spectrum of O_2 ($^1\Delta_g$) generated in the reaction of LAOOH and ONOO$^-$ recorded in the near-infrared region between 1200 and 1350 nm. A, LAOOH/ONOO$^-$, 1.0 mL of 100 mM LAOOH in methanol was injected into 2 mL of 100 mM ONOO$^-$ in 67% D$_2$O at a flow rate of 0.7 mL/min at 37 °C; B, H$_2$O$_2$/hypochlorite, 2 mL of 0.3 M hypochlorite was injected into 1 mL of 3 M H$_2$O$_2$ at a flow rate of 1.4 mL/min at room temperature; C, thermodissociation of 15 mM DMNO$_2$ in chloroform at 40 °C; and D, H$_2$O$_2$/molybdate, 1 mL of 0.5 M MoO$_4^{2-}$ solution (0.1 M HCO$_3^-$, 0.1 M CO$_3^{2-}$) was mixed with 1 mL D$_2$O and with 0.1 mL of 10 M H$_2$O$_2$ at room temperature. All solutions were maintained under mixing.

and its endoperoxide (DPAO$_2$) were analyzed by gradient reverse-phase HPLC (Figure 5A) and electrospray ionization tandem mass spectrometry (Figure 5C and 5D). The mass spectrum of DPA recorded in the positive mode exhibits a major [M]$^+$ ion at $m/z = 330$ (Figure 5C), corresponding to the positively charged molecular ion. The spectrum of DPAO$_2$ displays, as expected, an intense [M+H]$^+$ ion at $m/z = 363$ (Figure 5D). The incubation of LAOOH (final concentration, 50 mM) and peroxynitrite (final concentration, 50 mM) in the presence of 60 mM DPA resulted in the formation of DPAO$_2$ (Figure 5B*d*), as proved by co-injection of DPAO$_2$ (Figure 5B*e*) and by its mass spectrum (Figure 5D). For comparison, we show the formation of DPAO$_2$ when 60 mM DPA was reacted with

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Figure 5. Chemical detection of O$_2$ ($^1\Delta_g$) by the formation of DPAO$_2$. A, *a*: DPA 0.48 mM, *b*: DPAO$_2$ 0.012 mM and *c**: 60 mM DPA incubated with 5 mM DMNO$_2$; B, *a**: Control, 60 mM DPA *b**: 60 mM DPA incubated with 50 mM LAOOH, *c**: 60 mM DPA incubated 50 mM ONOO$^-$, *d**: 60 mM DPA incubated with 50 mM LAOOH and 50 mM ONOO$^-$, *e**: aliquot of *d* spiked with pure DPAO$_2$; C, Mass spectra of DPA, and D, Mass spectra of DPAO$_2$. * Incubated samples were diluted 125 times before analysis by HPLC. Conditions: all solutions were incubated under mixing at 37 °C for 1 h, in a biphasic system composed of chloroform and D$_2$O (1:1, v/v) and protected from light.

O$_2$ ($^1\Delta_g$) generated by thermal decomposition of 5 mM DMNO$_2$ (Figure 5A*c*). In contrast, neither LAOOH nor ONOO$^-$ alone led to the formation of DPAO$_2$ (Figure 5B*b* and 5B*c*, respectively), as compared to the control (DPA) (Figure 5B*a*). Quantitative measurements of DPAO$_2$ in the reaction of LAOOH and ONOO$^-$ (Figure 6) yielded an estimated O$_2$ ($^1\Delta_g$) 1.2%, which is close to the values found by photoemission measurements.

Characterization of Labeled Singlet Oxygen Generated in the Reaction of LA^{18}O^{18}OH and Peroxynitrite, using 9,10-Diphenylanthracene. Figure 7 shows the mass spectra of both LAOOH (Figure 7A) and LA^{18}O^{18}OH (Figure 7B). The mass spectrum of LAOOH displays a molecular ion [M−H]$^-$ at m/z 311 and the mass spectrum of LA^{18}O^{18}OH exhibits an

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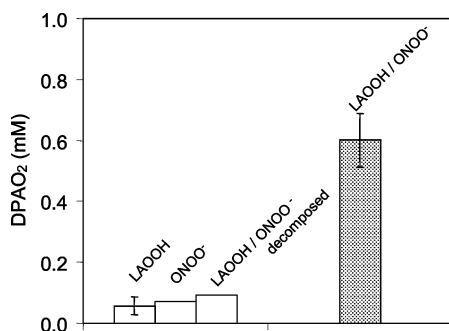
Figure 6. Amount of DPAO$_2$ detected in the reaction of LAOOH and ONOO$^-$ in a biphasic system. LAOOH (50 mM) in chloroform and D$_2$O (1:1, v/v) was incubated with 50 mM peroxynitrite in the presence of 60 mM DPA under mixing at 37 °C for 1 h. An aliquot of the chloroform phase was dried under a nitrogen stream, dissolved in acetone: acetonitrile (1:5, v/v) solution and analyzed by HPLC.

intense ion peak at *m/z* 315, corresponding to the labeled hydroperoxide molecular ion [M− H]$^-$. The increase by four atomic mass units (amu) for the molecular ion of LA^{18}O^{18}OH indicates that two atoms of ^{18}O were successfully incorporated into the hydroperoxide. The detection of the ion peak at *m/z* 311 (LA^{16}O^{16}OH) demonstrates that LA^{18}O^{18}OH is 90% pure.

Labeled O$_2$ ($^1\Delta_g$) (^{18}[O$_2$ ($^1\Delta_g$)]) generated through the reaction of LA^{18}O^{18}OH and peroxynitrite was chemically trapped with 9,10-diphenylanthracene (DPA) generating the corresponding labeled endoperoxide (DPA^{18}O^{18}O) (eq 7) and detected by HPLC−MS/MS in the MRM mode (Figure 7C). The tandem mass spectra (MS2 full scan) of the labeled endoperoxide was recorded, by selecting in the first analyzer (MS1) the molecular ion of DPA^{18}O^{18}O at *m/z* 367 (Figure 7D). As expected the endoperoxide showed an intense fragment ion at *m/z* 330 corresponding to the loss of one labeled oxygen molecule.

The generation of DPA^{18}O^{18}O in the incubation of LA^{18}O^{18}OH with peroxynitrite clearly demonstrated the formation of ^{18}[O$_2$ ($^1\Delta_g$)] that probably occurs via the combination of ^{18}O-labeled peroxyl radical of linoleic acid (LA^{18}O^{18}O$^\bullet$). According to Russell's mechanism, the bimolecular reaction of LA^{18}O^{18}O$^\bullet$ would generate O$_2$ ($^1\Delta_g$) containing 18-oxygen atoms. Besides the oxygen production, the decomposition products arising from the Russell mechanism, ketone and alcohol were also characterized by HPLC mass spectrometry (not shown).
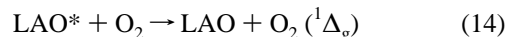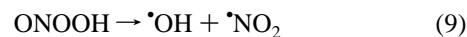
Oxygen-18 tracer studies with mass spectrometry[70,71] and electron spin resonance spectroscopy,[72] using various ^{18}O-labeled and unlabeled peroxyl radicals containing or not the α-hydrogen, demonstrated that evolution of oxygen occurs during the interaction between the radicals via head to head mechanism. 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 (O$_2$ ($^1\Delta_g$), 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.

Singlet Oxygen Formation. Peroxynitrite (ONOO$^-$) and its conjugated acid (ONOOH, pK_a = 6.8) are strong oxidizing agents. Peroxynitrous acid is a powerful oxidizing agent with estimated one and two-electron reduction potentials of $E^{\circ\prime}$ (ONOOH, H$^+$/$^\bullet$NO$_2$, H$_2$O) = 1.6−1.7 V and $E^{\circ\prime}$(ONOOH, H$^+$/

NO$_2^-$, H$_2$O) = 1.3−1.4 V, respectively.[73] In addition, it is reported that, upon protonation, ONOO$^-$ can undergo decomposition via homolytic O−O cleavage to generate nitrogen dioxide radical ($^\bullet$NO$_2$) and hydroxyl radical ($^\bullet$OH) in approximately 30% yields (eq 8 and 9).[63−65] At physiological conditions where normally the concentration of bicarbonate is high, ONOO$^-$ reacts rapidly with CO$_2$ (*k* = 3 × 10^4 M^{-1} s^{-1}) forming an unstable nitrosoperoxycarbonate anion adduct (ONOOCO$_2^-$), which decomposes giving carbonate radical anion (CO$_3^{\bullet-}$) and $^\bullet$NO$_2$ in approximately 35% yields (eq 10).[51,74−76] All of these radical species generated from peroxynitrite are highly oxidizing agents. Hydroxyl radical is considered to be one of the most powerful oxidants, with E° ($^\bullet$OH, H$^+$/H$_2$O) = 2.31 V at pH 7.0[77] reacting rapidly with most organic compounds (∼10^9 M^{-1} s^{-1}). It is well-known that $^\bullet$OH stimulates lipid peroxidation by H$^\bullet$ abstraction from unsaturated fatty acids. The carbonate radical anion has also a high reduction potential which is close to that of $^\bullet$OH at pH 7.0, E° (CO$_3^{\bullet-}$/CO$_3^{2-}$) = 1.78 V, and therefore being capabable of oxidizing a variety of bimolecules.[78,79] Indeed CO$_3^{\bullet-}$ is reported to react with thiols by one-eletron oxidation mechanism generating thiol-derived radicals.[79] The nitrogen dioxide radical is also a moderately potent oxidant (E°= 0.99 V) capable of reacting by hydrogen atom abstraction as well.[75,78,80] On the other hand, the standard reduction potential calculated for ROO$^\bullet$/ROOH is approximately 1.0 V.[81] Considering this information, it is thermodynamically probable that the reaction of ONOO$^-$ with lipid hydroperoxides generates peroxyl radicals. Therefore, we can expect that LAOO$^\bullet$ are produced by one-electron oxidation of LAOOH mediated by $^\bullet$OH (eq 11) and CO$_3^{\bullet-}$ (eq 12).

The peroxyl radicals formed, combine via a tetraoxide intermediate, as proposed by Russell, which decomposes generating O$_2$ ($^1\Delta_g$) and the corresponding alcohol and a ketone (eq 13 and Scheme 1A). Alternatively, the tetraoxide intermediate can also decompose, generating a triplet ketone in the excited state (LAO*, Scheme 1B), which produces O$_2$ ($^1\Delta_g$) by energy transfer to the ground-state oxygen (eq 14)

$$ONOO^- + H^+ \rightleftharpoons ONOOH \tag{8}$$

$$ONOOH \rightarrow {}^\bullet OH + {}^\bullet NO_2 \tag{9}$$

$$ONOO^- + CO_2 \rightarrow [ONOOCO_2^-] \rightarrow {}^\bullet NO_2 + CO_3^{\bullet-} \tag{10}$$

$$LAOOH + {}^\bullet OH \rightarrow LAOO^\bullet + H_2O \tag{11}$$

$$LAOOH + CO_3^{\bullet-} \rightarrow LAOO^\bullet + CO_3^{2-} + H^+ \tag{12}$$

$$2\,LAOO^\bullet \rightleftharpoons LAOOOOLA \rightarrow LAO + LAOH + O_2\,({}^1\Delta_g) \tag{13}$$

$$LAO^* + O_2 \rightarrow LAO + O_2\,({}^1\Delta_g) \tag{14}$$

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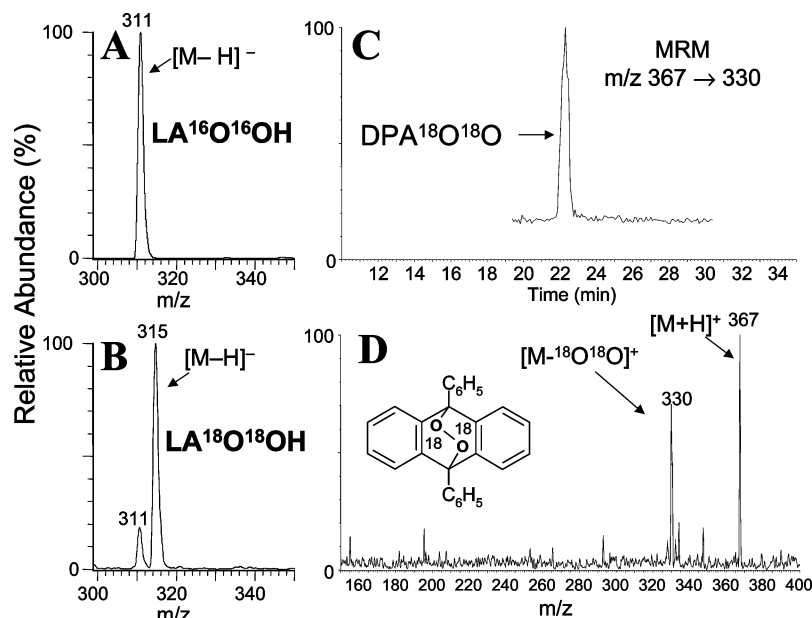
Figure 7. Characterization of labeled O_2 ($^1\Delta_g$) produced in the reaction of $LA^{18}O^{18}OH$ and peroxynitrite, by chemical trapping with DPA. $LA^{18}O^{18}OH$ and ^{18}O-labeled DPA endoperoxide ($DPA^{18}O^{18}O$) were detected by HPLC coupled to tandem mass spectrometry: mass spectrum of (A) the unlabeled $LA^{16}O^{16}OH$ and (B) ^{18}O-labeled LAOOH ($LA^{18}O^{18}OH$); C, chromatogram of the corresponding ^{18}O-labeled DPA endoperoxide by HPLC coupled to tandem mass spectrometry in the MRM mode ($DPA^{18}O^{18}O$, m/z 367→330); and D, MS2 full scan acquired by selecting in the first analyzer the ion with $m/z = 367$. $LA^{18}O^{18}OH$ (100 mM) was injected (final concentration, 13 mM) in 100 mM peroxynitrite and 60 mM of DPA in D_2O:chloroform (1:1, v/v) under mixing at 37 °C, final pH 11.

Conclusions

Taken together, these novel observations serve as important evidence of O_2 ($^1\Delta_g$) production in the reaction of LAOOH and peroxynitrite. Inspired by common chemical features shared by the Russell mechanism[23] and the reaction system studied here, we envisage an O_2 ($^1\Delta_g$) formation mechanism via the intermediate tetraoxide-configuration, involving two molecules of LAOO• (eq 13 or 14). This is an additional reaction involved in the process of lipid peroxidation, providing further insights into the potential involvement of O_2 ($^1\Delta_g$) in oxidative reactions mediated by peroxynitrite in biological systems.