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Cytochrome *c* Reacts with Cholesterol Hydroperoxides To Produce Lipid- and Protein-Derived Radicals

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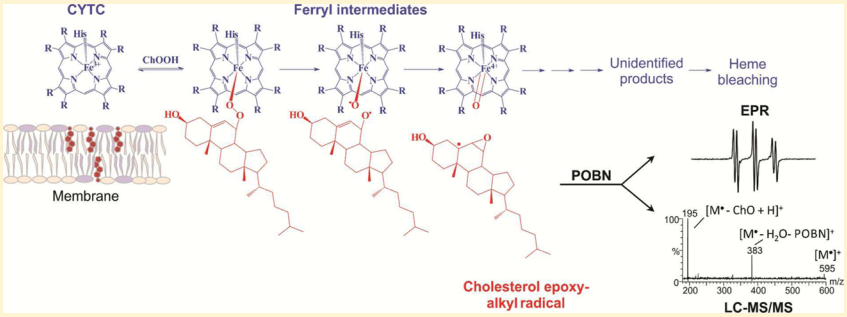
Supporting Information



ABSTRACT: Lipid peroxidation is a well-known process that has been implicated in many diseases. Recent evidence has shown that mitochondrial cholesterol levels are increased under specific conditions, making it an important target for peroxidation inside the mitochondria. Cholesterol peroxidation generates, as primary products, several hydroperoxides (ChOOH), which can react with transition metals and metalloproteins. In this sense, cytochrome *c* (CYTC), a heme protein largely found in the mitochondria, becomes a candidate to react with ChOOH. Using CYTC associated with SDS micelles to mimic mitochondrial conditions, we show that ChOOH induces dose-dependent CYTC Soret band bleaching, indicating that it is using ChOOH as a substrate. This reaction leads to protein oligomerization, suggesting the formation of a protein radical that, subsequently, recombines, giving dimers, trimers, and tetramers. EPR experiments confirmed the production of carbon-centered radicals from both protein and lipid in the presence of ChOOH. Similar results were obtained with linoleic acid hydroperoxides (LAOOH). In addition, replacing SDS micelles by cardiolipin-containing liposomes as the mitochondrial mimetic led to similar results with either ChOOH or LAOOH. Importantly, kinetic experiments show that CYTC bleaching is faster with ChOOH than with H_2O_2, suggesting that these hydroperoxides could be relevant substrates for CYTC peroxidase-like activity in biological media. Altogether, these results show that CYTC induces homolytic cleavage of lipid-derived hydroperoxides, producing lipid and protein radicals.

Cholesterol is a neutral lipid highly enriched in the plasma membrane of eukaryotic cells, where it comprises approximately 20−25% of the total lipid pool.[1,2] Its distribution, however, is not uniform among other organelles. In mitochondria, for instance, cholesterol corresponds to only 0.5 to 3% of the total lipid pool under normal conditions.[1] Cholesterol is synthesized in the endoplasmic reticulum and then transported to the mitochondria, where it is metabolized into many steroids in a process strictly regulated in the cell.[2−5] The protein suggested to be involved in the trafficking process is steroidogenic acute regulatory protein (StAR), which contains a motif that targets mitochondria and another one that binds cholesterol.[3,5,6] StAR was recently shown to transport cholesterol-derived hydroperoxides into the mitochondria as well.[6] Under pathological conditions, such as cancer, Alzheimer's disease, atherosclerosis, and hepatic steatosis, cholesterol levels have been reported to increase in the mitochondria.[7−9] This increase could be associated with a malfunction in the trafficking process involving the StAR protein.[6] Knowing that cholesterol is susceptible to free radical oxidation and that mitochondria are the primary source of radicals, it is reasonable to assume that cholesterol may become an important target for peroxidation under these conditions.

Lipid peroxidation is an autocatalytic process that produces several reactive products, including hydroperoxides, epoxides, aldehydes, ketones, and singlet molecular oxygen.[10−12] During the past decade, many studies have been published showing the ability of such products to react with other biomolecules, especially proteins and nucleic acids.[13−20] The ability of metal-

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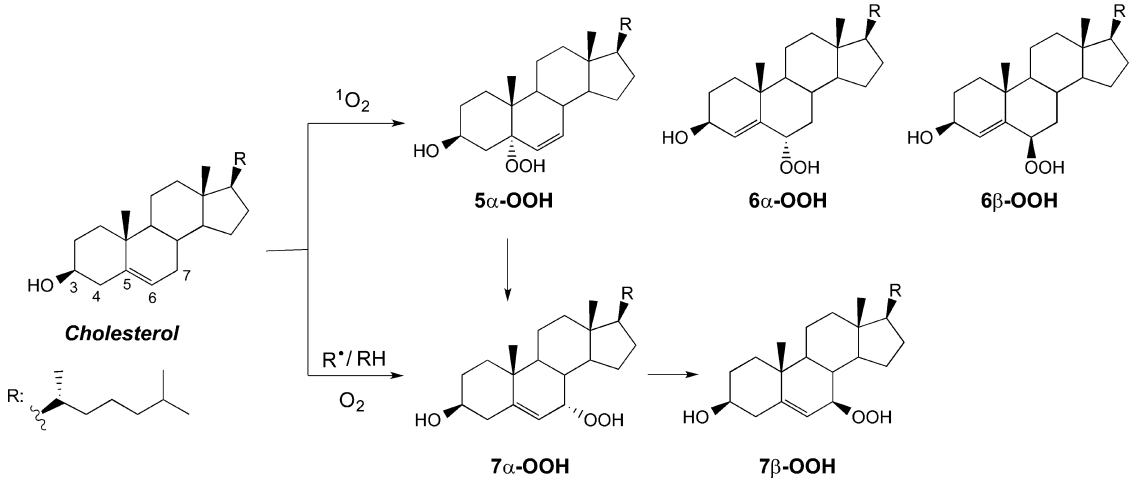
Scheme 1. Cholesterol Hydroperoxides Produced by Cholesterol Oxidation Mediated by Singlet Molecular Oxygen ($^1O_2$) or by Free Radicals ($R^•$)[a]



[a]Only the primary oxidation products are depicted (for details, see refs 10 and 32).

containing proteins to react with lipid hydroperoxides, generating a broad spectrum of products, has also been reported.[21−25] Hemeproteins, such as cytochrome *c* (CYTC), were shown to mediate free radical reactions with many peroxides, including, H_2O_2, *t*-BuOOH, cumene hydroperoxide, and lipid-derived peroxides.[23,24,26,27] The ability of CYTC to react with peroxides is usually attributed to its peroxidase activity, which consists of the reduction of the peroxide moiety via either one or two electrons.[27] These reactions have been reported to produce free radical species such as peroxyl and alkoxyl radicals.[28−31]

Cholesterol peroxidation generates, as primary products, several reactive hydroperoxides (Scheme 1).[10,32] The production of each species depends on several conditions, including which oxidant is being used and whether a hydrogen donor is present (for more details, see refs 10 and 32). In a scenario where cholesterol is accumulated in the mitochondria, the appearance of such hydroperoxides might become a major event. Therefore, their reaction with mitochondrial proteins, such as CYTC, can play an important role in the generation of free radicals inside the organelle.

With this in mind, we sought to study the reaction of cholesterol-derived hydroperoxides (ChOOH) with CYTC, a heme protein that is found peripherally bound to the mitochondrial inner membrane. Here, we employed SDS micelles and cardiolipin-containing liposomes as mimetic models to insert ChOOH and anchor CYTC in a similar manner as that used previously.[20,33] In this situation, CYTC is assumed to possess a similar structure and physicochemical properties as those of the native species found in the mitochondria.[33] By using electron paramagnetic resonance (EPR), we show that CYTC reacts with ChOOH, generating carbon-centered radicals on both lipid and protein. These protein radicals recombine, producing high molecular weight species (i.e., dimers, trimers, and tetramers). To evaluate the possible relevance of the reaction of CYT C with ChOOH in biological media, we compared its rate with those of other relevant peroxides, such as H_2O_2 and linoleic acid hydroperoxide (LAOOH).

MATERIALS AND METHODS

Chemicals. Bovine heart cytochrome *c* (Fe^{3+}), ammonium bicarbonate (NH_4HCO_3), cholesterol (cholest-5-en-3-ol), sodium dodecyl sulfate (SDS), silica gel 60 (230−400 mesh), methylene blue, potassium monobasic phosphate (KH_2PO_4), potassium dibasic phosphate (K_2HPO_4), potassium chloride (KCl), potassium cyanide (KCN), *t*-butyl hydroperoxide (*t*-BuOOH), and hydrogen peroxide (H_2O_2) were purchased from Sigma (St. Louis, MO). Isopropanol and acetonitrile were purchased from J. T. Baker. Dioleoylphosphatidylcholine (DOPC) and tetraoleoylcardiolipin (TOCL) were obtained from Avanti Polar Lipids (Alabaster, AL). Alfa-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) was obtained from the OMRF (Oklahoma City, OK). 3,5-Dibromo-4-nitrosobenzenesulfonate (DBNBS) was synthesized according to Kaur et al.[34] All other reagents were of analytical grade. Stock solutions of ammonium bicarbonate buffer (pH 7.4) were freshly prepared in ultrapure water (Millipore Milli-Q system), and the pH was adjusted to 7.4 prior to use. CYTC concentration was checked prior to each experiment as described before ($\varepsilon_{410\ nm}$ = 106.1 $mM^{-1}\ cm^{-1}$).[20,35]

Synthesis of Cholesterol Hydroperoxides (ChOOH) by the Photooxidation of Cholesterol. ChOOH was synthesized as described by Uemi and co-workers.[36] Briefly, cholesterol (200 mg) was dissolved in 20 mL of chloroform in a 100 mL round-bottomed flask, and 250 μL of methylene blue solution (10 mM in methanol) were added. The solution was ice-cooled and irradiated using two tungsten lamps (500 and 300 W) for 2.5 h under continuous stirring in an oxygen-saturated atmosphere. The mixture of ChOOH isomers was purified by flash column chromatography using silica gel 60 (230−400 mesh). The column was equilibrated with hexane, and a gradient of hexane and ethyl ether was used. After purification, the solvent was evaporated and the hydroperoxides were resuspended in isopropanol, quantified by iodometry, and stored at −80 °C for further use. ChOOH's identities were confirmed by RP-HPLC.

Synthesis of Linoleic Acid Hydroperoxides (LAOOH) by Photooxidation of Linoleic Acid. Linoleic acid hydroperoxide was synthesized as previously described.[37,38] Briefly, linoleic acid (0.5 g) was dissolved in 25 mL of chloroform in a

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50 mL round-bottomed flask, and 0.1 mL of methylene blue solution (100 mM) was added. The solution was ice-cooled and irradiated using two tungsten lamps (500 and 300 W) for 4.5 h under continuous stirring in an oxygen-saturated atmosphere. The mixture of LAOOH isomers (isomers containing hydroperoxides at C-9, -10, -12, and -13) was purified by flash column chromatography.[38] After purification, the solvent was evaporated and LAOOH was resuspended in methanol, quantified by iodometry, and stored at −80 °C for further use.

Liposomes Preparation. Unilamellar vesicle liposomes containing cardiolipin and LAOOH or ChOOH were prepared by an extrusion technique.[24] Dry films of a mixture containing dioleoylphosphatidylcholine (DOPC) and tetraoleoylcardiolipin (TOCL) were prepared from stock solutions in methanol. The solvent was evaporated under a nitrogen atmosphere, and the film that formed was left for 1 h under vacuum to remove traces of organic solvents. The lipid films were resuspended in HEPES buffer (20 mM) with 0.4 mM DTPA and mixed for 1 min in a vortex. The final lipid concentration in liposomes was 2.0 mM (1 mM DOPC and 1 mM TOCL). Alternatively, lipid hydroperoxides-containing liposomes were prepared at a final lipid concentration of 2.08 mM (1 mM DOPC, 1 mM TOCL, and 0.08 mM LAOOH or ChOOH). Unilamelar vesicles with an approximate diameter of 100 nm were prepared by extrusion through polycarbonate membranes (Avestin). The samples were extruded 21 times through the membrane.

CYTC Incubation with SDS Micelles. The reaction between CYTC and ChOOH in the presence of SDS micelles was performed as described previously.[20] Briefly, ammonium bicarbonate buffer (10 mM), pH 7.4, was mixed with SDS (8 mM final concentration) followed by ChOOH (varying concentrations). After 5 min, CYTC (5 μM) was added to the solution, and the reaction was carried out at room temperature (25 ± 0.5 °C) in a Varian Cary 50 Bio spectrophotometer (10 mm path length cuvette). As previously characterized, the interaction of CYTC with SDS micelles, in the concentrations used in our study, induces conformational changes and the displacement of Met^{80} from the axial coordination of the heme group.[33] These alterations are also observed when CYTC interacts with cardiolipin, and it is suggested to be the protein conformation found in the mitochondria.

Transient-State Kinetics. Transient-state kinetic studies were performed with a stopped-flow spectrophotometer (Applied Photophysics SX-18MV) at (25.0 ± 0.5 °C) using the single-mixing mode. CYTC (0.8 μM) in bicarbonate buffer (25 mM) in the presence of SDS micelles was allowed to react with various concentrations of ChOOH, which were in at least 5-fold excess to the protein. CYTC was monitored by the decay of the Soret band at 407 nm. The rate constants for CYTC bleaching promoted by H_2O_2 and LAOOH were determined as controls and compared to those reported in the literature.[27] Previoulsy, Belikova and co-workers used a competitive method and assumed that the peroxidase cycle is irreversible and that the reaction between CYTC and ROOH is the rate-determining step of the whole cycle.[27] Here, we assumed that CYTC reaction with ROOH is the rate-determining step of CYTC bleaching, which was monitored. Indeed, CYTC bleaching is likely caused by rapid radical reactions secondary to the reaction of CYTC with ROOH. The k_{obs} values were determined using the single-curve-fit equation of the instrument's software. Six to ten determinations of k_{obs} were performed for each substrate concentration.

Detection of POBN and DBNBS Radical Adducts by EPR. Unless otherwise stated, ammonium bicarbonate buffer (25 mM), pH 7.4, was mixed with SDS (8 mM), cholesterol hydroperoxide (1 mM), DTPA (0.1 mM), and spin traps POBN (100 mM), DMPO (350 mM), or DBNBS (10 mM). In some experiments, SDS micelles were substituted by cardiolipin-containing liposomes. Reactions were started by CYTC (100 μM) addition and incubated at 25 °C for 5 min before the EPR analysis. Aliquots (100 μL) were transferred to a quartz flat cell, and EPR spectra were recorded at room temperature (21 ± 1 °C) on a Bruker ER 200 D-SRC upgraded to an EMX instrument. The instrumental conditions were as follows: frequency, 9.7 GHz; receiver gain, 6.32×10^4; microwave power, 20 mW; modulation amplitude, 1 G; time constant, 163.84 ms; and conversion time, 163.84 ms. Four single scans were accumulated to improve the signal-to-noise ratio. In some experiments, CYTC (1 mM) was preincubated with cyanide (10 mM) for 5 min before its addition to the reaction mixtures at a final concentration of 100 μM.

POBN Lipid Radical Adduct Analysis by LC-ESI-MS/MS. POBN lipid radical adducts were separated on a C18 column (150 × 3.0 mm, 2.6 μm, Kinetex, Phenomenex) using a gradient of acetonitrile (solvent B) and water (solvent A), both containing formic acid (0.1% v/v). For the analysis, 10−30 μL of the reaction mixture was injected into the column. Elution was started by keeping 10% B for 5 min, then increasing from 10 to 27% B in 5 min, 27 to 70% in 15 min, 70 to 95% in 3 min, keeping at 95% for 17 min, and finally returning to 10% in 1 min. The flow rate was set to 0.2 mL/min. The eluent was monitored with a UV detector at 270 nm and with the mass spectrometer utilizing electrospray ionization (ESI) in positive ion mode (Quattro II triple quadrupole, Micromass, Manchester, UK). The initial 10 min of analysis containing unreacted POBN reagent was discarded to avoid overload of POBN into the MS. The source temperature of the mass spectrometer was held at 150 °C, the desolvation temperature was set at 200 °C, and the capillary and the electrode potentials were set to 2.5 and 0.5 kV, respectively. POBN radical adducts derived from cholesterol hydroperoxides were analyzed with a cone voltage and collision energy of 40 V and 30 eV, respectively. Linoleic acid hydroperoxide−POBN adducts were analyzed with a cone voltage and collision energy of 20 V and 15 eV, respectively. Full scan data from 100 to 800 m/z was acquired. Data were processed by means of the Mass Lynx NT data system.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE electrophoresis was performed to check the formation of CYTC oligomers.[20] Briefly, CYTC incubated in the presence and absence of ChOOH was submitted to electrophoresis in a 15% acrylamide gel under nonreducing conditions. Gels were prepared containing acrylamide (15%), Tris-HCl buffer (0.4 M), ammonium persulfate (0.1%), and SDS (0.1%), pH 8.8. Samples were diluted four times with Laemmli buffer containing Tris-HCl buffer 248 mM (pH 6.8), β-mercaptoethanol (16% v/v), glycerol (40% v/v), SDS (8% w/v), and bromophenol blue (0.04% w/v) and heated at 95 °C for 5 min for denaturation before loading onto the SDS-PAGE gel. After the electrophoretic run, the samples were silver-stained.

RESULTS

ChOOH Induces Concentration-Dependent CYTC Soret Band Bleaching. CYTC was incubated with ChOOH

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in bicarbonate buffered media (pH 7.4), and the resulting UV−vis spectra were recorded after 10 min of incubation (Figure 1).

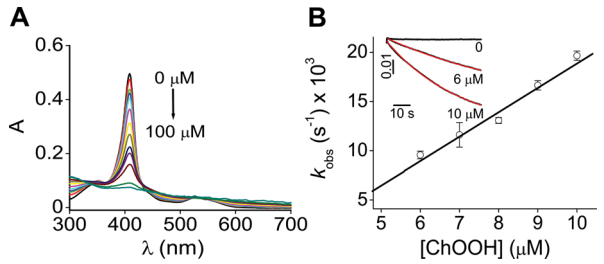

Figure 1. (A) UV−vis scans of CYTC in the presence of different concentrations of ChOOH. All experimental conditions contained bicarbonate buffer (10 mM), pH 7.4, SDS (8 mM), and CYTC (5 μM). ChOOH concentrations ranged from 5 to 100 μM. Scans were registered after 10 min of incubation with ChOOH. Black line represents the control conditions (without ChOOH), and subsequent lines represent each concentration of ChOOH used. (B) Determination of the rate constant for the reaction between ChOOH and CYTC. Pseudo-first-order rate constants (k_{obs}) were determined upon mixing CYTC (0.8 μM) with SDS (8 mM), DTPA (0.1 mM), bicarbonate buffer (25 mM), and ChOOH (at the indicated concentrations), pH 7.4. The reaction time course was monitored at 408 nm at 25 °C for 60 s. The inset graph shows typical traces obtained in the absence (0) and presence of ChOOH (6 and 10 μM). The k_{obs} values were determined using the single-curve-fit equation of the instrument's software (red line). The rate constant was calculated from the slope using linear least-squares regression analysis (black line). The values shown are the mean ± SD obtained from three independent experiments.

The Soret band of CYTC has its maximum absorption at 408 nm when CYTC is incubated in the presence of SDS (8 mM) (black line, Figure 1A).[33] The addition of increasing amounts of ChOOH (5 to 100 μM) induces dose-dependent Soret band bleaching centered at 408 nm (Figure 1A), suggesting that the heme group is being compromised. It is noteworthy that no increase in the absorbance at 550 nm was observed in these experiments, suggesting that the heme was not reduced to its ferrous form.[39] This is an indication that CYTC is mediating free radical reactions using ChOOH as substrate and that these reactions end up oxidizing the heme group.

CYTC Reacts with Lipid Hydroperoxides Faster Than with H_2O_2. We studied the kinetic parameters of the interaction of CYTC (0.8 μM) with SDS micelles with a stopped-flow instrument, following the heme absorbance shift from 410 to 408 nm to confirm the low-spin state of the protein.[33] The interaction of CYTC and SDS did not follow simple second-order kinetics, and the plot of k_{obs} against SDS concentration was a rectangular hyperbola (Figure S2). This saturation behavior indicates a binding interaction between CYTC and SDS micelles. A value of the dissociation constant (K) of 3.1 mM was estimated directly from the hyperbolic equation using a nonlinear least-squares fit to the data. We confirmed that the SDS concentration of 8 mM used in our experiments was enough to displace Met^{80} from the axial coordination at CYTC's active site, generating a species that is likely more similar to that found under *in vivo* conditions.[33]

Next, we determined the reaction of micelle-associated ChOOH (4−10 μM) with CYTC by following heme decay at 408 nm, since the spectroscopic characterization of CYTC compounds I and II is possible only at extremes of pH.[40] Typical pseudo-first-order kinetics was observed for each ChOOH concentration employed (Figure 1B, inset). Plotting the obtained k_{obs} values against the ChOOH concentration provided a straight line, whose slope provided the rate constant of CYTC bleaching: $(2.5 \pm 0.3) \times 10^3$ M^{-1} s^{-1} (pH 7.4, 25 °C) (Figure 1B). This rate was about 2 orders of magnitude higher than that for H_2O_2 (Table 1). For comparison, we also determined the rate constant of CYTC bleaching induced by LAOOH under our experimental conditions (Figure S3). It is noteworthy that the values of the rate constants for CYTC−heme bleaching promoted by SDS micelle-associated peroxides (Table 1) are quite similar to those previously determined for CYTC−heme oxidation by the same peroxides using amplex red oxidation.[27] This similarity supports the assumption that the oxidation of CYTC−heme promoted by the reaction with the hydroperoxide is the rate-limiting step in its bleaching (see Materials and Methods for more details). Since organic hydroperoxides can oxidize CYTC−heme more effectively than H_2O_2, we can infer that reaction with lipid hydroperoxides may be yet another important regulatory function of CYTC in mitochondria.

Table 1. Rate Constants for CYTC Bleaching Promoted by SDS Micelle-Associated Peroxides

R-OOH	k (M^{-1} s^{-1})[a]
H_2O_2	$2.09 \pm 0.02 \times 10^1$
LAOOH	$1.52 \pm 0.05 \times 10^4$
ChOOH	$2.50 \pm 0.20 \times 10^3$

[a]Values correspond to the average of three distinct determinations. Determinations were done as described in the Materials and Methods.

CYTC Reacts with ChOOH To Produce Protein- and Lipid-Derived Carbon-Centered Radicals. EPR spin trapping experiments were performed to confirm the participation of free radicals in CYTC bleaching by ChOOH.[29] The composite EPR spectrum obtained using the spin trap DBNBS (10 mM) was ChOOH concentration-dependent (Figure 2). The immobilized nitroxide detected in the complete reaction mixture (filled circles in Figure 2) is likely due to DBNBS/•Tyr-CYTC radical adducts, as previously reported for the reaction of CYTC with hydrogen peroxide (Scheme S1).[23] The other components of the composite spectra can be attributed to a mobile triplet (aN = 13.6 G) produced from the reaction between DBNBS and a ChOOH-derived tertiary carbon-centered radical (crosses in Figure 2) and to a mobile triplet of doublets (aN = 14.1 G; aH = 7.2 G) (open circles in Figure 2) resulting from the reaction of a ChOOH-derived secondary carbon-centered radical adduct of DBNBS (Scheme S1).[41]

The production of ChOOH-derived radicals was analyzed using another spin trap, POBN, which has been successfully employed for the detection of lipid-derived radicals.[42] CYTC was incubated with ChOOH, LAOOH, or *t*-BuOOH in the presence of SDS micelles and POBN with subsequent EPR analysis (Figure 3). Under control conditions, i.e., in the absence of CYTC, a reasonable signal was detected with both ChOOH and LAOOH but not with *t*-BuOOH, probably due to the thermal instability of the lipid hydroperoxides. However, addition of CYTC to the mixtures containing hydroperoxides yielded stronger EPR signals, apparently corresponding to more than one radical adduct. The hyperfine constants of the predominant spectra were approximately 2.6 and 15.7 G for aH

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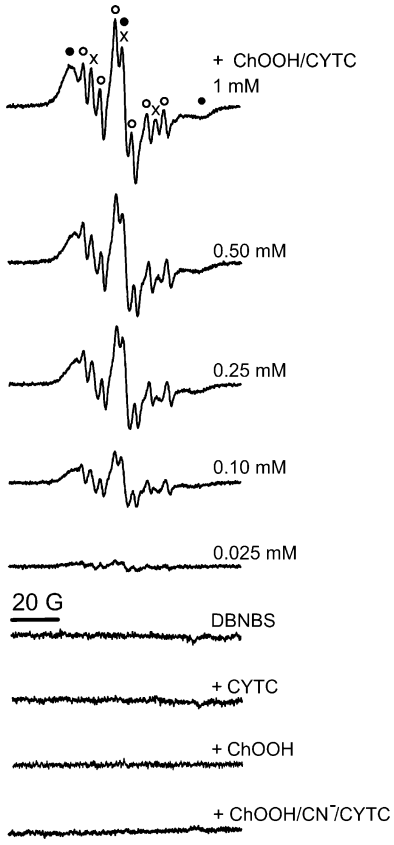

Figure 2. Representative spectra of DBNBS radical adducts produced during the reaction between CYTC and ChOOH. CYTC (100 μM) was added to SDS micelles prepared by mixing SDS (8 mM), DBNBS (10 mM), ChOOH (0−1 mM), and DTPA (0.1 mM) in bicarbonate buffer (25 mM), pH 7.4. The mixtures were incubated at 25 °C. After 5 min, an aliquot was transferred to a quartz flat cell, and the EPR spectrum was recorded. In some experiments, CYTC (1 mM) was preincubated with cyanide (10 mM) for 5 min before its addition to the reaction mixtures. The filled circles, open circles, and crosses mark the spectral lines of protein radical, secondary carbon-centered lipid radical, and tertiary carbon-centered lipid radical, respectively. Spectra were accumulated four times and are representative of three independent experiments.

and aN, respectively, indicating trapping of lipid-derived carbon-centered radicals (Scheme S1).[43,44]

To prove that these signals were not artifacts arising from the use of of SDS micelles, the experiments were repeated with cardiolipin-containing liposomes. As shown in Figure 4, both membrane mimetics provided similar results with 1 mM lipid peroxides. In contrast, incubating CYTC with a lower concentration of LAOOH (5 μM; the same concentration used in ref 27) produced an EPR signal barely distinguishable from noise (Figure 4). Liposomes were also prepared with a combination of DOPC, TOCL, and LAOOH or ChOOH (at a final concentration of 0.08 mM). As expected, the addition of CYTC in the incubation mixtures containing POBN and the liposome-inserted lipid peroxides also rendered EPR signals with hyperfine constants characteristics of carbon-centered radical adducts (Figure S2). Taken together, the EPR spin-trapping experiments suggest that CYTC, in contact with SDS micelles or with cardiolipin-containing liposomes, can split both ChOOH and LAOOH through a homolytic mechanism (Scheme 2).

In an attempt to trap primary alkoxyl radicals, DMPO (at a high concentration of 350 mM) was added to CYTC and SDS micelles in the presence of ChOOH (1 mM). Under our experimental conditions, DMPO adducts were not detected (data not shown). Accordingly, Belikova and co-workers[27] did not trap EPR signals derived from LAOOH in CYTC/liposomes complexes by employing DMPO (500 mM) as the spin trap. These results may indicate that alkoxyl radicals derived from ChOOH rearrange to epoxy allylic radicals faster than they react with DMPO.[45]

Reaction of CYTC with ChOOH Induces Protein Oligomerization. We performed SDS-PAGE experiments in order to test the hypothesis that CYTC oligomerization is induced upon reaction with ChOOH (Figure 5). Native CYTC gives two bands in the SDS-PAGE under reductive conditions, which remained unaltered under our experimental conditions (Figure 5, first lane). The high molecular weight band in the gels corresponds to a CYTC dimer, which is already present in small amounts in the purified protein (see ref 20 and the reactant brochure at www.sigmaaldrich.com). The incubation of CYTC with either 150 or 300 μM ChOOH induces the appearance of species with molecular weights corresponding to CYTC dimer, trimer, and tetramer in a concentration-dependent manner (Figure 5). These oligomers can be formed by the recombination of protein-centered radicals (e.g., dityrosine cross-links) produced upon CYTC reaction with ChOOH (Figure 2). Further analyses are being conducted to characterize these oligomers.

Identification of the POBN−Lipid Adducts by LC-MS/MS. To identify the POBN-lipid radical adducts produced from ChOOH, we performed MS analysis,[42] which showed the

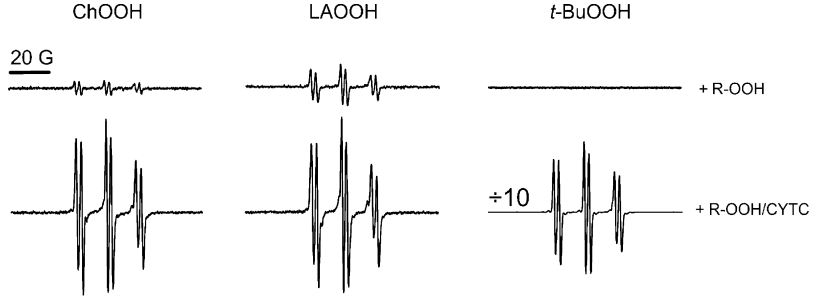

Figure 3. Representative spectra of POBN radical adducts produced during the reaction between CYTC and ChOOH. CYTC (100 μM) was added to SDS micelles prepared by mixing SDS (8 mM), POBN (100 mM), ChOOH (1 mM), LAOOH (1 mM), *t*-BuOOH (1 mM), and DTPA (0.1 mM) in bicarbonate buffer (25 mM), pH 7.4. The mixture was incubated at 25 °C. After 5 min, an aliquot was transferred to a quartz flat cell, and the EPR spectrum was recorded. Spectra were accumulated four times and are representative of three independent experiments.

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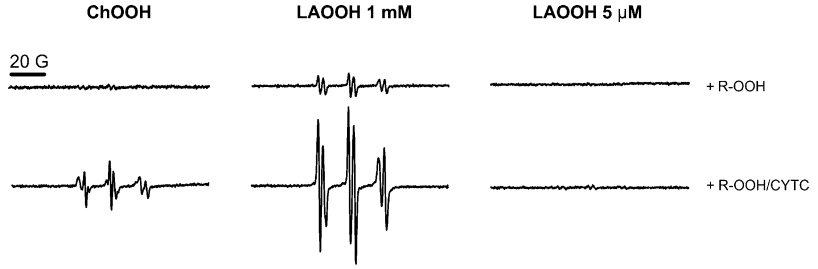
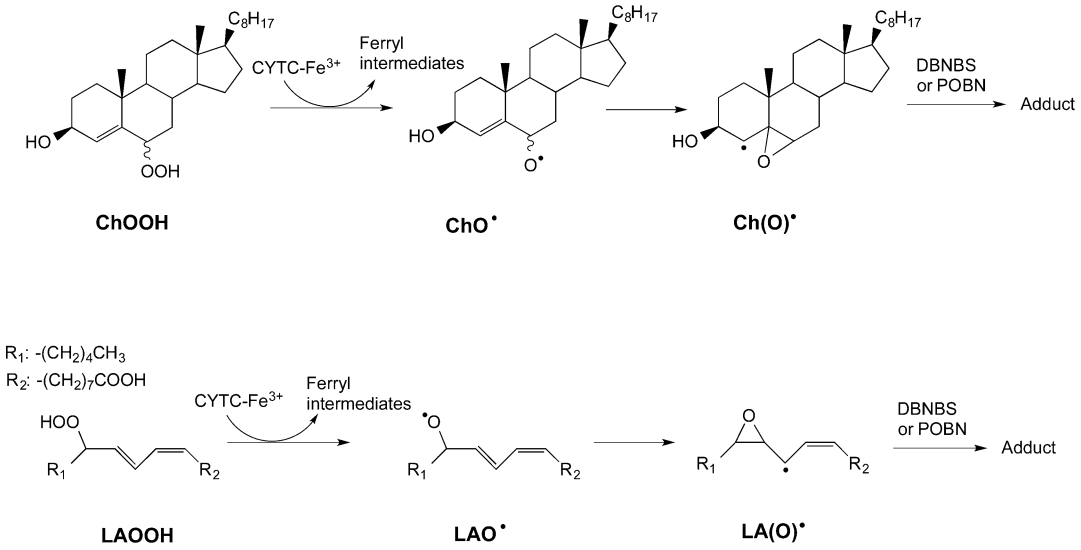


Figure 4. Representative spectra of POBN radical adducts produced during the reaction between CYTC and organic hydroperoxides in liposomes. CYTC (50 μM) was added to cardiolipin-containing liposomes (prepared as described in the Materials and Methods), POBN (100 mM), ChOOH (1 mM), LAOOH (5 μM and 1 mM), and DTPA (0.1 mM) in bicarbonate buffer (25 mM), pH 7.4. The mixture was incubated at 25 °C. After 5 min, an aliquot was transferred to a quartz flat cell, and the EPR spectrum was recorded. Spectra were accumulated four times and are representative of three independent experiments.

Scheme 2. Homolytic Cleavage of the O−O Bond in Lipid Hydroperoxides Mediated by CYTC[a]



[a]The hydroperoxide is cleaved, generating an alkoxyl radical (ChO• or LAO•) that rearranges and producing a carbon-centered radical (Ch(O)• or LA(O)•). This carbon-centered radical, in turn, reacts with spin traps (DBNBS or POBN), forming an adduct that is detectable by EPR or LC-MS/MS. The tertiary ChOOH-derived radical identified in Figure 2 could be attributed to the reaction with the 7-OOH isomer, and the secondary radical could be attributed to the 5-OOH or 6-OOH (ChOOH) isomer. The hydroperoxide isomers at C-6 and C-13 were chosen for ChOOH and LAOOH, respectively, as an example, but the reaction mechanism is the same for all isomers.

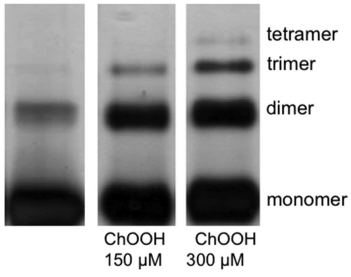


Figure 5. Representative detection of CYTC oligomers produced during the reaction of CYTC with ChOOH by SDS-PAGE. Incubation mixtures contained SDS (8 mM), CYTC (30 μM), and DPTA (0.1 mM) in bicarbonate buffer (10 mM), pH 7.4. Reactions were incubated for 10 min at 25 °C. Lane A represents control conditions containing only CYTC (without ChOOH). Lanes B and C represent conditions in the presence of 150 and 300 μM ChOOH, respectively. Gels are representative of three independent experiments.

presence of a covalent adduct characteristic of a POBN/carbon-centered radical adduct (Figure 6C). The pseudomolecular ion with m/z 595 at 21.87 min is visualized in the selected-ion monitoring (SIM) chromatogram (Figure 6A). The MS spectrum corresponding to this peak is depicted in Figure 6B, where $[M^{\bullet}]^+ = 595$, $[M^{\bullet} - POBN - H_2O]^+ = 383$, and $[M^{\bullet} - ChOOH]^+ = 195$. Similar results were obtained for the reaction between LAOOH and CYTC (Figures 6D−F). The likely POBN/•ChOOH radical adduct is illustrated in Figure 6C. Figure 6D shows an extracted ion chromatogram from the pseudomolecular ion of m/z = 489 at 18.9 min. MS spectrum corresponding to this peak is shown in Figure 6E, where it is possible to identify the following species: $[M^{\bullet}]^+ = 489$, $[M^{\bullet} - H_2O]^+ = 471$, and $[M^{\bullet} - LAO]^+ = 195$. These results suggest that CYTC reacts with both ChOOH or LAOOH through a homolytic pathway, generating an alkoxyl radical that rearranges spontaneously to an epoxy-alkyl radical[45] that can be trapped by POBN.

DISCUSSION

CYTC is peripherally bound to the inner membrane of the mitochondria, which increases the possibility of its reaction with lipid-derived hydroperoxides. Such a reaction could occur by one- or two-electron mechanisms.[27] The first mechanism

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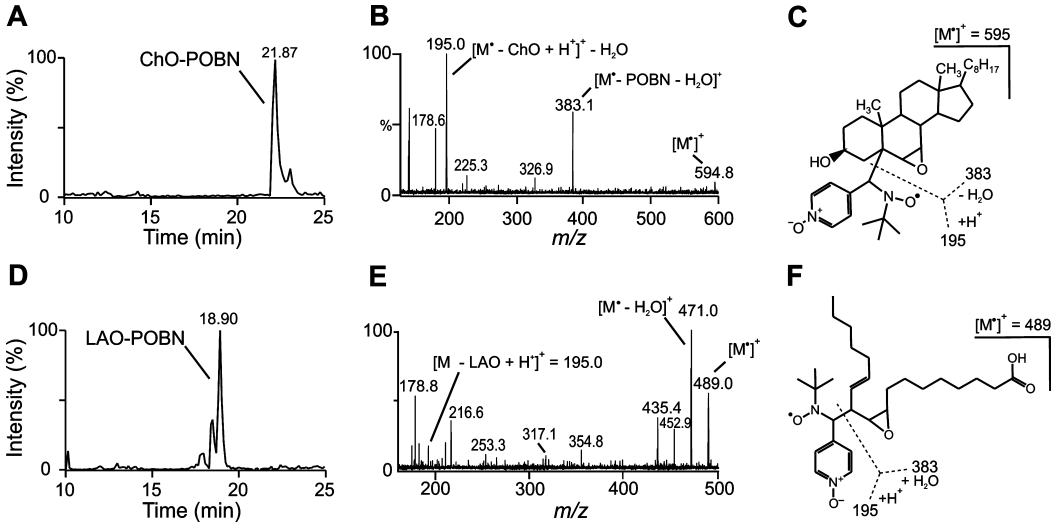

Figure 6. HPLC-MS/MS analyses of POBN−lipid radical adducts produced during the reaction of CYTC with lipid hydroperoxides. Experimental conditions are the same as those described in Figure 3. After EPR analyses, samples of CYTC incubated with CHOOH or LAOOH were analyzed by LC-MS as described in the Materials and Methods. (A) Representative extracted ion chromatogram obtained for ChOOH and CYTC. The peak at 21.87 min corresponds to $[M^{\bullet} + H]^+ = 595$, which is the ion corresponding to the epoxy-alkyl radical of ChOOH trapped by POBN. (B) Representative MS spectrum from the peak at 21.87 min: $[M^{\bullet} + H]^+ = 595$, $[M^{\bullet} + H - POBN - H_2O]^+ = 383$, and $[M^{\bullet} + H - ChOOH]^+ = 195$. (C) Proposed structure of the POBN−ChOOH adduct. (D) Representative extracted ion chromatogram obtained for LAOOH and CYTC. The peak at 18.9 min corresponds to $[M^{\bullet} + H]^+ = 489$, which is the ion corresponding to the epoxy-alkyl radical of LAOOH trapped by POBN. (E) Representative MS spectrum from the peak at 18.9 min: $[M^{\bullet} + H]^+ = 489$, $[M^{\bullet} + H - H_2O]^+ = 471$, and $[M^{\bullet} + H - LAOOH]^+ = 195$. (F) Proposed structure of the POBN−LAOOH adduct. These experiments were performed in triplicate.

consists of the homolytic cleavage of the O−O bond, generating the highly reactive alkoxyl radical.[29] The second mechanism consists of the heterolytic cleavage of the O−O bond, reducing the hydroperoxide group to the corresponding alcohol, which is the catalytic mechanism of several peroxidase enzymes.[46,47] Indeed, this mechanism has been suggested as an antioxidant mechanism of CYTC in the mitochondria.[27,48] Conversely, the homolytic mechanism is likely to be pro-oxidant because the cleavage product (alkoxyl radical) is more reactive than the hydroperoxide.[49] Here, we present evidence toward the CYTC-catalyzed homolytic cleavage of cholesterol hydroperoxides, producing lipid and protein-derived radicals (Figures 2−4). Reaction rates, determined by CYTC Soret band bleaching, demonstrate that CYTC associated with SDS micelles reacts with cholesterol hydroperoxides 10^2 to 10^3 faster compared to that with H_2O_2, confirming its higher reactivity toward lipid-derived hydroperoxides (Table 1).[27]

As an attempt to gain some insight into the mechanism of the reaction between CYTC and ChOOH, we studied the production of radical intermediates. Lipid radicals can be formed by the homolytic cleavage of the O−O bond of lipid hydroperoxides, generating an alkoxyl radical. However, alkoxyl radicals are highly unstable and rearrange spontaneously to the epoxide, generating a carbon-centered radical (Scheme 2).[45,50] Indeed, epoxy allylic radicals were trapped by POBN and DBNBS under our experimental conditions (Figures 2−4). Since the rearrangement of the alkoxyl radical to the epoxide is much faster than the reaction of alkoxyl radical with spin traps, the trapping of this species is difficult.[45,50,51] Accordingly, incubations containing DMPO (350 mM) under our experimental conditions did not trap any alkoxyl radicals in the reaction of CYTC and ChOOH (data not shown).

In a previous study, Belikova and co-workers did not detect any lipid-derived radicals in the reaction of CYTC with fatty acid hydroperoxides, supporting the idea that CYTC acts as an antioxidant.[27] However, it is highly probable that they could not detect any radicals in the reaction of CYTC with LAOOH using DMPO (500 mM) because fatty acid-derived alkoxyl radicals rapidly rearranged to the epoxy-alkyl radical in a manner similar to that of ChOOH (Scheme 2). In addition, the absence of signal in the presence of POBN could probably be due to the low concentration of the lipid hydroperoxide used in the assay. The later hypothesis was confirmed by EPR experiments showing that lipid alkyl radicals were barely detectable with the fatty acid hydroperoxide at 5 μM concentration (Figure 4). However, an intense signal attributed to a carbon-centered lipid-derived radical was observed when CYTC was incubated with higher concentrations of LAOOH (1 mM). Importantly, mass spectrometry analysis of the POBN adducts confirmed the formation of epoxy-alkyl radicals from both fatty acid and cholesterol hydroperoxides (Figure 6), showing that CYTC reacts with fatty acid or cholesterol hydroperoxides to produce alkoxyl radicals that rapidly rearrange to epoxy allylic radicals, propagating the oxidation reaction. This finding is in agreement with a previous study conducted by Barr and Mason,[29] in which they propose the formation of alkoxyl radicals as the primary radical formed upon reaction of CYTC with organic hydroperoxides.

In addition to lipid radicals, the reaction of CYTC with ChOOH also produced protein radicals. These radicals were detected using DBNBS for spin trapping (Figures 2), and their formation can be explained by the production of high-valent heme intermediates in both hetero- and homolytic cleavages of the hydroperoxide O−O bond. In analogy to the classic peroxidases, the heterolytic mechanism would occur with the two-electron oxidation of CYTC to compound I (Fe^{4+}=O porphyrin π-cation radical) with the reduction of the hydroperoxide to its corresponding hydroxide. However, CYTC is not a peroxidase enzyme *per se*, and our data point

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out that its return to the native form is not as effective as in other peroxidases.

In fact, the literature shows that CYTC ends the "peroxidase cycle" with either the heme group oxidized (Soret band bleaching) or with amino acids oxidized (i.e., tyrosine residues).[23,52] In line with these findings, the full recovery of the native protein after a peroxidase cycle has never been demonstrated. This fact is confirmed by the results depicted in Figure 1A, where the incubation of CYTC with increasing concentrations of ChOOH promoted concentration-dependent Soret band bleaching. In parallel, increasing concentrations of ChOOH also promoted a concentration-dependent increase in the EPR signal of both DBNBS/•lipid and DBNBS/•protein radical adducts (Figure 2), suggesting that oxidation of the heme group and appearance of the lipid and protein radicals are co-dependent processes. This hypothesis is further supported by the experiments where CYTC was preincubated with CN^-, a well-recognized general peroxidase inhibitor[53] that prevents CYTC reaction with ChOOH (Figure 2). Therefore, on the basis of the assumptions described above, the homolytic mechanism (Scheme 2) could explain the formation of both lipid and protein carbon-centered radicals and is probably the preferential route by which CYTC reacts with ChOOH. It should be pointed out that the heterolytic mechanism could also explain the formation of protein-derived carbon-centered radicals observed in Figure 2, but it fails to explain the formation of epoxy-alkyl radicals trapped by DBNBS and POBN and identified by MS (Figure 6).

Reaction of CYCT with ChOOH induces protein oligomerization, as depicted by the detection of dimers, trimers, and tetramers by SDS-PAGE (Figure 5). These oligomers can be formed through intermolecular cross-linking involving protein radicals. Tyrosine residues have been previously identified to be oxidized and dimerized upon CYTC reaction with peroxides.[23,52,54] Oxidation of tyrosine residues generates a phenoxyl radical, which, in turn, resonates in the aromatic ring, giving carbon-centered radicals.[55] These radicals, in turn, could be trapped by spin traps (Figures 2, S1, and S6) or recombine to produce protein dimers and, subsequently, oligomers, as observed by the formation of CYTC trimers and tetramers (Figure 5).[54,55]

Alternatively, protein oligomers could also result from protein hydrophobic interactions, generating strong noncovalent species resistant to SDS treatment.[56] This finding could also explain the oligomers observed under our conditions (Figure 5). Noncovalent CYTC oligomers (dimers and trimers) are known to be formed when CYTC is treated with ethanol (60% v/v),[57,58] but these species seem to be stable only at low temperatures (4 °C), being converted back to the monomers when heated at 70 °C for 5 min.[58] Furthermore, dimeric species have also been described for CYTC incubated with SDS (4 mM) for 10 min at 20 °C.[58] However, we were not able to detect increased dimerization of CYTC by SDS-PAGE in the presence of SDS only (Figure 5, line 1, and ref 20). It is important to mention that dimeric CYTC species are already present in control samples[20] incubated in the absence of SDS. Such species, however, migrate slightly differently from that of CYTC dimers upon ChOOH treatment (data not shown). Thus, further mass spectrometry experiments are being conducted in order to characterize the real nature of the CYTC oligomers seen in Figure 5.

In summary, our results corroborate available data showing that CYTC reacts with lipid-derived hydroperoxides with higher rate constants than that with other peroxides (i.e., H_2O_2).[27] Our data shows that CYTC reacts with cholesterol hydroperoxides (and other lipid-derived hydroperoxides such as LAOOH) through a homolytic mechanism, producing lipid and protein radicals and promoting CYTC inactivation and oligomerization. From an evolutionary perspective, CYTC evolved to transport electrons in the mitochondria through a redox cycle that involves the reduction and reoxidation of the heme iron ($Fe^{3+}/Fe^{2+}/Fe^{3+}$). Under conditions of enhanced oxidative stress in the mitochondria, lipid hydroperoxide production would be increased and reaction with CYTC would be more likely to occur. We speculate that under these circumstances CYTC may contribute to increasing the oxidative stress in the mitochondria and, ultimately, to apoptosis signaling.

■ ASSOCIATED CONTENT

Supporting Information

EPR spectra of lipid-derived hydroperoxides previously inserted in the liposome, kinetic data of the interaction of SDS with CYTC, and kinetic data of the reaction of H_2O_2 with CYTC. Schematic reactions of epoxy-alkyl or tyrosyl radicals with spin traps are also depicted. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/bi501409d.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ChOOH, cholesterol-derived hydroperoxides; CYTC, cytochrome *c*; SDS, sodium dodecyl sulfate; StAR, steroidogenic acute regulatory protein; *t*-BuOOH, *tert*-butyl hydroperoxide; LAOOH, linoleic acid-derived hydroperoxides; ROOH, hydroperoxides; ROS, reactive oxygen species; EPR, electron paramagnetic resonance; TOCL, tetraoleoylcardiolipin;

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DOPC, dioleoylphosphatidylcholine; CL, cardiolipin; DTPA, diethylenetriaminepentaacetic acid; PBN, alpha-phenyl *N*-*tert*-butyl nitrone; DMPO, 5,5-dimethyl-pyrroline *N*-oxide; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; KCN, potassium cyanide; MNP, 2-methyl-2-nitrosopropane; LC-MS, liquid chromatography coupled with mass spectrometry; ESI, electrospray ionization; SDS-PAGE, SDS polyacrylamide gel electrophoresis; HRP, horseradish peroxidase

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