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PII: S0891-5849(20)30058-7

DOI: https://doi.org/10.1016/j.freeradbiomed.2020.03.003

Reference: FRB 14628

To appear in: *Free Radical Biology and Medicine*

Received Date: 9 January 2020

Revised Date: 26 February 2020

Accepted Date: 2 March 2020

Please cite this article as: L.C.P. Gonçalves, Jú. Massari, S. Licciardi, F.M. Prado, E. Linares, A. Klassen, M.F.M. Tavares, O. Augusto, P. Di Mascio, E.J.H. Bechara, Singlet oxygen generation by the reaction of acrolein with peroxynitrite via a 2-hydroxyvinyl radical intermediate, *Free Radical Biology and Medicine* (2020), doi: https://doi.org/10.1016/j.freeradbiomed.2020.03.003.

Singlet oxygen generation by the reaction of acrolein with peroxynitrite via a 2-hydroxyvinyl radical intermediate

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Abstract

Acrolein (2-propenal) is an environmental pollutant, food contaminant, and endogenous toxic by-product formed in the thermal decomposition and peroxidation of lipids, proteins, and carbohydrates. Like other α,β-unsaturated aldehydes, acrolein undergoes Michael addition of nucleophiles such as basic amino acids residues of proteins and nucleobases, triggering aging associated disorders. Here, we show that acrolein is also a potential target of the potent biological oxidant, nitrosating and nitrating agent peroxynitrite. In vitro studies revealed the occurrence of 1,4-addition of peroxynitrite ($k_2 = 6 \times 10^3$ M^{-1} s^{-1}, pH 7.2, 25°C) to acrolein in air-equilibrated phosphate buffer. This is attested by acrolein concentration-dependent oxygen uptake, peroxynitrite consumption, and generation of formaldehyde and glyoxal as final products. These products are predicted to be originated from the Russell termination of $^{\bullet}$OOCH=CH(OH) radical which also includes molecular oxygen at the singlet delta state (O_2 $^1\Delta_g$). Accordingly, EPR spin trapping studies with the 2,6-nitrosobenzene-4-sulfonate ion (DBNBS) revealed a 6-line spectrum attributable to the 2-hydroxyvinyl radical adduct. Singlet oxygen was identified by its characteristic monomolecular IR emission at 1,270 nm in deuterated buffer, which was expectedly quenched upon addition of water and sodium azide. These data represent the first report on singlet oxygen creation from a vinylperoxyl radical, previously reported for alkyl- and formylperoxyl radicals, and may contribute to better understand the adverse acrolein behavior in vivo.

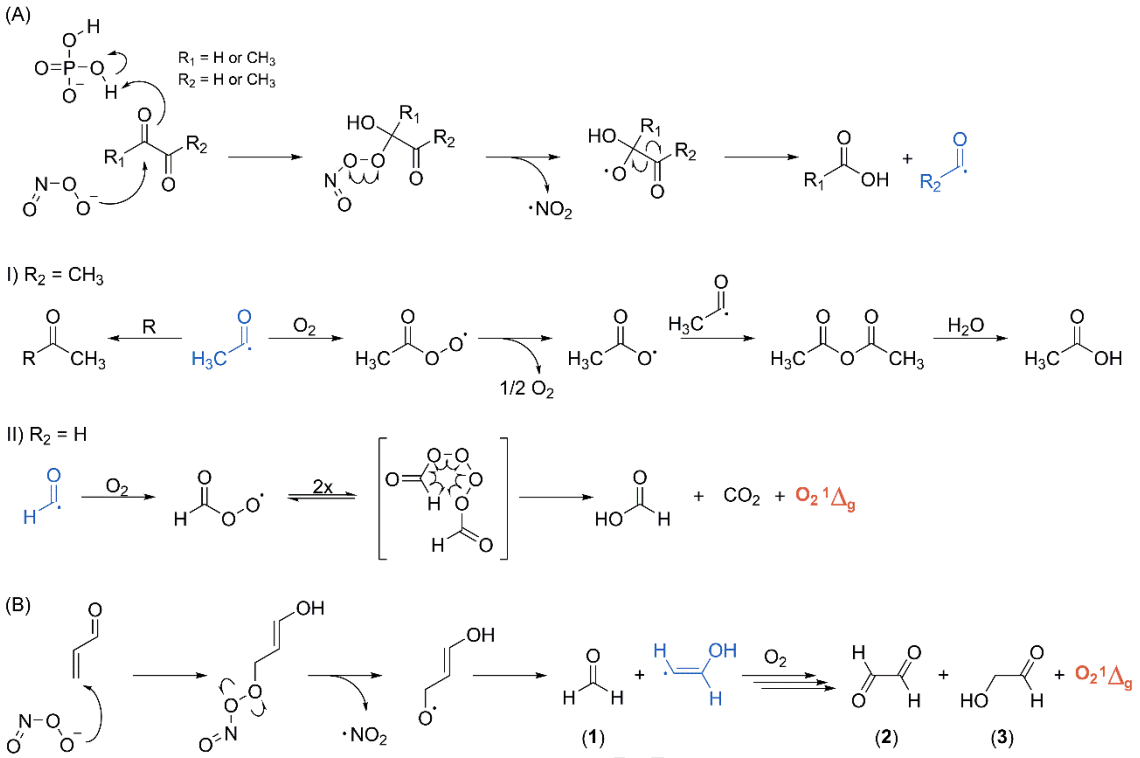
Keywords: acrolein, peroxynitrite, Russell reaction, singlet oxygen, carbonyl stress

Introduction

Short chain aldehydes (*e.g.*, formaldehyde, acetaldehyde, malondialdehyde, *n*-hexanal), 2-alkenals (*e.g.*, acrolein, crotonaldehyde, 4-hydroxy-2-nonenal), and α-dicarbonyls (*e.g.*, glyoxal, methylglyoxal, diacetyl, 4,5-dioxovaleric acid, 3-deoxyglucosone) are highly electrophilic endogenous species generated by the oxidation and thermal dehydration of lipids, proteins, and carbohydrates.[1-8] They can also be acquired by environmental exposure and food ingestion. Once produced in tissues, these reactive carbonyl catabolites undergo nucleophilic additions of the basic centers of protein amino acid residues (Cys, His, Lys, Arg) and DNA bases, leading to covalent modifications, aggregation, denaturation, and even activation of cell signals. When deleterious to the organism, accumulation of these adducts triggers a condition often named "carbonyl stress".[9] Carbonyl adducts have been detected in a plethora of acquired and innate diseases, among others, atherosclerosis, Alzheimer's disease, porphyria diseases, multiple sclerosis, autoimmune diseases, diabetes, food poisoning, and cancer.[10-17]

The peroxynitrite anion formed by the diffusion-controlled reaction ($k_2 \sim 10^{10}$ $M^{-1}s^{-1}$) between the superoxide anion radical ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}NO$) upon oxidative and nitrosative imbalance has been associated to different diseases.[18-22] Peroxynitrite not only acts as a one- and two-electron oxidant, a nitrating and nitrosating agent but it is also a strong nucleophile. Accordingly, the reaction of peroxynitrite with electrophilic and biologically relevant dicarbonyls, such as glyoxal, methylglyoxal and diacetyl, was well established. [23-25] In addition, singlet oxygen (O_2 $^1\Delta_g$), a potent biological oxidant, was detected in the reaction of glyoxal with peroxynitrite as a result from formyl radical annihilation by ground state oxygen and subsequent bimolecular Russell reaction of the formylperoxyl radical product (**Scheme 1A**).

The 2-alkenal acrolein (2-propenal) is a thermally processed food contaminant and endogenous toxin (metabolism and lipid peroxidation) implicated in various disorders including multiple sclerosis, Alzheimer's disease, spinal cord injury, cardiovascular disease, diabetes mellitus, and neuro-, hepato- and nephro-toxicity.[10, 26-28] Acrolein is as a powerful and harmful compound which can lead to protein-acrolein adduct with Arg, Lys, His, and Cys residues of proteins as well as with nucleobases, thereby disrupting the intracellular redox balance and driving mutagenesis and carcinogenesis.[11, 29, 30] Recently, it has also been detected in the aerosols of electronic cigarette devices.[31] The high reactivity of acrolein to nucleophiles lies on the electronic delocalization along the double and the carbonyl bonds, favoring 1,4-Michael addition.[30] Here we show that the nucleophilic addition of peroxynitrite to acrolein in aerated phosphate buffer produces oxygen in the excited singlet state through the formation of a carbon-centered radical bearing a germinal hydrogen atom which reacts with molecular oxygen via Russell reaction mechanism (**Scheme 1B**). If occurring in cell sites where both peroxynitrite and acrolein increase, singlet oxygen could potentially exert noxious biological responses.[32]



Scheme 1. (A) Reaction mechanisms and final products of the reaction of peroxynitrite with (A) diacetyl (R_1 = R_2 = CH_3),[23] methylglyoxal (R_1 = H, R_2 = CH_3)[25] and glyoxal (R_1 = R_2 = H)[24] in aerated phosphate buffer, pH 7.2. (B) Proposed simplified mechanism of oxidation of acrolein by peroxynitrite and expected products: (1) formic acid, (2) glyoxal, (3) glycolaldehyde and singlet oxygen (O_2 $^1\Delta_g$).

Experimental details

General information

All chemicals were purchased of the purest grade available from Sigma-Aldrich, Fluka and Merck and used without further purification, unless otherwise stated. Stock solutions and buffers were prepared using deionized water (18.2 MΩ cm at 25 ºC, TOC ≤ 4 ppb, Milli-Q, Millipore) and pretreated with Chelex-100 to remove metal contaminants.

Preparation of peroxynitrite

Peroxynitrite (300.0 – 450.0 mmol L^{-1}) was prepared from $NaNO_2$ (0.60 mol L^{-1}) and H_2O_2 (0.70 mol L^{-1}) in HCl (0.60 mol L^{-1}) and NaOH (1.50 mol L^{-1}) in a quench-flow reactor as described previously.[33] Remaining H_2O_2 in the alkaline peroxynitrite solution was eliminated with the addition of MnO_2. Peroxynitrite and H_2O_2 concentrations were determined spectrophotometrically at 302 nm (ε = 1670 L mol^{-1} cm^{-1})[33, 34] and 240 nm (ε = 42 L mol^{-1} cm^{-1})[35], respectively. Peroxynitrite was aliquoted and stored at −80°C. The concentration of an aliquot of peroxynitrite was re-measured immediately before the experiment and kept on ice in the dark during the experiments.

Oxygen consumption

The rate of oxygen consumption was monitored in a Hansatech oxygraph equipped with a Clark type electrode. The measurement was recorded immediately after injection of the peroxynitrite solution (0.25 − 3.0 mmol L^{-1}) to the air-equilibrated solution of acrolein (0.50 − 10.0 mmol L^{-1}) in phosphate buffer (500.0 mmol L^{-1}, pH 7.2) at 25°C.

Peroxynitrite depletion

The effect of acrolein (0 − 40 mmol L^{-1}) in the decay rate of peroxynitrite (200 μmol L^{-1}) was monitored with a stopped-flow spectrophotometer (Applied Photophysics model SX-18V) at 302 nm. The temperature was kept constant at 25.0 ± 0.2 °C, and the pH values of the final reaction mixtures were confirmed by a simulated scaled up experiment under the same experimental conditions. Pseudo-first-order rates, k_{obs} (s^{-1}) were determined with a liner regression adjust of ln *A vs* time (0 – 300 ms) plots. The results are the average of of 9−12 measurements. The apparent second-order rate constants ($k_{2,app}$) were determined from the slopes of the plots of k_{obs} *vs* acrolein concentration. The actual k_2 value was calculated taking into account the degree of

acrolein hydration in the buffered solution (93%)[36]. The rate of peroxynitrite decay in the buffer solution (k_0), was subtracted from k_{obs} before plotting the data against pH or phosphate buffer concentration. All data were fitted using Origin 8 (OriginLab).

EPR spin trapping

The spin trap 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) was synthesized as previously described[37] and employed in the experiments using a Bruker EMX EPR spectrometer. Spectra were recorded after 1.5 min and 5 min of reagents incubation in phosphate buffer (500.0 mmol L^{-1}, pH 7.2) pretreated with Chelex-100 at 22°C. The final pH values were measured in order to check for subtle pH changes that might result from the addition of the alkaline stock solutions of peroxynitrite to the buffered reaction mixtures. No pH change was observed. The instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 0.1 mT; time constant, 81.92 ms; and receiver gain, 1×10^{-5}. Computer simulation of spectra was performed using the program P.E.S.T written by Duling.[38]

Detection of singlet oxygen

Reaction mixtures of acrolein (0.05 – 10.0 mmol L^{-1}) and peroxynitrite (1.0 mmol L^{-1}) in deuterated 500 mM phosphate buffer, pD approximately 7.4, at 25°C, were monitored in an ultrasensitive photocounting equipment aiming to detect the monomolecular photoemission of $O_2(^1\Delta_g)$ in the near-infrared (NIR) region (λ = 1,270 nm), as described previously.[39] All reactions were carried out in a quartz cuvette under constant stirring at room temperature. The light emission was immediately recorded by a FLSP 920 photon counter (Edinburgh Instruments, Edinburgh, UK) consisting of two UV-Visible Hamamatsu detectors R9110, maintained at −20 °C by a CO1 thermoelectric cooler (Edinburgh Instruments). The monomol light emission of O_2 ($^1\Delta_g$) at 1,270 nm was monitored over time using a detector coupled to the device, a

Hamamatsu H10330A-45 apparatus (Hamamatsu city, Japan), preceded by a monochromator. Peroxynitrite was infused to the reaction mixture in the cuvette after 50 s using a syringe with tubing for all experiments. Quenching of the emission intensity of the reaction of acrolein (0.1 mmol L^{-1}) with peroxynitrite (1 mmol L^{-1}) by water (20%) and sodium azide (NaN_3, 0.125 mmol L^{-1}) confirmed the singlet nature of the molecular oxygen. The NIR spectrum of the reaction of acrolein (0.1 mmol L^{-1}) with peroxynitrite (1 mmol L^{-1}) was filtered with a diffraction grate and the signal acquired digitally with a computer. To determine the O_2 ($^1\Delta_g$) yield, the *N*,*N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide endoperoxide ($DHPNO_2$, 10 mmol L^{-1}) was used as standard. The NIR reference spectrum of O_2 ($^1\Delta_g$) at 1,270 nm was produced by thermal decomposition of $DHPNO_2$ at 37 °C.[40] The presence of hydroquinone as stabilizer in the Sigma acrolein did not affect the singlet oxygen emission at the concentration range employed in our experiments.

Product Analysis

The expected aldehydes (formaldehyde and glyoxal) and carboxylic acids (formic and acetic acids) products formed in the reaction of acrolein with peroxynitrite were analyzed in a SCIEX PA800 capillary electrophoresis system equipped with a UV filter wheel detector accommodating a 254 nm filter (10 nm bandwidth) for indirect detection. A solution of 3,5-dinitrobenzoic acid (10 mmol L^{-1}) containing cetyltrimethylammonium bromide (0.20 mmol L^{-1}) was employed as background electrolyte (final pH = 4.5). Fused-silica capillaries with dimensions of 50 cm total length (40 cm effective lengh) and 75 µm i.d. × 375 µm o.d. were used. Aliquots of the reaction mixtures obtained by mixing peroxynitrite (1.45 mmol L^{-1}) and acrolein (3 mmol L^{-1}) adjusted to pH 7.2 − 7.5 were injected hydrodynamically at 3 kPa for 3 s and the separation of products was observed at a constant applied voltage (−20 kV).

Aldehydes were previously derivatized with bisulfite to convert them into the respective sulfonate adducts.[41] Standards of sodium nitrite, sodium nitrate, and carboxylic acids at 10 mmol L^{-1} each as well as the derivatized aldehydes were injected separately and as spiked final reaction mixtures to aid peak identification.

Results and Discussion

Peroxynitrite decay

The second order rate constant, pH profile and phosphate dependence of the nucleophilic addition of peroxynitrite to acrolein were determined by tracing the fast decay of peroxynitrite at 302 nm by stopped-flow absorption spectroscopy in air-equilibrated phosphate buffer (500 mmol L^{-1}, pH 7.2) at 25 °C (**Figure 1**, **Figure S1**). The high phosphate concentration is needed to guarantee no variation in the pH of the reaction mixture upon addition of the peroxynitrite stock solution containing 1.0 mmol L^{-1} NaOH. The pseudo-first order rate constants (k_{obs}) were found to increase linearly with the initial concentration of acrolein, leading to the apparent second order rate constant ($k_{2,app}$) 4.0 × 10^2 $M^{-1}s^{-1}$ (**Figure 1A**). The fraction of hydrated acrolein in aqueous medium is 93%[36]; hence, the k_2 value for the reaction can be estimated as 6.0 × 10^3 $M^{-1}s^{-1}$. Therefore, acrolein is ~10-fold more reactive than monoalkanals like ethanal, propanal, and isobutanal (~300 – 700 $M^{-1}s^{-1}$)[42, 43] and one order of magnitude less reactive than HCO_3^-/CO_2 (3 − 6 × 10^4 $M^{-1}s^{-1}$)[44]. Yet, its reactivity is close to reactivity of diacetyl (k_2 = 1.0 × 10^4 $M^{-1}s^{-1}$)[23]. These values are in accordance with the expected relative electrophilicity of the carbonyl reactants: carbon dioxide>glyoxals>acrolein>monoaldehydes.

The well-known general acid catalysis occurrence by the dihydrogen phosphate anion in the peroxynitrite addition to carbonyls[23, 25, 45] was verified for the reaction

of peroxynitrite with acrolein (**Figure 1B**). The k_{obs}-k_0 values increase linearly approx. 8-fold with the increase of the phosphate concentration (50 − 1000 mM, pH 7.2) at 25 °C, being k_0 related to the decomposition of peroxynitrite alone. The spontaneous decomposition rate of peroxynitrite was not significantly affected by the phosphate concentration. The bell-shaped pH profile of the peroxynitrite/acrolein reaction has a maximum at pH ~ 6.8 in the range 6.2 to 8.2 (**Figure 1C**), and this result corroborates the proposed phosphate catalysis. It roughly mirrors the overlap of the titration curve of the peroxynitrous acid providing nucleophilic peroxynitrite anion (pK_a ONOOH/$ONOO^-$ = 6.8, increasing to over 7.0 at higher ionic strength)[23, 33] with increased dissociation and loss of catalytic $H_2PO_4^-$. All together these data are related to those reported by Yang et al.[45] for the general-acid catalyzed addition of peroxynitrite to diacetyl and other carbonyls. The bell-shaped pH profile and the linear phosphate concentration dependence of the reaction rate points to a kinetic equation expressed as

$$\text{rate} = k_2[\text{acrolein}][H_2PO_4^-][ONOO^-]$$

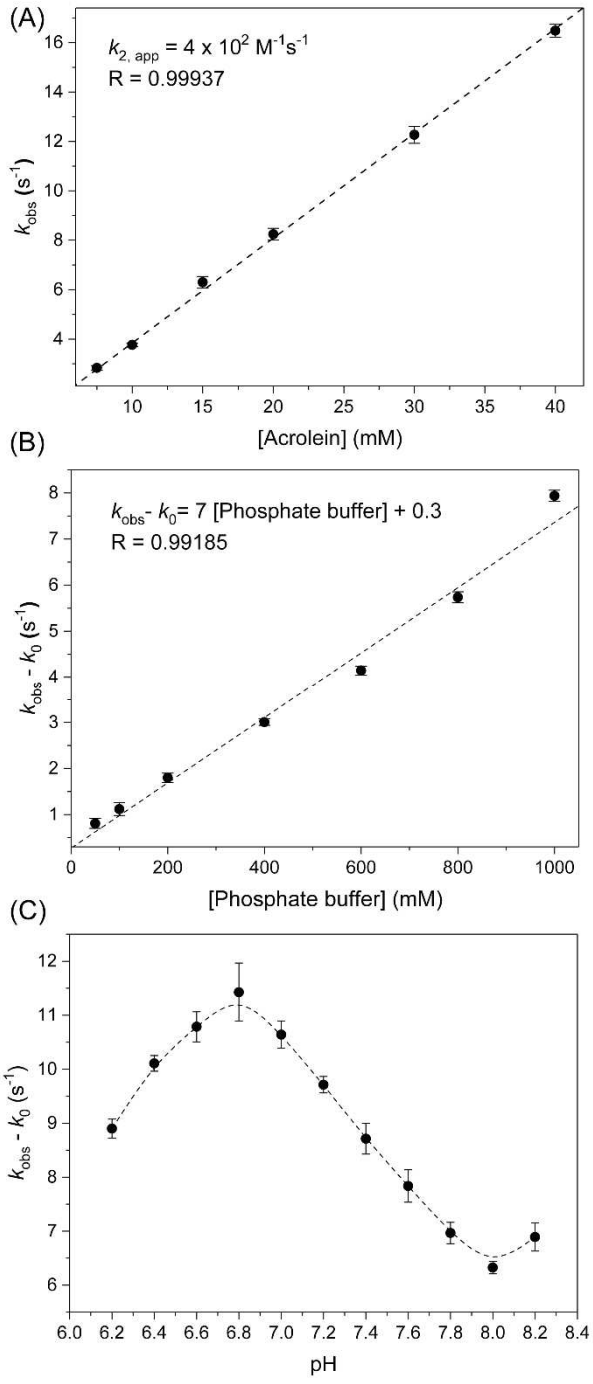


Figure 1. Stopped-flow kinetics of the peroxynitrite decay in the presence of acrolein. (A) Pseudo-first-order rate constants (k_{obs}) for the decay of peroxynitrite (200 μmol L^{-1}) in the presence of acrolein (0 − 40 mmol L^{-1}) in phosphate buffer (500 mmol L^{-1}, pH 7.2). Calculation of the apparent second-order rate constant of peroxynitrite addition to acrolein ($k_{2,app}$). (B) Catalytic effect of phosphate anion on the observed rate constant of peroxynitrite (200 μmol L^{-1}) reaction with acrolein (20 mmol L^{-1}) in phosphate buffer pH 7.2. The ionic strength was corrected upon addition of NaCl. (C) pH profile of the

reaction of peroxynitrite (200 μmol L^{-1}) with acrolein (20 mmol L^{-1}) in phosphate buffer (700 mmol L^{-1}). All experiments were performed at 25 °C.

EPR spin trapping studies

EPR spin trapping studies with 10 mM DBNBS were performed in phosphate buffer (500 mmol L^{-1}, pH 7.2) treated with Chelex-100. The reaction was initiated by the addition of peroxynitrite to the reaction mixture containing acrolein, DBNBS and buffer. After 1.5 min of incubation at 22 °C, somewhat distorted six lines spectra were recorded (**Figure 2**) and the values of the hyperfine coupling constants were estimated from spectral analysis and simulation (a_N = 13.33 G and the a_H = 6.45 G). A six-line spectrum is expected for a DBNBS radical adduct of the •CH=CH(OH) hydroxyvinyl radical, a possible intermediate in the mechanism of acrolein oxidation by peroxynitrite (**Scheme 1B**). The formation of this radical was previously reported in gas phase studies of the reaction of the hydroxyl radical with acrolein.[46]

The EPR signal was intensified with the increase in the concentration of acrolein (**Figure 2A**). Conversely, addition of the bicarbonate/CO_2 pair to the reaction mixture decreased the EPR signal intensity (**Figure 2B**), indicating competition of carbon dioxide with acrolein for the peroxynitrite anion. Control experiments in the absence of either acrolein or peroxynitrite did not give significant response, as well as hydrogen peroxide addition to acrolein (**Figure S2**). It should be noted that the lines of the EPR spectra are distorted and broad, suggesting superposition of two radical adducts, most likely the *cis* and *trans* forms of the 2-hydroxyvinyl radical. Indeed, attempts to split the lines of the EPR spectra by changing the instrumental conditions (smaller modulation amplitudes) failed (data not shown).

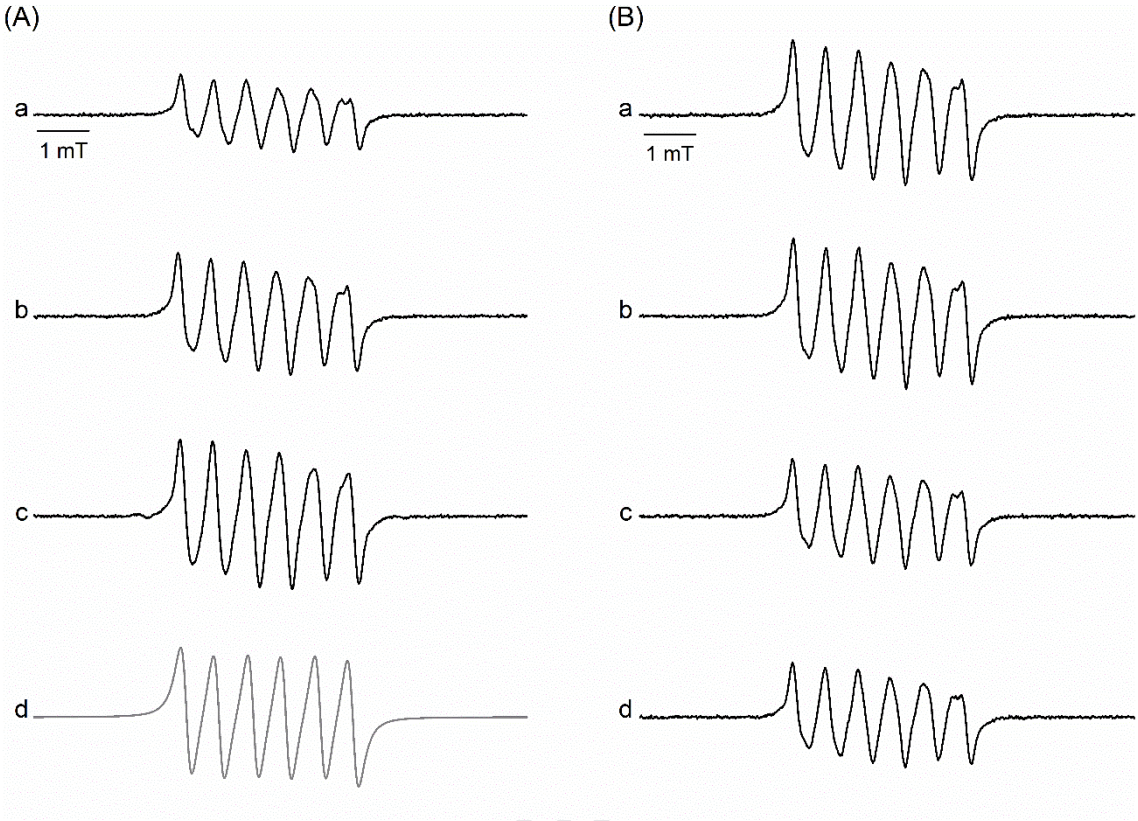


Figure 2. EPR spin trapping studies with DBNBS (10 mmol L^{-1}) of the acrolein/peroxynitrite reaction in phosphate buffer (500 mmol L^{-1}, pH 7.2) after incubation for 1.5 min at 22 °C. (A) Spectra of peroxynitrite (1 mmol L^{-1}) with 5 (a), 10 (b), and 20 mmol L^{-1} acrolein (c). Computer simulation in (d). (B) Effect of carbonate on the amplitude of the EPR signal of the adduct obtained from acrolein (10 mmol L^{-1}) and peroxynitrite (1 mmol L^{-1}) without (a) and with 5 (b), 10 (c) and 25 mmol L^{-1} sodium bicarbonate (d).

Oxygen uptake

Addition of peroxynitrite (0 − 3 mmol L^{-1}) to acrolein (0 − 10 mmol L^{-1}) in phosphate buffer (500 mmol L^{-1}, pH 7.2) at 25 °C resulted in the consumption of a fraction of the dissolved oxygen in less than one minute (**Figure 3**). Dissolved oxygen concentration values were estimated assuming that the oxygen concentration in 500 mmol L^{-1} phosphate buffer is near that reported for 500 mmol L^{-1} NaCl at 25 °C, *i.e.*, ~212 µmol L^{-1}.[47]. Increasing the acrolein concentration to 10 mmol L^{-1} in the presence of 1 mmol L^{-1} peroxynitrite, increased oxygen uptake to a saturation value of ~80 µmol L^{-1}

oxygen (**Figure 3A**) whereas proportionally less oxygen was consumed with equal concentrations of peroxynitrite and acrolein (3 mmol L^{-1}) 170 µmol L^{-1}. The non-linear response of oxygen uptake upon increasing the concentration of acrolein may be due to the well-known tendency of oxygen-exposed acrolein to undergo polymerization (**Figure 3A-3C**). In addition, the Clark electrode used to measure has a low response time, which may be response for distortions in the data. The effect of the concentration of acrolein and peroxynitrite on the rate of O_2 uptake was determined by the initial rate fit of each curve shown in the **Figure 3A-3B**. The plot of rough initial rate vs. concentration of acrolein and peroxynitrite (**Figure 3C**) may confirm a first order dependence of oxygen consumption on the peroxynitrite concentration whereas do not respond linearly to increasing acrolein concentration probably due to competing acrolein polymerization.

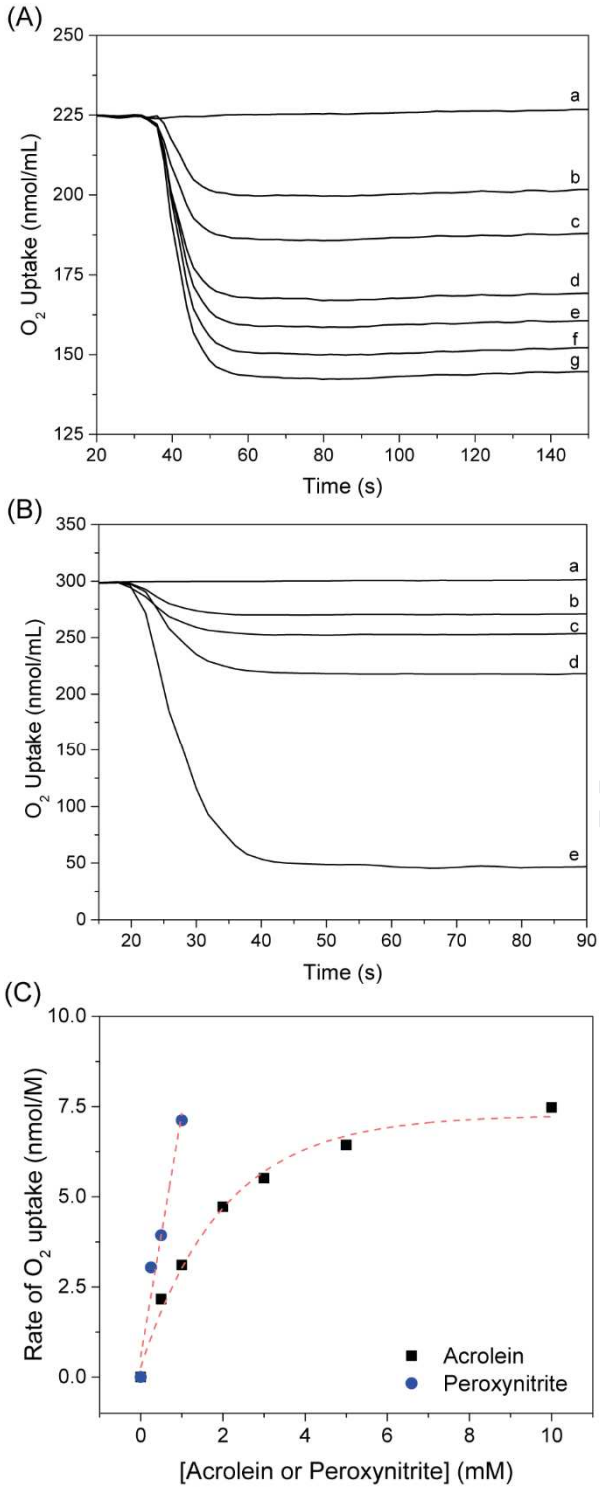


Figure 3. Time courses of oxygen uptake during the reaction of peroxynitrite with acrolein in air-equilibrated phosphate buffer (500 mmol L^{-1}, pH 7.2) at 25 °C. (A) Peroxynitrite (1 mmol L^{-1}) in the absence of acrolein (a) and in the presence of 0.50 (b), 1.00 (c), 2.00 (d), 3.00 (e), 5.00 (f) and 10.00 mmol L^{-1} acrolein (g). (B) Acrolein (3 mmol L^{-1}) in the absence of peroxynitrite (a) and in the presence of 0.25 (b), 0.50 (c),

1.00 (d) and 3.00 mmol L^{-1} peroxynitrite (e). (C) Effect of the concentration of acrolein (black square) or peroxynitrite (blue circle) on the rate of O_2 uptake (nmol/mL/s).

Assuming the 1,4-Michael addition of peroxyntrite to acrolein as previously reported for alkanals and α-dicarbonyls, the carbon-centered radical originated by C1-C2 acrolein cleavage, •CH=CH(OH) (**Scheme 1B**), is expected to add molecular oxygen yielding the 2-hydroxyvinylperoxyl radical, [CH(OO•)=C(H)OH]. The latter radical is expected to undergo the bimolecular Russell reaction to form glycolaldehyde [CH_2(OH)-C(O)H], glyoxal [C(O)H-C(O)H] and excited molecular oxygen O_2 ($^1\Delta_g$) state ranging 3 to 14% yields [48]. Therefore, considering the stoichiometry of peroxynitrite to consumed oxygen as 1.0 to 0.5, the fraction of peroxynitrite that reacted with acrolein in **Figure 3A** is minimally 8% when calculated over initial 10 mM acrolein at the experimental conditions stated in the figure legend. Parallel isomerization of peroxynitrite to the nitrate, reformation of acrolein and dimerization of the •CH=CH(OH) radical *pari passu* with oxygen depletion may contribute to the calculated low yield of oxygen consumption.

Product analysis

Formaldehyde, glycolaldehyde, and glyoxal are expected to be formed upon the 1,4-Michael addition of peroxynitrite to acrolein. Conversely, any unexpected contribution of peroxynitrite addition to the aldehyde group would result in formate and vinyl radical, whose products after oxygen addition would include acetate and acetaldehyde. Electrophoresis separation and quantification of the final products generated by the peroxynitrite/acrolein reaction were conducted to verify which final products were formed. Aldehyde products are electrophoretically silent; thus, final reaction mixtures were pre-derivatized with sodium bisulfite to the corresponding active sulfonate anions. Authentic formaldehyde and glyoxal were used to attest the putative products (**Figure**

4A-B) whereas formate and acetate were used as reference compounds to rule out peroxynitrite addition to the carbonyl group (**Figure S3**). In order to detect any expected increase of the ratio nitrite/nitrate originated by lateral formation of the $^{\bullet}NO_2$ radical during homolyzes of the peroxonitrous carbonyl intermediate, nitrite and nitrate peaks were also monitored in the reference electropherogram. These anions are contaminants of the peroxynitrite solutions and by-products formed upon hydrolysis of $^{\bullet}NO_2$.

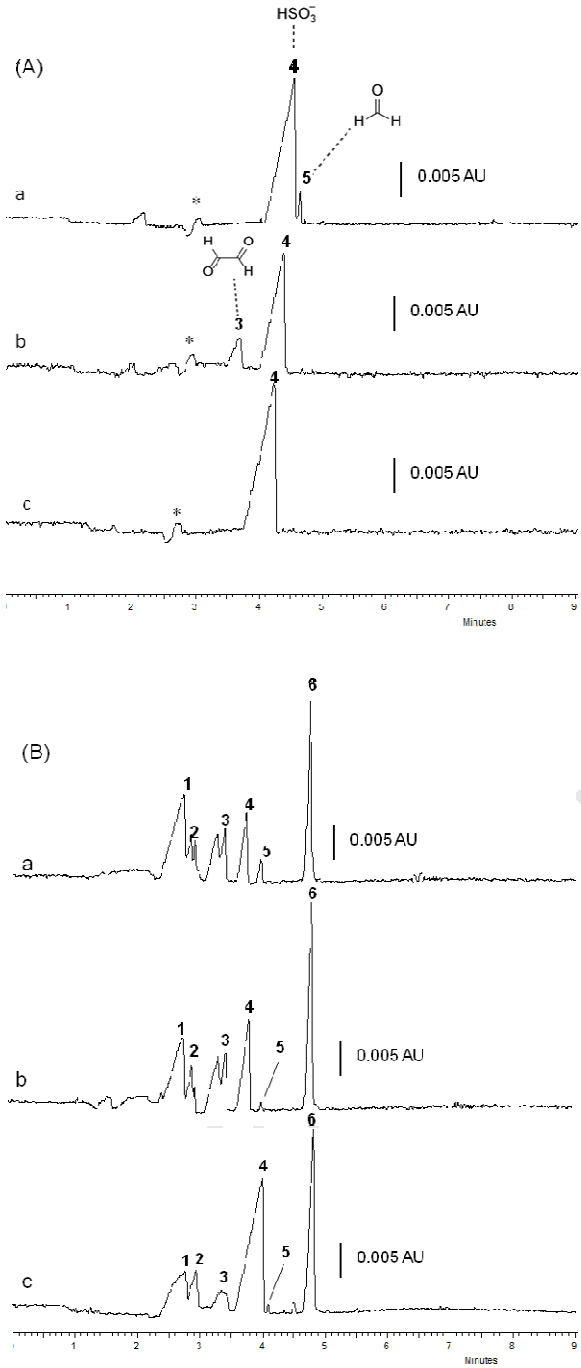


Figure 4. Analysis of the reaction products of the reaction of acrolein (3 mmol L^{-1}) with peroxynitrite (1.45 mmol L^{-1}) in pH 7.2-7.5. (A) Electropherograms of formaldehyde (a), glyoxal (b), and sodium bisulfite (c). (B) Electropherograms of the final reaction mixtures doped glyoxal and formaldehyde (a), the final reaction mixtures doped glyoxal (b) and the final reaction mixture without addition of reference compounds (c). Reaction products were previously derivatized with sodium bisulfite. Peak identification: (1) nitrite, (2) nitrate, (3) glyoxal, (4) bisulfite, (5) formaldehyde, (6) phosphate, (*) system peak. Standard concentration: 10 mmol L^{-1}. Experimental conditions: see methods.

The electropherograms of the final reaction mixture in comparison to that obtained from the standard mixture of analytes (**Figure 4A-B**) clearly show the presence of formaldehyde and glyoxal, two of the final products of the 1,4-Michael addition of peroxynitrite to acrolein. Accordingly, the electropherograms fingered the absence of the formate and acetate ions, expected to be formed from peroxynitrite addition to C1-acrolein (**Figure S3**).

Detection of singlet oxygen

Singlet oxygen exert deleterious effects to biomolecules and supramolecular cell structures due to its electrophilicity and diversity of chemical targets (such as double bonds, sulfur atoms, and heterocyclic rings).[32] To examine singlet oxygen O_2 ($^1\Delta_g$) generation during the oxidation of acrolein by peroxynitrite, we monitored its characteristic monomolecular emission in the NIR region ($\lambda_=$ 1,270 nm)[49] running the reaction in deuterated phosphate buffer, pD 7.4 (**Figure 5**). Addition of peroxynitrite to acrolein showed a clear light emission that did not appear in the absence of any of the reagents. Indeed, he light emission attributed to the minor conversion of peroxynitrite to peroxynitrate, whose decomposition yields very low yields of singlet oxygen was not observed under our experimental conditions (**Figure 5A**).[50] On the other hand, addition of small amounts of either water or sodium azide to the reaction mixture in D_2O resulted in quenching of the singlet oxygen NIR emission as expected (**Figure 5B**).[51] Increments of acrolein concentration decreased the signal (**Figure 5C**), what may be attributed to the increase in the water content of commercial acrolein (90%). The emission spectrum from the acrolein/peroxynitrite system was recorded and compared to that obtained from an already reported singlet oxygen generator, the endoperoxide $DHPNO_2$ [40] (**Figure 5D**). The singlet oxygen yield obtained from 0.1 mmol L^{-1} acrolein and 1 mmol L^{-1} peroxynitrite was calculated on the basis of the

emission area obtained from the decomposition of $DHPNO_2$ (10 mmol L^{-1}) at 1,270 nm (**Figure S4**).[40] The calculated value , 6.1 %, is in range expected for reactions involving the Russel reaction mechanism (3-14%).[48] Thus, the well-known Russell reaction by annihilation of geminal hydrogen-lacking radical is here unveiled also to occur with a vinylperoxyl radical, not only alkylperoxyl and acylperoxyl radicals. [24, 32]

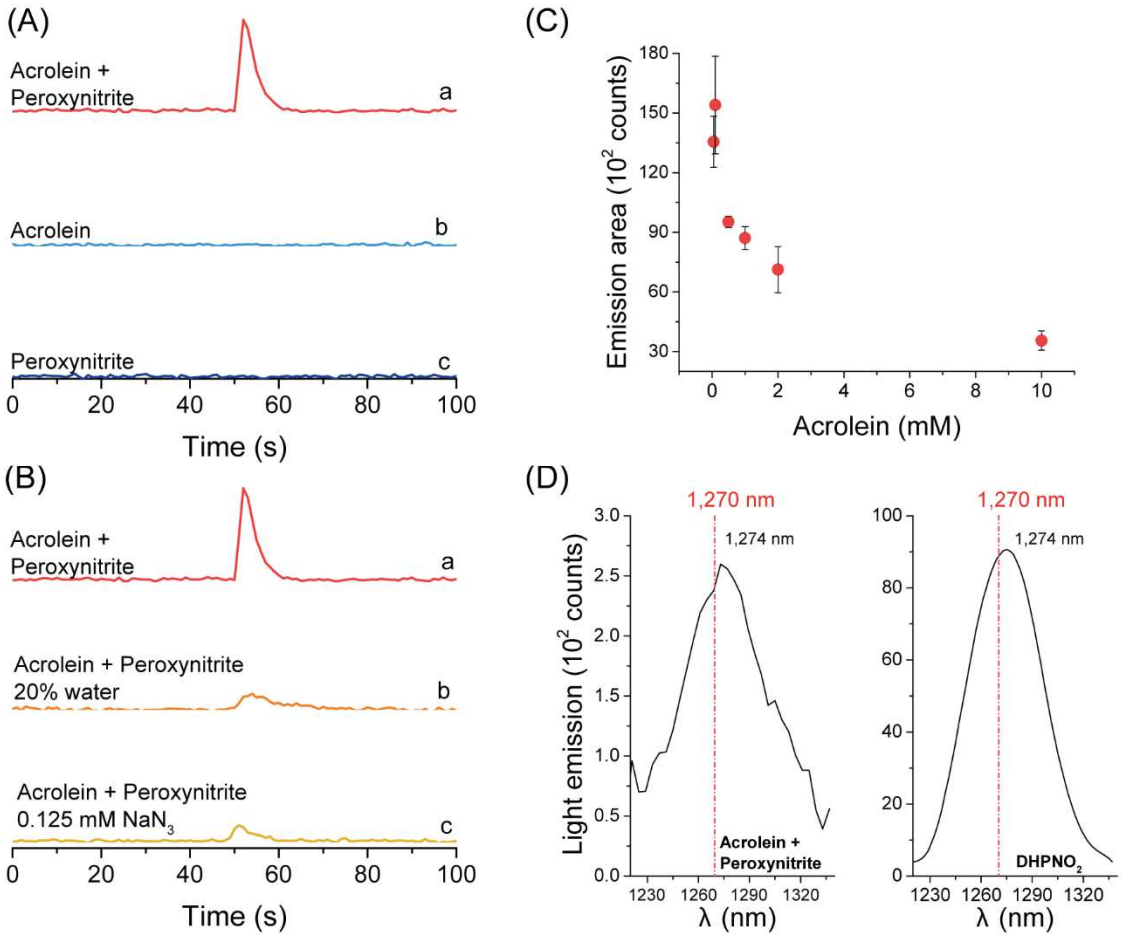


Figure 5. Near infrared analysis at 1,270 nm of singlet oxygen O_2 ($^1\Delta_g$) generated by the acrolein/peroxynitrite system in D_2O-prepared phosphate buffer. (A) Light emission produced in the reaction of peroxynitrite (1.0 mmol L^{-1}) with acrolein (0.1 mmol L^{-1}) (a), in acrolein alone (b) and peroxynitrite alone (c). (B) Chemical quenching of the reaction (a) by water (b) and sodium azide (NaN_3) (c). (C) Effect of the concentration of acrolein in the excited oxygen signal. (D) Spectra of the reaction peroxynitrite/acrolein

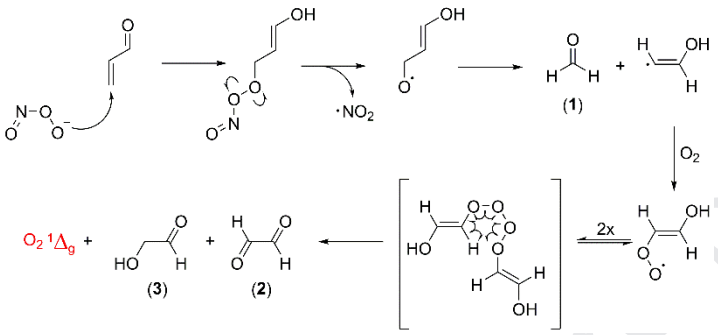
and the standard endoperoxide $DHPNO_2$ (**Figure S4**). Peroxynitrite was added to the reaction mixture after 50 s in all experiments.

Conclusions

Current research on the toxic effects of acrolein to the human health focuses essentially on its irreversible 1,4-Michael attachment to amino and thiolate groups of proteins and to DNA, disregarding other biological nucleophiles. In this study, we show that acrolein may act as a potential biological acceptor of peroxynitrite (k_2 = 6.0 x 10^3 $M^{-1}s^{-1}$ at 25 °C, pH 7.4; optimum pH ~ 6.8) ultimately yielding formaldehyde, glyoxal, and singlet oxygen. The aldehydes were analyzed by capillary electrophoresis after derivatization with bisulfite. The absence of formate and acetate anions in the spent reaction mixtures precluded the expected nucleophilic addition of peroxynitrite to the carbonyl group of acrolein. The reaction was shown to be catalyzed by the dihydrogen phosphate anion and to proceed via carbon- and oxygen-centered radicals. It terminates via the Russell reaction of a secondary peroxyl radical [CH_2($OO^{\bullet}$)-CH(O] or a vinylperoxyl radical [CH($OO^{\bullet}$)=CH(OH) yielding molecular oxygen in the excited singlet state (**Scheme 2**). Formation of the latter was confirmed in deuterated buffer by the characteristic NIR monomolecular emission at 1,270 nm and by quenching of the ultra-weak red chemiluminescence by H_2O and NaN_3. These data predict production of biologically active singlet oxygen, albeit at very low yields, from the nucleophilic addition of peroxynitrite to many putatively toxic alkanals and alkenals.

Although less reactive than CO_2 and α-oxoaldehydes like methylglyoxal and glyoxal,[24] acrolein is about ten-fold more reactive than alkanals and reported to be more reactive than other α,β-unsaturated alkenals[12]. Despite being found in tissues at much lower concentrations than the bicarbonate/carbon dioxide pair, one must keep in mind that in cell compartments, the reactions are governed not only by thermodynamic

and kinetic factors but also by spatial and temporal factors.[52] This argument also applies to the higher concentrations of phosphate in this study (500 mM) as beyond redox potentials and rate constants, reagent compartmentalization and diffusion rates also matters. In fact, various protein and DNA adducts of acrolein and of other carbonyls have been isolated and identified in biological samples from experimental animals and humans portraying redox unbalance.[53]



Scheme 2. Proposed mechanism for the reaction of acrolein with peroxynitrite yielding singlet oxygen ($O_2\ ^1\Delta_g$).

Acknowledgements

This work was supported by the São Paulo Research Foundation – FAPESP (EJHB, 2017/22501-2; LCPG, 2018/25842-8; OA and PDM, 2013/07937-8, PDM 2012/12663-1, MFMT 2017/27059-6), the Brazilian National Council for Scientific and Technological Development – CNPq (EJHB 444237/2014-3, EJHB 306460/2016-5). FMP, EL, OA and PDM are members of NAP Redoxoma (PRPUSP), and the CEPID Redoxoma (FAPESP).

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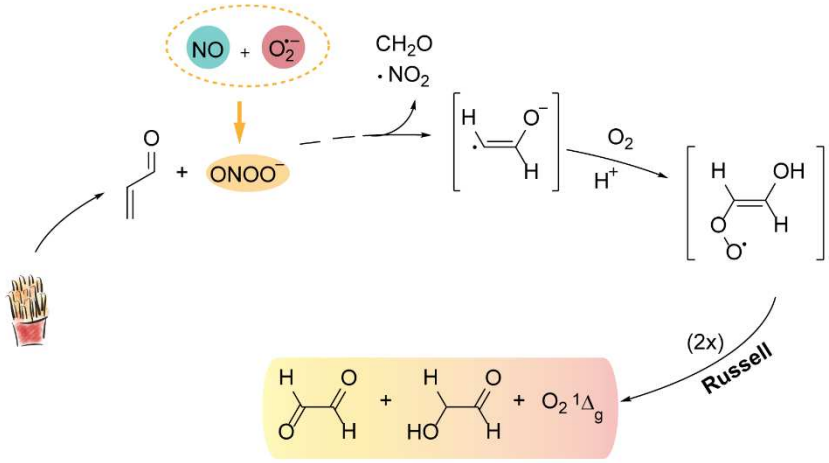
Graphical abstract



Highlights

- Acrolein is an endogenous toxicant, also found in thermoprocessed food.
- In aerated medium acrolein adds peroxynitrite yielding formaldehyde and glyoxal.
- Phosphate catalyses the reaction accompanied by singlet oxygen emission.
- EPR spin trapping studies disclose a putative 2-hydroxyvinyl radical.
- Russell reaction of the vinyloxyl radical explains singlet oxygen generation.