Nitric Oxide Inhibits Peroxidase Activity of Cytochrome c-Cardiolipin Complex and Blocks Cardiolipin Oxidation*

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The increased production of NO during the early stages of apoptosis indicates its potential involvement in the regulation of programmed cell death through yet to be identified mechanisms. Recently, an important role for catalytically competent peroxidase form of pentacoordinate cytochrome c (cyt c) in a complex with a mitochondria-specific phospholipid, cardiolipin (CL), has been demonstrated during execution of the apoptotic program. Because the cyt c-CL complex acts as CL oxidase and selectively oxidizes CL in apoptotic cells in a reaction dependent on the generation of protein-derived (tyrosyl) radicals, we hypothesized that binding and nitrosylation of cyt c regulates CL oxidation. Here we demonstrate by low temperature electron paramagnetic resonance spectroscopy that CL facilitated interactions of ferro- and ferri-states of cyt c with NO and NO⁺, respectively, to yield a mixture of penta- and hexa-coordinate nitrosylated cyt c. In the nitrosylated cyt c-CL complex, NO chemically reacted with H₂O₂-activated peroxidase intermediates resulting in their reduction. A dose-dependent quenching of H₂O₂-induced protein-derived radicals by NO donors was shown using direct electron paramagnetic resonance measurements as well as immuno-spin trapping with antibodies against protein 5,5-dimethyl-1-pyrroline N-oxide-nitrooxide adducts. In the presence of NO donors, H₂O₂-induced oligomeric forms of cyt c positively stained for 3-nitrotyrosine confirming the reactivity of NO against protein 5,5-dimethyl-1-pyrroline N-oxide-nitrooxide adducts. Given the importance of CL oxidation in apoptosis, mass spectrometry analysis was utilized to assess the effects of NO on oxidation of 1,1',2,2'-tertalinoleoyl cardiolipin. NO effectively inhibited 1,1',2,2'-tertalinoleoyl cardiolipin oxidation catalyzed by the peroxidase activity of cyt c. Thus, NO can act as a regulator of peroxidase activity of cyt c-CL complexes.

Cyt c is an essential component of the protein assembly in the intermembrane space of mitochondria where it shuttles electrons between complex III (ubiquinol:cytochrome c reductase) and complex IV (cytochrome c oxidase). The electron transporting function of cyt c is effectively accommodated by its hexa-coordinate arrangement of heme iron, whereby Met⁶⁸ and His¹⁴ represent the two axial ligands (1). The hexa-coordinate organization of heme-iron, however, hinders binding and chemical interactions of cyt c with small molecules such as NO, O₂, CO, and H₂O₂; therefore cyt c in solution exhibits only a marginal peroxidase activity (2–6). The hindrance can be eliminated, and the redox catalytic reactivity can be conferred on cyt c by converting its hexa-coordinate arrangement into a penta-coordinate form by full or partial unfolding of the protein with strong denaturing agents such as guanidinium chloride or chemical modification of Met⁶⁸ (7, 8). Similar structural perturbations accompanied by an exchange and a loss of axial ligands and an increase of peroxidase activity were observed when cyt c interacted with negatively charged phospholipid membranes (3–5, 9–11). The structure of cyt c bound to the anionic membranes and its peroxidase activity strongly depend on the experimental conditions, such as membrane composition, protein-to-lipid ratio, ionic strength, and pH (1, 5, 7–12). Denatured cyt c was shown to display an increased NO binding and a significant peroxidase activity in the presence of H₂O₂ (7, 8, 13–15). Chen et al. (13) reported NO consumption by HOCl-modified cyt c. However, the studies were performed in model systems under conditions and/or treatments hardly compatible with known physiological or pathophysiological roles of cyt c.

NO was shown to play an important role in the regulation of mitochondrial respiration (16). One established mechanism of this regulatory function is reversible inhibition of cytochrome c oxidase (17). The discovery of mitochondrial nitric-oxide synthase emphasized the significance of NO in mitochondrial functions (18, 19), although NO can also reach mitochondria from external sources because of its hydrophobic nature and facile diffusion in biomembranes (20, 21). An increased production of NO has been demonstrated during early stages of apoptosis indicating its potential involvement in regulation of programmed cell death (22, 23). One possible pathway is the interactions of NO with superoxide generated by disrupted electron transport yielding a very reactive oxidant, peroxynitrite, that may cause mitochondrial damage during apoptosis (20, 21, 24). However, the protective effects of NO in apoptosis also were demonstrated (25).

Our previous work has identified CL as a physiologically relevant regulator of peroxidase activity of cyt c markedly enhanced during apoptosis (5). Specific peroxidation of CL catalyzed by the peroxidase activity of CL-bound cyt c molecules contributes to mitochondrial outer membrane permeabilization and the release of proapoptotic factors from the intermembrane space of mitochondria into the cytosol, a critical step in the intrinsic pathway of apoptosis.

The cyt c-CL complex has very different structural and catalytic properties than cyt c in solution. Therefore its interactions with NO may differ substantially from those of isolated cyt c. We were specifically...
interested in the NO regulation of peroxidase activity of cyt c-CL complex because of its role in induction of apoptosis. Although several recent studies indicate that CL-induced unfolding of cyt c is associated with the formation of its penta-coordinate state (5, 9), interactions of the cyt c-CL complex with NO and its effects on the peroxidase activity of the complex remain unknown. Here we report, for the first time, that CL facilitates the binding and chemical interactions of NO with cyt c and its reactive intermediates formed in the presence of H$_2$O$_2$. Thus, NO can act as a regulator of the H$_2$O$_2$-dependent peroxidase activity of the cyt c-CL complex.

**EXPERIMENTAL PROCEDURES**

**Materials**

Horse heart cytochrome c (type C-7752), potassium phosphate, HEPES-Na, diethylentraminepentaaetic acid (DTPA), ascorbate, etoposide (VP16, demethylpipodophyllotoxin-ethylidene-glucopyranoside), hydrogen peroxide, 3,3’-diaminobenzidine (DAB), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), microperoxidase 11 (MP-11), phospholipase A$_2$, and catalase were from Sigma-Aldrich. Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine) was obtained from Invitrogen. 1,1-Xylidine NONOate was purchased from Invitrogen. 1,1-Dimethyl-2-trinitro-4-phenoxazine) was obtained from Molecular Probes (Eugene, OR). HPLC grade solvents, fetal bovine serum, RPMI 1640 with and without phenol red, sodium phosphate buffer, pH 7.4, diethylentriaminepentaacetic acid (DTPA), ascorbate, etoposide (VP16, demethylepipodophyllotoxin-ethylenediene-glucopyranoside), horse heart cytochrome c (type C-7752), potassium phosphate, HEPES-Na, diethylentriaminepentaacetic acid (DTPA), ascorbate, etoposide (VP16, demethylepipodophyllotoxin-ethylenediene-glucopyranoside), 0.5 G; receiver gain, 103; time constant, 0.03 s; scan time, 8 min. The spectra were recorded at 77 K under the following instrumental settings: center field, 3200 G; scan range, 500 G; modulation amplitude, 5 GHz; microwave power, 10 milliwatt; time constant, 0.1 s; scan time, 4 min; receiver gain, 5 $\times$ 105.

**Flux NO**

![Flux NO](image)

**Flux NO**

where Flux$_{NO}$ was computed from Equation 1.

The inhibition constant (IC$_{50}$) was determined as the concentration of an NO donor (or Angeli’s salt) that caused a 2-fold decrease of the cyt c activity. Corresponding half-inhibiting NO concentration was estimated from IC$_{50}$ of NO donor according to Equation 2.

**Low Temperature EPR Measurements**

Nitrosylation of cyt c was performed in the presence of DOPC-TOCL liposomes with different content of CL (total lipid, 8 mM) in 25 mM sodium phosphate buffer, pH 7.4.

For cyt c nitrosylation by NO donors, ferro-cyt c (100 $\mu$M) was incubated for 15 min with liposomes in the presence of 500 $\mu$M NO donor (DEANOate at room temperature or PAPANONOate at 37 °C) under N2$_2$. When Angeli’s salt (750 $\mu$M) was used as a donor of nitrosyl (HNO, pK$_a$ = 11.4), it was incubated with ferri-cyt c (200 $\mu$M) for 10 min at room temperature. The reaction was stopped by freezing the samples in liquid nitrogen. The spectra were recorded at 77 K under the following conditions: center field, 3230 G; sweep width, 500 G; modulation amplitude, 5 G; microwave power, 10 milliwatt; time constant, 0.1 s; scan time, 4 min; receiver gain, 5 $\times$ 105.

**Absorbance Spectra of Ferri-cytochrome c**

Cyt c (5 $\mu$M) was incubated for 5 min with DOPC-TOCL liposomes (1:1; total lipid 5 mM) preincubated with DOPC/TOCL liposomes (1:1; total lipid 5 mM) for 1 min at room temperature. EPR spectra from frozen samples were detected at 77 K under the following conditions: center field, 3230 G; sweep width, 100 G; field modulation, 5 G; microwave power, 1 milliwatt; receiver gain, 2 $\times$ 105; time constant, 0.1 s; time scan, 4 min.

**Peroxidase Activity Measurements**

Etoposide-based Assay—EPR spectra of etoposide phenoxyl radical were recorded at 25 °C in gas-permeable Teflon tubing (inner diameter, 0.8 mm; thickness, 0.013; Alpha Wire Corp., Elizabeth, NJ). The tubing (length, ~6 cm) was filled with 60 $\mu$l of sample, double-folded, and placed in an open 3.0-mm-internal diameter EPR quartz tube. The spectra were recorded under the following EPR conditions: center field, 3350 G; sweep width, 50 G; microwave power, 10 milliwatt; field modulation, 0.5 G; receiver gain, 103; time constant, 0.03 s; scan time, 8 min. The time course of etoposide radical signal was obtained by repeated scanning of the field corresponding to a part of the EPR signal (sweep width, 5 G; the other instrumental conditions were the same).

To obtain a solution of etoposide phenoxyl radicals free of the radical-generating system, horseradish peroxidase (40 $\mu$g/ml) was incubated with etoposide (200 $\mu$M) and H$_2$O$_2$ (100 $\mu$M) for 4 min; then the solution was quickly passed through an Amicon Ultra PL-30 filter (Millipore Co.,
NO Regulates Cardiolipin Oxidation by Cytochrome c

France). The filtrate containing etoposide phenoxyl radicals was used immediately to monitor its interactions with reactive nitrogen species.

Peroxidase-catalyzed Oxidation—Peroxidase-catalyzed oxidation of DAB was monitored spectrophotometrically (absorbance at 470 nm) for 10 min in 20 mM HEPES- Na buffer at room temperature (28).

PAGE of Cyt c-Cardiolipin Complexes

Cyt c-Fe(III) (30 μM) was incubated with DOPC:TOCL liposomes in the presence of H₂O₂ (200 μM each 15 min). The peroxidase reaction was stopped by 1400 units/ml catalase. Then the loading buffer was added, and the samples were immediately loaded onto a gel. SDS-PAGE was run using 12.5% running gel and 4% stacking gel as described in Ref. 29. The gels were stained with Coomassie R-250 Brilliant Blue.

Western Blotting of Cyt c-CL

For blotting, the proteins were electrophoretically transferred to nitrocellulose membrane, and the membrane was incubated with anti-DMPO or anti-nitrotyrosine antibodies. Incubation with anti-DMPO antibodies was performed essentially as described in Ref. 30. In the case of anti-nitrotyrosine antibodies, nonspecific binding was blocked by the incubation in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 of anti-nitrotyrosine antibodies, nonspecific binding was blocked by the incubation in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 (TBS-T) with 3% bovine serum albumin for 1.5 h at room temperature. The membrane was then incubated with anti-nitrotyrosine antibodies (1:5000 in TBS-T with 1% bovine serum albumin) overnight at 4 °C. After washing five times with TBS-T, the membrane was incubated with the secondary antibody conjugated with alkaline phosphatase for 1 h at room temperature. After washing three times with TBS-T, the bands were visualized using chemiluminescent substrate for the detection of alkaline phosphatase, Lumi-Phos™ WB.

Detection of NO

NO was quantitated amperometrically using a NO-selective electrode (Iso-NO; 2-mm shielded sensor: PPI, Sarasota, FL). The samples were incubated at room temperature in a reaction chamber under continuous stirring. Changes in current output (pA) were recorded, and NO was quantified using a standard curve generated by the addition of NaNO₂ in nitrite-free water under reducing conditions (KI/H₂SO₄).

Lipid Extraction and Two-dimensional HPTLC Analysis

Lipids were extracted from liposomes using the Folch procedure (31). The lipid extracts were separated and analyzed by HPTLC (high performance thin layer chromatography) as previously described (32). Lipid phosphorus was determined as reported in Ref. 33.

Phospholipid Hydroperoxides

Phospholipid hydroperoxides were determined by fluorescence HPLC of products formed in MP-11-catalyzed reactions with a fluorogenic substrate, Amplex Red, according to our newly developed protocol (5, 34). Oxidized phospholipids were hydrolyzed by porcine pancreatic phospholipase A₂ (0.2 unit/μL) in 25 mM phosphate buffer containing 1.0 mM Ca²⁺, 0.5 mM EDTA, and 0.5 mM SDS (pH 8.0 at room temperature for 30 min). After that, 50 μL Amplex Red and MP-11 (1.0 μg/μL) were added, and the samples were incubated at 4 °C for 40 min. The reaction was terminated by the addition of 100 μL of a stop reagent (10 mM HCl, 4 mM butylated hydroxytoluene in ethanol). After centrifugation at 15,000 × g for 5 min, aliquots of supernatant (5 μL) were injected into Eclipse XDB-C18 column (5 μm, 150 × 4.6 mm). The mobile phase was composed of 25 mM NaH₂PO₄, pH 7.0, methanol (60:40, v/v). The flow rate was 1 ml/min. The resorufin (an Amplex Red oxidation product) fluorescence was measured at 590 nm after excitation at 560 nm. Shimadzu LC-100AT vp HPLC system equipped with a fluorescence detector (RF-10Axl) and an autosampler (SIL-10AD vp) was used. The data were processed and stored in digital form with Class-VP software.

ESI Tandem Mass Spectrometry

ESI (electrospray ionization) tandem mass spectrometry of TLCL oxidation products was performed by direct infusion into a Finnigan MAT TSQ 70 mass spectrometer with a triple-quadrupole tandem mass spectrometry analyzer (Thermo Electron Co.). Sheath flow was adjusted to 5 μL/min, and the solvent consisted of chloroform:methanol (2:1, v/v). The electrospray probe was operated at a voltage differential of −3.5 kV in the negative ion mode. Mass spectra for doubly and singly charged CL were obtained by scanning in the range of 400–950 and 1200–1800 m/z, respectively, every 1–1.5 s and summing individual spectra. The source temperature was maintained at 70 °C.

Statistical Analyses

The data are expressed as the means ± S.D. of at least triplicate determinations. Changes in variables were analyzed by one-way analysis of variance for multiple comparisons. The differences were considered significant at p < 0.05.

RESULTS

Cyt c Heme Nitrosylation—We used low temperature (77 K) EPR spectroscopy to study the effects of CL on nitrosylation of cyt c. Only weak predominantly hexa-coordinate EPR signal was observed from a system in which cyt c-Fe(II) and an NO donor, DEANOate, were incubated with DOPC liposomes lacking TOCL. The spectrum of hexa-coordinate NO-heme complex showed the characteristic shape with rhombic symmetry around the paramagnetic center. There were peaks at g = 2.068 and 1.986 and, in between, an “inverted S shape” (35). Because of the broadness of the peaks and their overlapping, the g values must be considered only as tentative. Incubation of ferro-cyt c with DOPC liposomes containing TOCL (at 1:1 molar ratio) in the presence of DEANOate resulted in a marked increase of the amount of nitrosylated cyt c, as well as the appearance of a signal from a penta-coordinate heme-iron complex. In the presence of TOCL, a new three-line signal with a splitting of 17 G emerged at g = 2.009, and a new low field peak also appeared at g ≈ 2.094. Based on the results presented on Fig. 1, the percentage of penta-coordinate cyt c can be estimated by subtraction of spectrum a from spectrum b or c. The estimate shows that ~20% of NO-heme was penta-coordinate at cyt c:TOCL = 15 (TOCL = 1.5 mM, Fig. 1A, panel b), and its content increased to more than 40% at cyt c:TOCL = 40 (TOCL = 4 mM, Fig. 1A, panel c).

Similarly, the incubation of a nitroxyl donor, Angeli’s salt (26, 36), with ferri-cyt c-TOCL complexes caused the formation of typical EPR signals of heme-Fe(II)-nitrosylated species (Fig. 1B). In this case too, the increased amount of nitrosylated cyt c with markedly expressed signal of its penta-coordinate form was observed in the presence of TOCL (Fig. 1B, panels b–d).

The complex of NO with ferri-cyt c is not detectable by EPR because the NO-ferri-porphyrin complex is EPR silent (37). However, optical absorption spectroscopy readily detected the interaction of cyt c-Fe(III) with NO (Fig. 2). The spectrum of ferri-cyt c-TOCL complexes showed significant changes after the addition of 100 μM of DEANOate (Fig. 2), indicative of the Fe(III)-NO complex formation (15, 22, 37). The Soret maximum shifted from 409 to 415 nm, and a new peak at 562 nm was observed. The effect was not detectable with solubilized cyt c in the absence of DOPC:TOCL liposomes.
not likely due to competitive binding to the complex of H2O2 because the effect was observed at equimolar concentrations of NO and H2O2. Similarly, NO gas dissolved in aqueous solution effectively quenched the EPR signal of nitrosylated cyt c, which is a distinctly weaker ligand for ferro-cyt than NO (38, 39), yet the effect was observed at equimolar concentrations of NO and H2O2.

Conversely, when H2O2 was incubated with cyt c, a typical EPR signal of protein-derived radicals of the activated peroxidase was induced (Fig. 3B) (3, 40). The signal was quenched by either an NO donor, DAENOate, or by a nitrosyl anion donor, Angeli’s salt. Similarly, NO gas dissolved in aqueous solution effectively quenched protein-derived radicals of cyt c-CL complexes induced by H2O2. Neither H2O2-induced protein derived radicals nor their quenching were detectable in the absence of TOCL.

Combined, these results demonstrate that CL facilitates binding of NO with cyt c. Thus, heme of cyt c in the complex with CL is accessible for nitrosylation by NO and HNO both in its ferro- and ferri-states.

**NO Regulates Cardiolipin Oxidation by Cytochrome c**

**FIGURE 1.** CL facilitates cyt c nitrosylation as evidenced by EPR spectroscopy. EPR spectra of nitrosylated cyt c in TOCL complexes formed in the presence of an NO donor, DAENOate (A) or a nitrosyl generator Angeli’s salt (B). Panels a–c, EPR spectra of nitrosylated ferro-cyt c at different TOCL concentrations (in A, panel a, 0.2 mM; panel b, 1.5 mM; panel c, 4 mM; in B, panel a, 0.75 mM; panel b, 3.5 mM; panel c, 7 mM). Panels d, dependence of integral intensity of EPR spectra of nitrosylated cyt c-Fe(II) on the cardioperoxidase ratio. Note that an increase of TOCL resulted not only in increased amounts of nitrosylated cyt c, but also in a larger contribution of penta-coordinate form in the total signal of nitrosylated cyt c. Cyt c (A, 100 µM ferrous; B, 200 µM ferric) was incubated with DOPC-TOCL liposomes (with varied ratios of two phospholipids, the total phospholipid concentration 8 mM) in the presence of 500 µM DAENOate 15 min (A, N2 conditions) or of 750 µM Angeli’s salt 10 min (B) at room temperature in 25 mM sodium phosphate buffer, pH 7.4, containing 100 µM DTPA. The reaction was stopped by freezing the samples in liquid nitrogen. The spectra were recorded at 77 K. The relative gain is indicated on the spectra.

**FIGURE 2.** Optical spectra of nitrosylated cyt c-CL complexes. Absorbance spectra of cyt c-Fe(III)-CL (solid line) and cyt c-Fe(III)-CL 10 min after the addition of DEANOate (dotted line). The inset shows the 500–600 nm region of the spectra for control (solid line) and nitrosylated (dotted line) cyt c. Ferri-cyt c (5 µM) was incubated for 5 min with DOPC-TOCL liposomes (1:1; total lipid 200 µM) in 25 mM sodium phosphate buffer, pH 7.4, 100 µM DTPA. Then 100 µM DEANOate was added, and the spectrum was recorded 6 min thereafter.

**FIGURE 3.** Interactions of H2O2 with nitrosylated cyt c-TOCL as evidenced by EPR spectroscopy. A, EPR spectra of nitrosylated cyt c-Fe(III)-TOCL in the presence and absence of H2O2. The addition of H2O2 to nitrosylated cyt c-TOCL resulted in a decrease of EPR signal. Panel a, spectrum of ferri-cyt c (200 µM) plus TOCL (DOPC-TOCL liposomes, 1:1; total lipid, 5 mM) after incubation with 750 µM Angeli’s salt (10 min). Panel b, the same sample, but 750 µM H2O2 was added 20 s before freezing of the sample. Effect of DEANOate on protein-derived radicals of cyt c-Fe(III)-TOCL. The inset shows a typical EPR spectrum of H2O2-induced protein-derived radicals of cyt c-TOCL. Concentration-dependent quenching of H2O2-induced protein-derived radicals of ferri-cyt c-TOCL by DEANOate. Ferri-cyt c (200 µM) was incubated with DOPC-TOCL liposomes (1:1; 5 mM total lipid) and DEANOate for 15 min at room temperature in 25 mM sodium phosphate buffer, pH 7.4, 100 µM DTPA; then H2O2 (1 mM) was added. The reaction was stopped after 20 s by freezing the samples in liquid nitrogen.

H2O2-induced Protein-derived (Tyrosyl) Radicals of Cyt c-CL Complexes Are Quenched by NO Donors—Incubation of cyt c-CL complexes with H2O2 caused the formation of multiple protein bands revealed by Coomassie Blue staining on PAGE gels. These bands corresponded to different oligomeric forms of cyt c: dimers, trimers, tetramers, pentamers, and hexamers.
The protocol has been shown to be specific for protein-derived, likely tyrosyl, radicals in different activated hemoproteins (30, 41), including cyt c (13).

Using a Western blotting technique with antibodies against DMPO, we found that the antibodies readily interacted with all of the bands corresponding to different oligomeric forms of cyt c (Fig. 4B). This indicates that the presence of the immobilized DMPO nitroxide adducts likely formed on tyrosyl radicals of activated cyt c. High molecular weight aggregates contained more DMPO adducts than low molecular weight aggregates. A concentration-dependent decrease of DMPO spin adducts in protein (tyrosyl) radicals was observed in the presence of an NO donor, PAPANONOate (Fig. 4B). Notably, PAPANONOate also caused a dose-dependent inhibition of the formation of high molecular weight aggregates of cyt c and increased the proportion of its monomeric form revealed by PAGE (Figs. 4A and 5A).

To more definitively characterize the involvement of tyrosyl radical intermediates, we performed Western blotting using anti-3-nitrotyrosine antibodies (13, 42, 43). Oligomeric forms of cyt c, in particular its high molecular mass aggregates, revealed a positive staining for 3-nitrotyrosine when PAPANONOate was added to the incubation system (particularly at high concentrations) (Fig. 5B). In a separate series of low temperature EPR experiments, we confirmed that PAPANONOate was able to quench H$_2$O$_2$-induced signals of protein-derived radicals, similarly to the effects of DEANOate (Fig. 3B).

Maximal concentrations of PAPANONOate (1 mM) were not sufficient to completely block oxidation of cyt c and formation of tyrosyl radicals and oligomers, at high concentrations of H$_2$O$_2$ employed (800 μM H$_2$O$_2$, 200 μM, four times). Nevertheless, the increase of the monomeric form of the protein is quite apparent in Figs. 4 and 5.

Thus, protein-derived, likely tyrosyl, radicals are produced as reactive intermediates of catalytically competent peroxidase cyt c-CL complexes. Because NO interacts with these radical intermediates, we further studied whether NO acted as an inhibitor of peroxidase activity of cyt c-CL complexes.

**Inhibition of Peroxidase Activity of cyt c-CL Complexes**—We tested the peroxidase activity of cyt c-Fe(III)-CL complexes in the presence and absence of NO donors using two different protocols (Fig. 6). In the first one, we used EPR spectroscopy to monitor the formation of etoposide phenoxyl radicals during one-electron oxidation of etoposide (44). The addition of H$_2$O$_2$ to cyt c-TOCL complexes in the presence of etoposide produced a characteristic EPR signal of etoposide phenoxyl radical (Fig. 6A) whose magnitude monotonously increased, reached a plateau after 4 min of recording, and did not decay over a subsequent 5-min period. In the absence of TOCL, the magnitude of the signal was ~50 times less. In contrast, preincubation of cyt c-CL with DEANOate caused a progressive decrease of the magnitude of the signal (IC$_{50}$ $\approx$ 25 ± 5 μM, the corresponding half-inhibiting NO concentration, ~4 μM). Because the lifetime of etoposide phenoxyl radicals is on the order of several min at μM concentrations (45), we demonstrated, in separate experiments, that NO did not directly interact with the etoposide radicals. This was shown as a lack of any effect of DEANOate on the EPR signal of the etoposide radical detectable after its separation from the generating system, horseradish peroxidase/H$_2$O$_2$, by ultrafiltration/centrifugation.

Second, we used cyt c-CL-peroxidase-catalyzed oxidation of DAB (28) and found that DEANOate caused inhibition of DAB oxidation in a dose-dependent manner with IC$_{50}$ = 10 ± 3 μM (the corresponding half-inhibiting NO concentration, ~2.5 μM) (Fig. 6B). These results demonstrate that NO acts as an effective inhibitor of peroxidase activity of cyt c-CL complex.

**NO Consumption by cyt c-CL Peroxidase**—We further assessed consumption of NO during incubation of ferri-cyt c-CL complexes using an...
NO Regulates Cardiolipin Oxidation by Cytochrome c

FIGURE 6. Effects of NO donors on the peroxidase activity of cyt c-TLCL. A, a cytochrome c-selective assay of cyt c-Fe(III)/TLCL peroxidase activity. Panel a, EPR spectrum of cyt c-Fe(III)/TLCL peroxidase activity. Panel b, panel c, dependence of cyt c-Fe(III)/TLCL peroxidase activity upon cyt c concentration. Cyt c (5 μM) was incubated with liposomes (200 μM total lipid; DOPC:TOCL = 1:1) for 1 min at room temperature in 25 mM sodium phosphate buffer, pH 7.4, containing 100 μM DTPA; then DEANOate was added, and the incubation was continued for additional 15 min. After the addition of etoposide (100 μM) to the incubation mixture, the peroxidase reaction was initiated by H2O2 (100 μM), and the time course (4 min) of cyt c-Fe(III)/TLCL peroxidase was recorded in 1 min after H2O2 addition. B, oxidation of 3,3′-diaminobenzidine by cyt c-Fe(III)/TLCL peroxidase. Cyt c (30 μM) was incubated with liposomes (200 mM HEPES containing 100 μM total lipid) for 5 min at room temperature and mixed with different concentrations of DEANOate. DEANOate was incubated 15 min in buffer before addition to cyt c-Fe(III)/TLCL complex. After the addition of 1.2 mM 3,3′-diaminobenzidine peroxidase reaction was initiated by 200 μM H2O2. The absorbance of 3,3′-diaminobenzidine was measured in 10-min intervals at room temperature (470 nm).

NO-selective electrode (13, 37). Concentration of NO in solution as measured by NO electrode current initially increased linearly after the addition of DEANOate and then came to saturation as NO production equaled NO consumption as a result of NO interaction with oxygen and cyt c-CL peroxidase. An initial increase of NO concentration computed from NO electrode current in the absence of H2O2 agreed very well with the initial NO production value computed from the concentration of DEANOate (0.101 μM/s versus 0.105 μM/s). A steady-state NO concentration value of ~7–8 μM in the absence of H2O2 also agrees with the computed NO production rate and the reported bimolecular rate of NO oxidation in the saturation oxygen conditions (k2 = 10^3 M^-1 s^-1) (27). An initial rate of increase of NO concentration and saturation NO concentration value were markedly inhibited upon the addition of cyt c-TOCL and H2O2 to the incubation system (Fig. 7). NO consumption in the peroxidase reaction of cyt c-CL complex computed from the changes of slopes of initial current increases in Fig. 7 was ~0.03 μM/s, corresponding to a bimolecular rate constant of peroxidase reaction of ~10 M^-1 s^-1 comparable with estimations of the peroxidase activity of cyt c-CL in similar conditions performed by other methods. H2O2-induced NO consumption did not take place when cyt c and DOPC liposomes were incubated with DEANOate in the absence of TOCL. At ratios of cyt c/NO ≤ 3:1, no significant quenching of NO response was observed in the absence of H2O2 (cyt c/CL = 1:25). At high ratios of cyt c to NO (≥10:1), a significant inhibition of NO release occurred likely because of direct binding of NO to heme iron (data not shown). Thus, peroxidase function of cyt c-CL complex may be viewed as a regulatory mechanism to control NO levels in mitochondria.

Inhibition of Cardiolipin Oxidation—A physiologically important peroxidase function of the cyt c-CL complex is its ability to induce peroxidative modifications of CL, particularly important in the execution of an apoptotic program (5). Therefore, we tested whether CL oxidation was affected by NO. Mass spectrometry assessment of TLCL oxidation by cyt c-TLCL complexes revealed a significant accumulation of different hydroxyl-, hydroperoxy-, and hydroxy/hydroperoxy-derivatives of TLCL as evidenced by the appearance of signals with m/z 731.3, 739.3, 755.3, 755.5, 770.9, and 786.9 as compared with only one signal at m/z 723.8 for nonoxynized doubly charged ion of TLCL. In the presence of PAPANONOate, the signals of oxidized derivatives of TLCL were almost completely suppressed (Fig. 8A). We further employed our newly developed Amplex Red-based protocol with fluorescence HPLC detection of products to quantitatively assess TLCL oxidation by cyt c-H2O2 (Fig. 8B). We found that 1 h of incubation of cyt c-TLCL in the presence of H2O2 resulted in accumulation of (165 ± 25) pmol CL-OH/nmol TLCL. When the incubation was performed in the presence of PAPANONOate, only 30 ± 4 pmol CL-OH/ nmol TLCL were formed in the reaction. This indicates that NO acted as an effective inhibitor of TLCL peroxidation catalyzed by the peroxidase activity of cyt c. No nitrated derivatives of CL were observed in mass spectrometry experiments, indicating that quenching of lipid centered radicals by NO is a relatively unimportant antioxidant mechanism compared with NO interaction with oxoferyl reactive intermediates of the peroxidase reactions and quenching of protein-centered radicals.

**DISCUSSION**

Numerous studies on protein unfolding/refolding employed cyt c as a favorite object (1, 7, 46–48). These established that the native hexa-coordinate state includes axial ligation of Met60 to the heme that can be ruptured and replaced by His26 and His33 under destabilizing conditions (1, 47). Five cooperative and hierarchic folding units of different stabilities have been revealed in horse heart cyt c such that substructures of...
higher stability are dependent on the unfolding of lower stability substructures whereby destabilization of Met\(^{80}\)-containing domain displays the lowest stability (46, 48). Different physical treatments and chemical modifiers including strong oxidants of Met\(^{80}\) (hypochlorous acid and singlet oxygen) have been shown to cause destabilization of cyt \(c\) resulting in appearance of its peroxidase activity (8, 49). One can expect that Met\(^{80}\) in cyt \(c\)/H\(_{18528}\)/CL will be much more exposed to solvent and more prone to interaction with some other oxidants, for example, peroxynitrite (43, 50, 51). So, modification of peroxidase activity of cyt c-CL complex by peroxynitrite may be due not only to the nitration of tyrosine residues but as well to the oxidation of Met\(^{80}\) in the cyt c-CL complex. Further, several anionic phospholipids, particularly CL, were demonstrated to cause destabilization of cyt \(c\) (5, 9, 10). However, the physiological relevance of these findings remained poorly understood.

We have recently reported that the formation of cyt c-CL complexes occurs during apoptosis, resulting in marked enhancement of peroxi-
NO Regulates Cardiolipin Oxidation by Cytochrome c

Our measurements of optical spectra of cyt c-CL complexes showed that stable complexes between CL-bound ferri-cyt c and NO are formed at rather high NO concentrations of \(-8–10\ \text{\mu M}\), whereas a strong inhibition of lipid peroxidation by cyt c-CL is achieved at lower \((-2–4\ \text{\mu M})\) concentrations of NO. Thus, Fe-NO complex formation is a relatively unimportant mechanism of NO regulation of peroxidase activity of cyt c-CL, compared with the reduction by NO of the peroxidase reactive intermediates: heme-centered compounds I and II and protein-centered radicals generated in the presence of \(\text{H}_2\text{O}_2\) or organic (lipid) hydroperoxides. Although stable protein-derived radicals were observed in our EPR experiments, no measurable concentrations of compounds I and II were detected. This is in contrast to other heme proteins displaying peroxidase activity such as myoglobin, horseradish peroxidase, and myeloperoxidase, where long-lived compound I and II species are readily detectable in the presence of \(\text{H}_2\text{O}_2\) (53, 57, 59, 60). Apparently, oxoferryl reactive intermediates are very short-lived in the case of cyt c-CL catalyzed peroxidase reaction, and their formation is immediately followed by the oxidation of protein-centered (tyrosine) residues in close vicinity of cyt c heme and formation of protein radicals (probably Tyr and Trp centered).

Recently, stimulation of myeloperoxidase by low micromolar NO concentrations was demonstrated (57). MPO exerts specific catalytic and thermodynamic properties (58, 63). This peroxidase has very unusual reactivities of compounds I and II. Compound I/compound II couple of MPO has a very high redox potential of \(+1.35\ \text{V}\) and is able to oxidize halide anions to their respective haloperoxidic acids (63). Compound II/ferric MPO is much less active, and its reduction constitutes the limiting step of MPO peroxidase activity (57, 63). Nitric oxide effectively reduces compound II of MPO and thus at low concentrations stimulates peroxidase activity of MPO. There are no indications so far that compounds I and II of cyt c have different reactivities; they are also short lived (they are not observed spectroscopically), possibly because of the effective tyrosine oxidation. Thus, it is unlikely that nitric oxide will have an activation effect on the peroxidase activity of cyt c as has been shown for MPO.

A demonstrated strong inhibitory effect of NO on the peroxidase activity of cyt c-CL complex combined with its increased amount in mitochondria suggest that NO may regulate lipid peroxidation and apoptotic activity of CL membrane-bound cyt c during apoptosis. Notably, heme-nitrosylated cyt c has been detected in the cytosol of apoptotic cells (22, 23). Moreover, intramitochondrial origin of cyt c nitrosylation has been established along with its sensitivity to expression of Bcl-2 proteins and the importance for apoptosis. However, specific mechanisms involved in nitrosylation of hexa-coordinate heme of cyt c remained controversial. Our results clearly indicate that NO is capable, in the absence of \(\text{H}_2\text{O}_2\) or other sources of oxidizing equivalents, to effectively nitrosylate heme in cyt c-CL complexes. It is tempting to speculate that formation of such complexes is a prerequisite for the production of heme-nitrosylated cyt c in mitochondria and its subsequent release into the cytosol of apoptotic cells.

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MAY 26, 2006•VOLUME 281•NUMBER 21
JOURNAL OF BIOLOGICAL CHEMISTRY 14561

The peroxidase activity was directed toward oxidation of CL and accumulation of oxidized CL and stimulated the release of pro-apoptotic factors, including cyt c, from mitochondria. Shidoji et al. (52) demonstrated that CL oxidation products, particularly CL hydroperoxides, did not effectively bind cyt c. Thus, interactions of cyt c with CL play an important role at very early mitochondrial stages of apoptosis because of the selective ability of the complex to catalyze CL oxidation. This raises the question of potential regulation of peroxidase activity of cyt c-CL complex in mitochondria.

NO has been known to act as an effective inhibitor of heme-peroxidas via both binding to their heme as well as quenching of the catalytic reactive intermediates, peroxidase compounds I and/or II (53, 54) and nitration of reactive protein radicals formed as a result of peroxidase reaction (13, 40, 43). Our previous studies showed that a dominant mechanism of the antioxidant effect of NO on the peroxidation reaction catalyzed by myoglobin and hemoglobin is NO reduction of oxo-ferryl reactive species (53). We also demonstrated the effectiveness of NO in inhibiting lipid peroxidation dependent on peroxidase activity of myoglobin and hemoglobin in several different types of cells (55, 56). NO has been shown to serve as one electron substrate for both Compound I and Compound II for a number of peroxidases such as myeloperoxidase (MPO) (57, 58), eosinophil peroxidase, lactoperoxidase (58), and horseradish peroxidase (59, 60). Recently, O’Donnell and co-workers (61) demonstrated that NADPH oxidase-dependent consumption of NO prevails over its MPO-catalyzed metabolism in the absence of \(\text{H}_2\text{O}_2\). However, the addition of \(\text{H}_2\text{O}_2\) caused a significant enhancement of MPO-mediated NO consumption (61). Moreover, Ischiropoulos et al. (60) demonstrated inhibiting effects of NO donor diethylamine on the peroxidase activity of \(\text{H}_2\text{O}_2\)-driven cyt c toward dihydroorhodamine 123. HOCl-oxidized cyt c exhibited a higher consumption of NO compared with native protein (13). Thus, it is likely that NO may act as an important regulator of peroxidase activity of the cyt c-CL complex in mitochondria.

Although the presence of NO in mitochondria has been established as diffused through membrane or produced by NO synthase (18, 20), its role in mitochondrial physiology is less clear. In fact, NO-dependent production of peroxynitrite has been associated with damage of mitochondrial electron transporters (20, 24). Formation of ONOO\(^-\) represents a very important pathway of NO reactivity. But in mitochondria not all of NO and \(\text{O}_2\) are converted to peroxynitrite. In mitochondria \(\text{O}_2\) will be effectively converted to \(\text{H}_2\text{O}_2\) by SOD (MnSOD in matrix and CuZnSOD in the intermembrane space). The enzymatic dismutation of \(\text{O}_2\) is characterized by rate constants comparable with that for peroxynitrite formation (21, 24). Superoxide is believed to be largely formed toward the matrix close to the location of MnSOD (20). On the other hand, NO may diffuse through the outer mitochondrial membrane. Thus, the sites of origin and the fluxes of the radicals may be spatially separated. Therefore, even at relatively high NO concentrations \((-1–2\ \text{\mu M})\), one can expect substantial \(\text{H}_2\text{O}_2\) production in mitochondria, allowing for the activation of peroxidase activity of cytochrome c-CL complex. Our results provide a reasonable explanation for potential beneficial effects of mitochondrial NO (nitric-oxide synthase) because of its regulatory role in the control of peroxidase activity of cyt c-CL complexes in mitochondria.

One can envision that NO interferes with the peroxidase activity of heme proteins via: 1) the formation of Fe-NO complexes (13–15), 2) the nitration of tyrosyl and other protein-derived radicals formed as catalytic intermediates of the peroxidase reaction (40, 42, 43), and 3) direct reduction of active oxo-ferryl species compound I and II (53, 57, 59). In addition, NO effectively scavenges lipid peroxyl and alkoxyl radicals, i.e., acts as an effective lipid antioxidant (21, 62).
NO Regulates Cardiolipin Oxidation by Cytochrome c