

Molecular Design of New Inhibitors of Peroxidase Activity of Cytochrome *c*/Cardiolipin Complexes: Fluorescent Oxadiazole-Derivatized Cardiolipin[†]

G. G. Borisenko,^{*,‡} A. A. Kapralov,[§] V. A. Tyurin,[§] A. Maeda,[§] D. A. Stoyanovsky,^{||} and V. E. Kagan^{*,§}

Center for Free Radical and Antioxidant Health and Departments of Environmental and Occupational Health and Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania, and Research Institute of Physico-Chemical Medicine, Moscow, Russian Federation

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ABSTRACT: Interaction of a mitochondria-specific anionic phospholipid, cardiolipin (CL), with an intermembrane protein, cytochrome *c* (cyt *c*), yields a peroxidase complex. During apoptosis, the complex induces accumulation of CL oxidation products that are essential for detachment of cyt *c* from the mitochondrial membrane, induction of permeability transition, and release of proapoptotic factors into the cytosol. Therefore, suppression of the peroxidase activity and prevention of CL oxidation may lead to discovery of new antiapoptotic drugs. Here, we report a new approach to regulate the cyt *c* peroxidase activity by using modified CL with an oxidizable and fluorescent 7-nitro-2,1,3-benzoxadiazole (NBD) moiety (NBD-CL). We demonstrate that NBD-CL forms high-affinity complexes with cyt *c* and blocks cyt *c*-catalyzed oxidation of several peroxidase substrates, cyt *c* self-oxidation, and, most importantly, inhibits cyt *c*-dependent oxidation of polyunsaturated tetralinoleoyl CL (TLCL) and accumulation of TLCL hydroperoxides. Electrospray ionization mass spectrometry and fluorescence analysis revealed that oxidation and cleavage of the NBD moiety of NBD-CL underlie the inhibition mechanism. We conclude that modified CL combining a nonoxidizable monounsaturated trioleoyl CL with a C₁₂-NBD fragment undergoes a regiospecific oxidation thereby representing a novel inhibitor of cyt *c* peroxidase activity.

The major physiological function of apoptosis is safe elimination of unwanted or irreparably damaged cells. Excessive apoptosis is usually associated with tissue degeneration during acute injury (e.g., ischemia, stroke, γ -irradiation) and chronic disease conditions (e.g., diabetes, cardiovascular and neurodegenerative diseases) (1–8). Stringent regulation of apoptosis is important to avoid massive cell loss. Therefore, significant efforts have been directed toward the development of new regulators acting at early stages of apoptosis, upstream of the point of no return, i.e., release of proapoptotic factors and caspase activation (9–16). In the early 1990s Radi et al. demonstrated that cyt *c*¹ can play a critical role in hydrogen peroxide induced lipid peroxidation

in isolated mitochondria (17, 18). We have recently documented that oxidation of a mitochondria-specific phospholipid, cardiolipin (CL), is a required step in the execution of the mitochondrial stage of apoptosis (19). This is achieved via the formation of a complex of cyt *c* with polyunsaturated molecular species of CL that confers peroxidase function on the former. The complex can oxidize small reducing substrates, protein tyrosines, and most importantly CL (19–22). Cyt *c*-driven accumulation of CL hydroperoxides is critical for the detachment of cyt *c* from the inner mitochondrial membrane, Bax-induced mitochondrial outer membrane permeabilization, and release of proapoptotic proteins, including cyt *c* into the cytosol (23–26). Therefore, suppression of the peroxidase activity and prevention of CL oxidation may lead to discovery of new antiapoptotic drugs (15, 27). This can be achieved via direct inhibition of the enzymatic activity, removal of cofactors feeding the peroxidase cycle (e.g., H₂O₂), or decreasing susceptibility of CL to oxidation (e.g., by biosynthesis of nonoxidizable species of monounsaturated/saturated CLs). Indeed, we demonstrated that mitochondria-targeted conjugates of nitroxides with hemigramicidin S were able to scavenge electrons from damaged carriers, suppress CL peroxidation, and protect cells against apoptosis induced by chemical proapoptotic agents or irradiation in vitro and in vivo (15, 27). We further found that enrichment of the CL pool of HL-60 cells with highly oxidizable docosahexaenoic acid (C_{22:6}) increased their sensitivity to apoptosis; in contrast, enrichment of cells with oleic acid (C_{18:1}) containing species resulted in their decreased sensitivity to apoptosis (19, 20).

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* Corresponding authors. V.E.K.: tel, 412-624-9479; fax, 412-624-9361; e-mail, kagan@pitt.edu. G.G.B.: tel/fax, +7(499)246-4293; e-mail, grigoryb@yahoo.com.

[‡] Research Institute of Physico-Chemical Medicine.

[§] Center for Free Radical and Antioxidant Health and Department of Environmental and Occupational Health, University of Pittsburgh.

^{||} Department of Surgery, University of Pittsburgh.

¹ Abbreviations: cyt *c*, cytochrome *c*; HRP, horseradish peroxidase; NBD, 7-nitro-2,1,3-benzoxadiazole; CL, cardiolipin; CL-OOH, cardiolipin hydroperoxides; NBD-CL, 1,1',2'-trioleoyl-2'-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]cardiolipin; C₁₂-NBD-PC, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine; C₆-NBD-PC, 1-oleoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine; TLCL, 1,1',2,2'-tetralinoleoylcardiolipin; TOCL, 1,1',2,2'-tetraoleoylcardiolipin; TMCL, 1,1',2,2'-tetramyristoylcardiolipin; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; tBOOH, *tert*-butyl hydroperoxide.

Here, we report a new approach to regulate the cyt *c* peroxidase activity by using modified CL containing an oxidizable moiety, rerouting the high oxidizing potential of the complex and “distracting” it from peroxidation of polyunsaturated CLs. We designed such an inhibitor on the basis of two components: nonoxidizable trioleoyl-CL and oxidizable and fluorescent 7-nitro-2,1,3-benzoxadiazole (NBD).

MATERIALS AND METHODS

Reagents. Horse heart cytochrome *c* (cyt *c*, type C-7752, >95%), horseradish peroxidase (HRP, type VI), etoposide (demethylepipodophyllotoxin ethylenedienetriphosphoride), 2',7'-dichlorofluorescein, 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD), diethylenetriaminepentaacetic acid (DTPA), taurine, glycine, hydrogen peroxide (H₂O₂), *tert*-butyl hydroperoxide (tBOOH), digitonin, and SDS were purchased from Sigma-Aldrich (St. Louis, MO). Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine) reagent was obtained from Molecular Probes (Eugene, OR). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,1',2,2'-tetralinoleoylcardiolipin (TLCL), 1,1',2,2'-tetraoleoylcardiolipin (TOCL), 1-oleoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine (C₆-NBD-PC), 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (C₁₂-NBD-PC), 1,1',2,2'-tetramyristoylcardiolipin (TMCL), 1,1',2,2'-tetralinoleoylcardiolipin (TLCL), and 1,1',2,2'-tetramyristoylcardiolipin (TMCL) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Mouse anti-cyt *c* antibody (clone 7H8.2C12) was obtained from BD Biosciences (Franklin Lakes, NJ), goat anti-mouse HRP conjugated antiserum West Femto was from ThermoFisher Scientific. 1,1',2-Trioleoyl-2'-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]cardiolipin (NBD-CL) was custom-synthesized by Avanti Polar Lipids, Inc. (Alabaster, AL).

Small Unilamellar Liposomes. Individual phospholipids, stored in chloroform, were mixed and dried under nitrogen. Then lipids were mixed in vortex in HEPES buffer (20 mM, pH 7.4) and sonicated three times for 30 s on ice. Liposomes were used immediately after preparation.

Distribution of NBD-CL between the inner and the outer leaflets of DOPC/TOCL liposomes was estimated by using a reducing agent, sodium dithionite, which can reduce NBD and quench its fluorescence in the outer leaflet only (28). DOPC/TOCL/NBD-CL liposomes (50:49:1) were treated with dithionite (200 μ M) for 1 min. NBD-CL fluorescence was analyzed before and after treatment by using a Shimadzu F5301-PC spectrofluorometer with excitation and emission wavelengths of 470 and 537 nm, respectively.

Isolation of Mitochondria. Mitochondria were isolated from freshly obtained livers of adult male mice according to ref 29. The preparation was carried out using MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.5). Mitoplasts were immediately prepared from freshly isolated mitochondria by a digitonin method according to Krebs et al. (30) and then depleted of cytochrome *c* as described in ref 31.

Incorporation of NBD-CL in Mitochondria. Mitochondria were incubated (35 min) with different concentrations of NBD-CL and washed from the excess of the phospholipid, and mitochondrial lipids were extracted according to Folch (32). Amounts of NBD-CL in the extracts were determined

by measuring NBD-CL fluorescence in chloroform and using a standard calibration curve.

Native Gel Electrophoresis. Agarose gel electrophoresis was run by using the horizontal gel system “Mupid-21” (Cosmo Bio) at 50 V in nondenaturing HEPES buffer (35 mM, pH 7.4) containing imidazole (43 mM). Samples of cyt *c* and liposomes were applied onto 0.8% agarose gel (120 pmol of protein per sample). Gels were stained for protein with 0.25% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid.

PAGE and Western Blotting Analysis. Cyt *c* and its aggregates were separated by 12.5% SDS-PAGE in Tris-glycine buffer. The separated proteins were electrotransferred to nitrocellulose membrane. After blocking with 5% nonfat milk dissolved in phosphate-buffered saline and Tween-20 (0.05%) (PBS-T) for 1 h, membrane was incubated overnight at 4 °C with primary antibodies (anti-cyt *c*). The membranes were washed several times and incubated with HRP-conjugated goat anti-mouse antiserum for 1 h at room temperature. The protein bands were visualized by using a SuperSignal West Pico chemiluminescent substrate (ThermoFisher Scientific). The density of bands was determined by scanning with an Epi Chemi II darkroom (UVP BioImaging Systems, Upland, CA).

Fluorescence Spectrometry. A PC1 spectrofluorometer (ISS Inc., Champaign, IL) equipped with xenon arc lamp as a light source was employed for fluorescence measurements of NBD-CL incorporated in DOPC/TOCL (1:1) liposomes in the amount of 1 mol %. Fluorescence emission spectra of NBD-labeled CL were detected by using an excitation wavelength of 470 nm and excitation and emission slits of 8 nm; fluorescence excitation spectra, by using an emission wavelength of 536 nm. Excited-state lifetime of NBD-CL was estimated by using a frequency domain method. An excitation wavelength of 470 nm, cutoff emission filter of 515 nm, and modulation frequency in the range of 1–150 MHz were employed to determine the phase delay and the modulation ratio. Data were analyzed by using 2',7'-dichlorofluorescein as a reference standard and “Vinci” software.

Assessment of Peroxidase Activity. Assessment of peroxidase activity with Amplex Red reagent was performed by measuring the fluorescence of resorufin, an oxidation product of Amplex Red. Cyt *c* (0.5 μ M) was incubated with liposomes for 10 min. Peroxidase reaction was started by addition of Amplex Red (5–100 μ M) and H₂O₂ (100 μ M) and was carried out for 30 min (reaction rate was linear in the entire time interval). Fluorescence was detected by employing an “Fusion α ” universal microplate analyzer and by using an excitation wavelength of 535 nm and an emission wavelength of 585 nm.

Peroxidase activity of mitochondria was determined in the presence of Amplex Red (50 μ M) and tBOOH (2 mM) after 10 min incubation. Fluorescence was measured using a Shimadzu RF5301-PC spectrofluorometer (excitation and emission wavelengths of 560 and 582 nm, respectively).

EPR Spectroscopy. EPR measurements were performed on a JEOL-RE1X spectrometer at 25 °C in gas-permeable Teflon tubings (0.8 mm i.d., 0.013 mm thickness) obtained from Alpha Wire Corp. (Elizabeth, NJ). Teflon tube was filled with 50 μ L of sample and placed in EPR quartz tube for the measurements. Etoposide phenoxyl radical spectra

were recorded under the following conditions: 335.3 mT, center field; 2 mT, sweep width; 0.04 mT, field modulation; 10 mW, microwave power; 0.1 s, time constant; 2 min, time scan. The time course of the etoposide radical EPR signal was obtained by repeated scanning of the field (0.15 mT, sweep width; 335.3 mT, center field) corresponding to a part of the EPR signal. Other instrumental conditions were as follows: 0.04 mT, field modulation; 10 mW, microwave power; 0.1 s, time constant; 20 s, time scan; internal mode of recording.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) of phospholipids and their oxidation products was analyzed by direct infusion into a linear ion-trap mass spectrometer LXQ (Thermo Electron, San Jose, CA). Samples in chloroform/methanol, 2:1 v/v (20 pmol/ μ L), were utilized directly for acquisition of ESI mass spectra at a flow rate of 5 μ L/min. The electrospray probe was operated at 5.0 kV in the negative ion mode. Source temperature was maintained at 150 °C. MSⁿ analysis was carried out with relative collision energy ranging from 20% to 40% and with an activation *q* value at 0.25 for collision-induced dissociation (CID) and a *q* value at 0.7 for the pulsed-Q dissociation technique. TMCL was used as the internal standard. Isotopic corrections were performed by entering the chemical composition of each species into the Qual browser of Xcalibur (operating system) and using the simulation of the isotopic distribution to make adjustments for the major peaks. Chemical structures of lipid molecular species were confirmed by comparing with the fragmentation patterns presented in the Lipid Map Data Base (www.lipidmaps.org).

In our experiments the structure of TLCL oxidized products was confirmed by MSⁿ fragmentation analysis with an additional approach using reduction of hydroperoxy derivatives by a mild reductant of organic hydroperoxides, triphenylphosphine. These results were reported in our previously published articles (19, 33). Further, additional quantitative controls were used to independently confirm the validity of our estimates. Specifically, quantitative analysis of low concentrations of TLCL (5 pmol/ μ L) and its oxidation products (less than 0.5 pmol/ μ L) was based on a combination of (1) lipid phosphorus determination by a micromethod (34) and (2) ESI-MS protocol where CL molecular species were quantified by comparing the ratio of the individual peak intensities with that of an internal standard TMCL, which was added to the sample before MS analysis. TMCL was used as internal standard, and TLCL was used as a reference standard (35). Additionally, NBD-CL was used as a reference standard in the presence of TMCL as an internal standard. The linear response and detection limit for CL were established by injection of calibration mixtures with different concentrations of TLCL or NBD-CL at a constant concentration of TMCL. Thus, the concentration of nonoxidized TLCL in the sample was confirmed by two independent methods: by lipid phosphorus determination and ESI-MS protocol. Both methods resulted in quantitatively the same concentration of TLCL. In experiments where TLCL was oxidized by cyt c/H₂O₂, the total amount of individual molecular species of oxidized TLCL (TLCLox) plus nonoxidized TLCL, which were estimated by MS protocol (using TMCL as internal standard and TLCL as reference standard), resulted in the same total value of CL that was independently

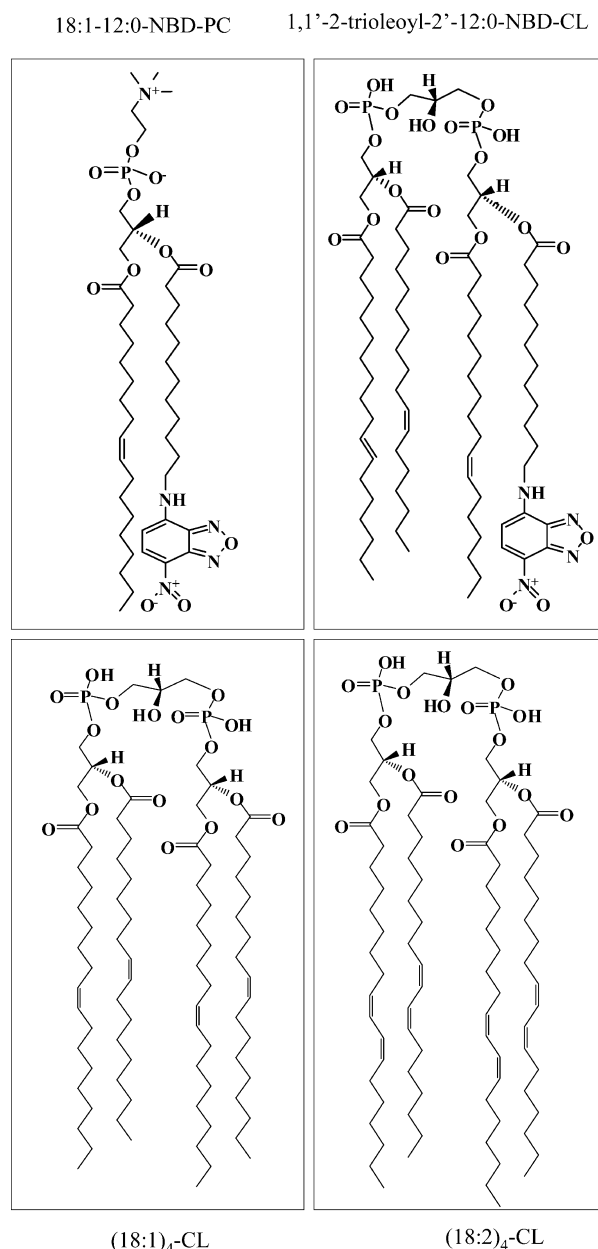


FIGURE 1: Structural formulas of CL, NBD-CL, and NBD-PC.

obtained by lipid phosphorus determination. These results indicate that although oxidized TLCL molecular species may have different ionization efficiency than nonoxidized TLCL, these differences in MS responses were negligible in the range of low concentrations employed (36).

Statistical Analysis. Data are expressed as means \pm SD values from at least three experiments. Changes in variables are analyzed by one-way ANOVA for multiple comparisons. Differences are considered significant at $p < 0.05$.

RESULTS

Interactions of Cyt c with NBD-CL. (A) *Native Gel Electrophoresis of Cyt c/Cardiolipin Complexes.* Interaction of cyt c with various anionic lipids (see Figure 1) can be assessed by bidirectional gel electrophoresis in native low density gel whereby cationic cyt c and negatively charged liposomes migrate in opposite directions. Binding of cyt c to lipids shifts the protein mobility and can be used for quantitative assessments of the strength of the interactions

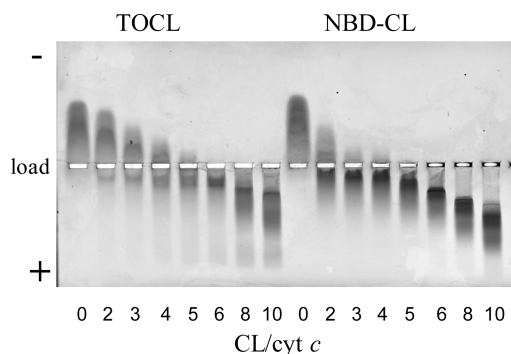


FIGURE 2: Native agarose gels of cyt *c* complexes with TOCL and NBD-CL at various anionic phospholipid/cyt *c* ratios. Samples of protein mixed with liposomes were loaded onto a 0.8% agarose gel (as indicated on the figure), and the electrophoresis was run in HEPES–imidazole buffer (35 mM HEPES, 43 mM imidazole, pH 7.4). Gels were stained with Coomassie Brilliant Blue R-250. Cyt *c*, 40 μ M; anionic phospholipid/cyt *c* ratios are indicated. Liposomes were made from PC premixed with equal amounts of TOCL or NBD-CL.

(19, 37). We applied this approach to analyze cyt *c* interactions with CL- and NBD-CL-containing liposomes (Figure 2). TOCL and NBD-TOCL were mixed with zwitterionic DOPC at a ratio of 1:1. In the presence of TOCL or NBD-TOCL, the migration profile of cyt *c* was changed. Zero electrophoretic mobility of cyt *c* in the presence of TOCL was achieved at a ratio of 5:1 (Figure 2). CL has a negative charge of 1.3 at pH 7.4 (38); hence, the cyt *c*/CL mixture had a zero net charge at this ratio. Zero electrophoretic mobility of NBD-CL/cyt *c* was observed at a ratio of 3:1, suggesting that NBD-CL was able to form more stable complexes with cyt *c* than nonmodified TOCL. Moreover, NBD-CL prevented migration of cyt *c* to the cathode even when the theoretical total charge of the complex was positive. This indicates that the NBD group could be involved in the charge distribution in the complex.

(B) *Quenching of NBD-CL Fluorescence upon Cyt *c* Binding to Membranes.* The absorbance spectrum of NBD-labeled CL in TOCL/DOPC liposomes had two characteristic maxima at 338 and 468 nm. The fluorescence spectrum had a maximum at 538 nm (Figure 3A, insert). Fluorescence lifetime was 5.0 ns as determined by a frequency-domain method (39). Thus, fluorescence properties of NBD-CL were very close to other NBD-labeled phospholipids (40). The location of the NBD molecule near the phosphoglycerol moiety of lipids in the membrane and the high sensitivity of its fluorescence to the local environment result in fluorescence decay upon protein binding to the membrane (41). Indeed, instant quenching of NBD-CL fluorescence was observed upon cyt *c* addition to liposomes (Figure 3A). To minimize NBD fluorescence self-quenching and direct interaction of NBD-CL with cyt *c*, only 1 mol % of NBD-CL was added to the lipid mixture (compare with 49 mol % of TOCL in CL/PC liposomes). The extent of quenching depended on the cyt *c*/CL ratio and reached the maximum value of $\sim 80\%$. NBD-CL was nearly equally distributed between inner and outer leaflets of the bilayer as was confirmed by quenching experiments with dithionite (28) (Figure 3B); therefore, a strong quenching effect suggests that cyt *c* affects NBD fluorescence on both sides of the bilayer. In the outer leaflet fluorescence can be altered due to direct interaction with the protein (via lipid segregation

and interaction with amino acid groups). Because NBD fluorescence emission spectrum overlaps with the absorbance spectrum of cyt *c* heme, an efficient fluorescence resonance energy transfer (FRET) from NBD to heme is anticipated upon protein–phospholipid binding. In the inner leaflet (which is physically inaccessible for cyt *c*), NBD fluorescence is quenched via the FRET mechanism. On the basis of quenching efficiency of NBD-CL in the inner leaflet we approximated the distance of the membrane-bound heme from the center of the bilayer to be in the range of 9–17 Å.

*Inhibition of Cyt *c* Peroxidase Activity by NBD-CL. (A) Effect of NBD-CL on Etoposide Radical Generation by the Cyt *c*/CL Complex.* The ability of cyt *c* to catalyze oxidation of various substrates is markedly augmented upon binding to CL-containing membranes (19–21). We tested whether NBD-CL could induce the peroxidase activity by analyzing cyt *c*-dependent production of etoposide phenoxyl radicals, oxidation of Amplex Red, protein aggregation, and generation of CL hydroperoxides.

One-electron oxidation of etoposide by peroxidases yields phenoxyl radical intermediates readily detectable by EPR spectroscopy. The radicals were generated by CL-bound (but not by free) cyt *c* (Figure 4), in line with previous demonstrations of the ability of the cyt *c*/CL complex to catalyze H_2O_2 -dependent one-electron oxidation of etoposide (21). Surprisingly, when TOCL was substituted for NBD-CL, etoposide radical production was completely blocked. Similarly, NBD-CL inhibited oxidation and chemiluminescence response from West Femto (ThermoFisher Scientific), a typical substrate of peroxidase reactions catalyzed by the cyt *c*/CL complex (Figure 4C).

(B) *Effect of NBD-CL on the H_2O_2 -Induced Oligomerization of Cyt *c*.* Formation of protein-centered radicals, likely tyrosyl radicals, has been previously documented in the course of peroxidase reaction of cyt *c* with H_2O_2 (42–44), particularly in the complex of cyt *c* with CL (19, 20). In the absence of reducing substrates, incubation of cyt *c* with TOCL-containing liposomes and H_2O_2 resulted in oligomerization of the protein and decrease of its monomeric form (Figure 5) due to radical recombination and dityrosine cross-linking (19, 21, 22). In the presence of NBD-CL, the monomeric form of cyt *c* was preserved (Figure 5, left panel). The higher the molar fraction of NBD-CL incorporated in the phospholipid vehicles the lower the amount of high molecular weight aggregates detected by PAGE analysis. Notably, oligomerization of cyt *c*/TOCL complexes in the presence of H_2O_2 was inhibited by etoposide proportionally to the amount of this reducing substrate (Figure 5, right panel). These results suggest that NBD-CL was acting similarly to reductants capable of competitive inhibition of oxidative oligomerization of the protein. Of note, NBD-CL was considerably more effective as compared to etoposide. The highest concentration of NBD-CL (25 mol %, 6.25 μ M) that caused marked protection against cyt *c* oligomerization, as evidenced by the amounts of the detectable monomeric form of cyt *c*, was essentially ineffective in the case of etoposide. Even at much higher concentrations, up to 200 μ M, the protective effects of etoposide were less pronounced than those of NBD-CL at 6.25 μ M.

(C) *Effect of NBD-CL on the Cyt *c*-Catalyzed Accumulation of Cardiolipin Hydroperoxides (CL-OOH).* In the presence of polyunsaturated CL, cyt *c* catalyzes H_2O_2 -

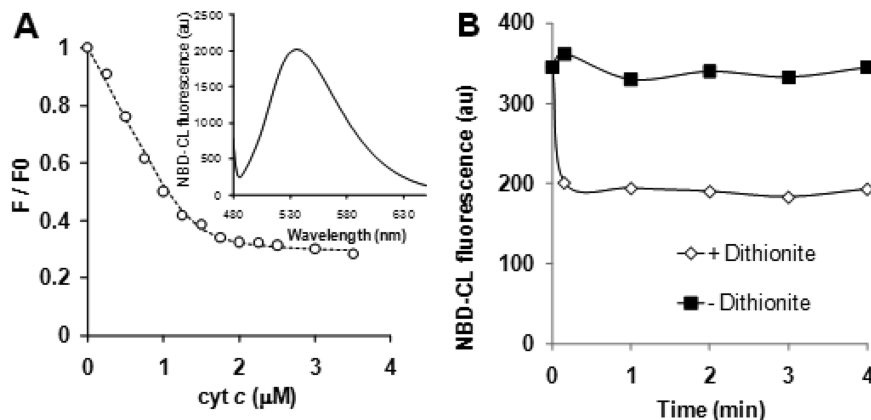


FIGURE 3: Quenching of NBD-CL fluorescence by cyt *c* (A) and dithionite (B). (A) Dependence of NBD-CL fluorescence on the concentration of cyt *c*. Incubation time 1 min. Insert: Fluorescence emission spectrum of NBD-CL in liposomes. (B) Time course of NBD-CL fluorescence in the presence of dithionite (200 mM). Excitation wavelength was 470 nm. Liposomes were prepared from DOPC/TOCL/NBD-CL at a ratio of 50:49:1. Phospholipid concentration was 20 μM for (A) and 50 μM for (B).

dependent peroxidation of CL with a characteristic pattern of products in ESI mass spectra (19, 33). As shown on Figure 6, peroxidation of tetralinoleoyl-CL (TLCL) by cyt *c*/H₂O₂ yielded oxidation products, which include mono-, di-, and trihydroperoxides as well as different hydroxy derivatives. In the presence of 50 mol % NBD-CL, the production of CL-OOH was inhibited 3-fold; similarly, the content of TLCL oxidation products with several hydroperoxy and/or hydroxy groups was markedly decreased (with the exception of CL with the monohydroxy group with *m/z* 731) (Figure 6). Thus, NBD-CL inhibited cyt *c*/CL-induced peroxidation of TLCL.

Mechanism of Cyt *c* and Peroxidase Inhibition by NBD-CL. (A) Analysis of the NBD-CL Inhibition Mechanism of cyt *c* Peroxidase Activity by Amplex Red Fluorescence Assay.

We further utilized Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine) as a substrate for the peroxidase activity of cyt *c*/CL complexes, catalyzing its oxidation to a highly fluorescent resorufin (45), in the presence and absence of NBD-CL. Expectedly, NBD-CL inhibited oxidation of Amplex Red by the cyt *c*/CL complex. Kinetic studies revealed the decreased maximum reaction rate and apparent affinity of the enzyme for the substrate in the presence of NBD-CL (Figure 7A,B). The extent of inhibition depended on the molar fraction of NBD-CL in the liposomes; the inhibitory effect was observed even at high Amplex Red concentrations (up to 100 μM). On the basis of Michaelis–Menten formalism, our results can be interpreted as a reversible mixed mechanism of inhibition. Interestingly, C₁₂-NBD-PC (for structure see Figure 1) also exerted a slight inhibitory effect, which was not observed in the presence of excess Amplex Red (Figure 7C). As can be seen from a Lineweaver–Burk plot, the affinity for Amplex Red (but not the reaction rate) decreased in the presence of C₁₂-NBD-PC (Figure 7D). Of note, C₆-NBD-PC did not affect the peroxidase activity of the cyt *c*/CL complex (Figure 7E,F).

(B) Mass spectrometry of NBD-CL and Its Products Formed by Cyt *c*/TLCL/H₂O₂. Combined, our results suggest that NBD-CL acted as a substrate for the peroxidase activity of the cyt *c*/CL complex. To further confirm this, we performed ESI-MS analysis of NBD-CL incubated with cyt *c* and TLCL-DOPC (see Figure 1) liposomes in the presence of H₂O₂. As shown in Figure 8A singly charged ions of

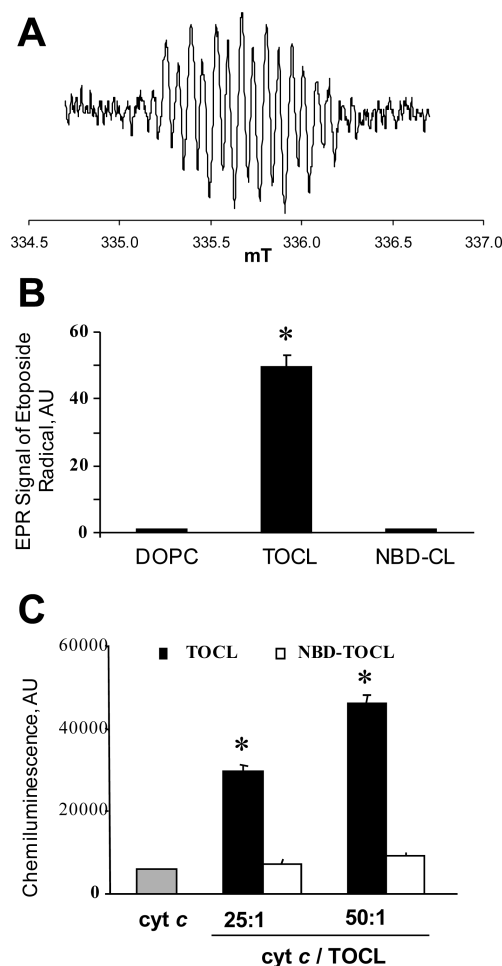


FIGURE 4: NBD-CL inhibits generation of etoposide phenoxyl radicals and chemiluminescence substrate oxidation by cyt *c*. (A) EPR spectrum of the etoposide phenoxyl radical generated by the cyt *c*/TOCL complex in the presence of H₂O₂. (B) Relative intensity of the EPR signal in the presence of liposomes of different composition: DOPC only, DOPC/TOCL (1:1), and DOPC/NBD-CL (1:1). Incubation conditions: phospholipid concentration 1 mM, cyt *c* 20 μM, etoposide 200 μM, H₂O₂ 100 μM, incubation time 4 min. (C) Effect of NBD-CL on oxidation of West Femto sensitive substrate by cyt *c*/H₂O₂. Conditions: 5 nM cyt *c* was mixed with liposomes (DOPC/TOCL, 1:1) containing either nonlabeled or labeled TOCL, total volume 100 μL. Reaction was started by addition of substrate solution already containing H₂O₂.

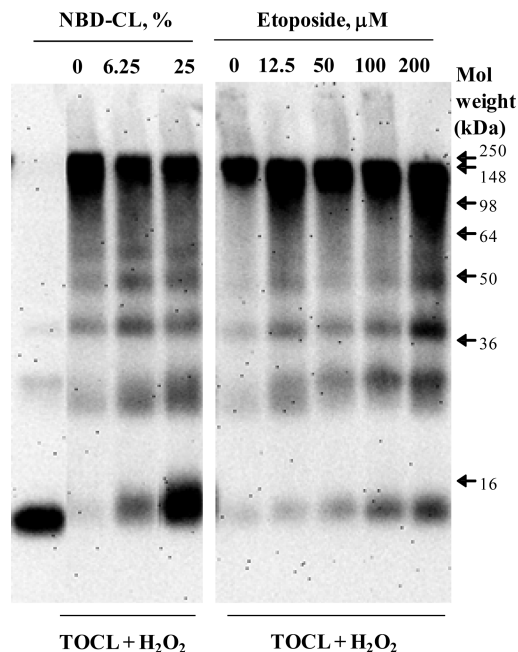


FIGURE 5: NBD-CL and etoposide inhibit H_2O_2 -dependent oligomerization of cyt *c*. Typical Western blots of cyt *c* and its oligomeric products after staining with anti-cyt *c* antibody. Cyt *c* ($1.5 \mu M$) was preincubated with liposomes (concentration of phospholipids $25 \mu M$) in HEPES buffer (20 mM , $\text{pH } 7.4$) for 15 min , and then aliquots of H_2O_2 (at a final concentration of $25 \mu M$) were added every 15 min during a 1 h interval at 37°C . Liposomes were prepared from DOPC/TOCL ($1:1$). Mol % of NBD-CL in liposomes and etoposide concentration were as shown above the gel.

TLCL and NBD-CL in negative mode were represented by two different molecular clusters with m/z 1447 and 1551 with their Na adducts at m/z 1469 and 1573, respectively. After the incubation, in addition to a signal corresponding to trioleoyl- C_{12} -NBD-CL (m/z 1551), a prominent signal with m/z 1388 appeared in the spectrum (Figure 8B, b) consistent with $[M - \text{trioleoyl-}C_{12}\text{-NH}_2\text{-CL} - H]^-$. Another peak at m/z 1410 represented its Na adduct. The identity of an ion at m/z 1388 was unambiguously supported by the structural analysis using CID tandem MS, which revealed fragments of glycerol backbone plus PO_4 (m/z 152), oleic acid (m/z 281), and aminomyristic acid (m/z 214); neither NBD nor NBD-myristic acid fragments were detected. ESI-MS analysis of NBD-CL before and after its incubation with cyt *c*/ H_2O_2 confirmed the formation of the NBD derivative with m/z 180 (Figure 8B, a). In addition, we observed trace amounts of an ion with m/z 1405, probably corresponding to a hydroxy derivative of the compound with m/z 1388 (trioleoyl- C_{12} -NHOH-CL). Quantitative analysis showed a drastic decomposition of the molecular ion of NBD-CL at m/z 1551 after incubation of liposomes with cyt *c*/ H_2O_2 (Figure 8C, a).

(C) H_2O_2 -Dependent Decay of NBD-CL Fluorescence in the Presence of Cyt *c*. In addition to instant fluorescence quenching upon binding of cyt *c* to NBD-CL/CL-containing liposomes, further fluorescence decay was induced by H_2O_2 (Figure 8C, b). The time course of H_2O_2 -dependent fluorescence decay was characterized by a small and rapid initial drop followed by a more pronounced and slower fluorescence reduction (Figure 8C, b). The effect of H_2O_2 was universally observed within a broad range of cyt *c* concentrations: from a relatively low protein content (cyt *c*/CL ratio of $1:100$,

when binding was associated with only 2% of initial fluorescence quenching) to a high protein level (cyt *c*/CL ratio of $1:4$, when cyt *c* binding sites were completely saturated and caused 75% quenching of initial fluorescence) (data not shown).

Incorporation of NBD-CL in Mitochondria and Assessment of Mitochondrial Peroxidase Activity. Incubation of NBD-CL with mitochondria was associated with the increase of its fluorescence. Quantitative assessments showed that incorporation of NBD-CL into mitochondria was concentration-dependent (data not shown). At 20 and $200 \mu M$ NBD-CL, around 0.5 and 8 nmol of NBD-CL/mg of protein (8% and 12% of total, respectively) were integrated. Using these two concentrations of NBD-CL, we assessed its effects on peroxidase activity of mitochondria induced by *tert*-butyl hydroperoxide (tBOOH). We employed measurements of fluorescence of resorufin produced upon oxidation of Amplex Red. Resorufin formation was only detected in the presence of both tBOOH and mitochondria (Figure 9A). NBD-CL suppressed peroxidase activity, and the inhibitory effect depended on the amount of NBD-CL incorporated in mitochondria (Figure 9B). Similar results were obtained using mitoplasts (data not shown).

DISCUSSION

Mitochondria are central to the execution of intrinsic apoptosis triggered by different damaging stimuli (46). Mitochondrial generation of ROS is important in this cell death pathway, and manipulations of ROS production were shown to cause changes in the sensitivity of cells to apoptosis (10, 47–49). Recently, we demonstrated that ROS can act via a cyt *c*-dependent mechanism whereby cyt *c* binds to mitochondrial CL, adopts a new function of a peroxidase, and catalyzes H_2O_2 -dependent oxidation of CL (19). CL oxidation products were shown to be essential for the mitochondrial permeability transition and release of proapoptotic factors from mitochondria (23, 25, 26). This implies that inhibition of CL oxidation may be a promising approach to regulate apoptosis. Indeed, we reported that prevention of the production of H_2O_2 , feeding the peroxidase cycle of cyt *c*/CL complexes, by mitochondria-targeted conjugates of hemigramicidin S with nitroxides inhibited CL oxidation and protected cells against apoptosis induced by different agents both in cultured cells and in vivo (15, 27). We further demonstrated that mitochondria-targeted donors of $NO\cdot$ activated by the peroxidase function of cyt *c*/CL complexes were also capable of preventing CL oxidation and decreasing sensitivity of cells to apoptosis (50). Here, we report yet another approach to inhibit CL peroxidation based on the utilization of modified CLs with functional moieties capable of “consuming” the high oxidizing potential of the peroxidase complex, thus “distracting” it from peroxidation of polyunsaturated CLs. We designed such an inhibitor on the basis of two components, nonoxidizable trioleoyl-CL and oxidizable and fluorescent 7-nitro-2,1,3-benzoxadiazole (NBD).

Using trioleoyl cardiolipin with C_{12} -NBD as the forth acyl chain (NBD-CL), we assessed the binding of cyt *c* to the modified CL. Native gel electrophoresis of mixtures of cyt *c* with various amounts of DOPC/NBD-CL liposomes clearly demonstrated that NBD-labeled CL can successfully compete with unlabeled CL for cyt *c*. Analysis of the cyt *c* effect on

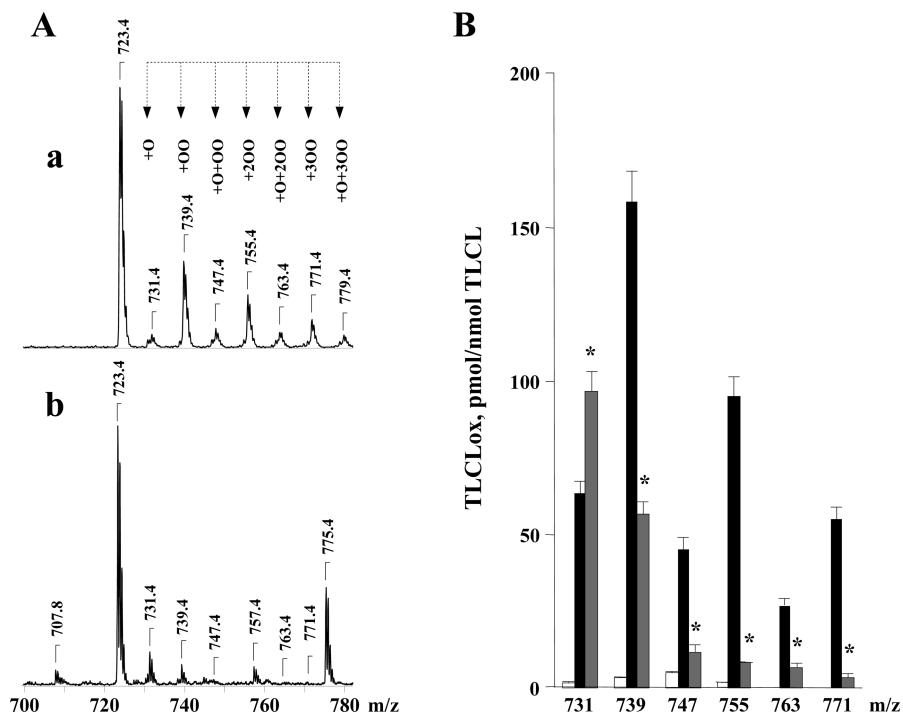


FIGURE 6: NBD-CL inhibits cyt *c*-dependent formation of cardiolipin hydroperoxides. (A, a, b) Typical mass spectra of TLCL and its H₂O₂-induced oxidation products formed in the presence of cyt *c*. Peaks at *m/z* 723 and 775 correspond to doubly charged TLCL and NBD-CL, respectively. TLCL oxidation products are represented by previously characterized monohydroxy (*m/z* 731), monohydroperoxy (*m/z* 739), monohydroxy/monohydroperoxy (*m/z* 747), dihydroperoxy (*m/z* 755), monohydroxy/dihydroperoxy (*m/z* 763), and trihydroperoxy (*m/z* 771) derivatives of TLCL (33). Cyt *c* (5 μ M) was incubated with DOPC/TLCL (1:1) liposomes (A, a) or DOPC/TLCL/NBD-CL (2:1:1) liposomes (A, b) (total phospholipid concentration was 500 μ M) in the presence of H₂O₂ (100 μ M) for 30 min at 37 °C. In the end of incubation, excess H₂O₂ was removed by addition of catalase (1.4 units/ μ L). (B) Quantitative analysis of oxygenated molecular species of TLCL. Key: open bars, control (TLCL/cyt *c*); filled black bars, after incubation of TLCL/cyt *c* with H₂O₂; filled gray bars, after incubation of TLCL/cyt *c* with H₂O₂ in the presence of NBD-CL. Lipid phosphorus was determined by a micromethod (34). CL molecular species were quantified by comparing the peak intensities with that of an internal CL standard, which was added to the sample before MS analysis. TMCL was used as an internal standard, and TLCL was used as a reference standard (35). The linear response and detection limit for TLCL were established by injection of calibration mixtures with different concentrations of TLCL and a constant concentration of TMCL. *, *p* < 0.05 versus cyt *c*/H₂O₂, *n* = 6 independent experiments.

the fluorescence of NBD-CL integrated into NBD-CL/TOCL/DOPC liposomes revealed an unusually strong quenching, which plateaued at ~80% upon gradual addition of cyt *c*. The amount of cyt *c* required for the maximal fluorescence quenching was 16 times lower than the amount of phospholipid available for protein binding in the outer leaflet. This again points to very avid cyt *c*/NBD-CL interactions. Although the complexity of mechanisms of NBD fluorescence quenching by cyt *c* does not permit unambiguous estimation of the cyt *c*/NBD-CL affinity constant, our data indicate that the lower limit for the binding constant of cyt *c*/CL is 5×10^8 M⁻¹.

A rough approximation of Forster radius for NBD-CL and heme yields values in the range from 28 to 36 Å that is shorter than the distance between NBD moieties located in the outer and inner leaflet (>38 Å). These *R*₀ values indicate that a quenching efficiency of 80% could be achieved only if (1) fluorescence of NBD-CL located in the outer membrane leaflet was completely quenched (by ~100%) at the saturated level of binding, that (2) fluorescence of inner leaflet NBD-CL was quenched by ~60%, and that (3) this NBD has to be at least within ~20 Å from the heme. Thus, heme has to be located within the membrane between the C7 atom of the acyl chain and the glycerol moiety of phospholipids. These data support the view that cyt *c* undergoes partial unfolding upon binding to the membrane whereby the heme (or at least its edge) is inserted in the lipid bilayer. Moreover,

these data indicate that the NBD moiety may physically interact with the protein and, most importantly, with the heme.

Analysis of the peroxidase activity of the cyt *c*/CL complex demonstrated that NBD-CL was able to inhibit oxidation of prototypical substrates, etoposide and Amplex Red, to an extent that no oxidation was observed upon complete substitution of TOCL for NBD-CL. H₂O₂-induced protein oligomerization via recombination of protein-centered radicals, likely tyrosyl radicals, was also markedly and competitively inhibited by both etoposide and NBD-CL. This suggests that, similar to etoposide, NBD-CL acted as a substrate for the peroxidase activity of cyt *c*/NBD-CL complexes. Kinetic analysis of Amplex Red oxidation revealed that NBD-CL's inhibitory effects were due to both competitive and noncompetitive mechanisms.

Finally, NBD-CL was able to block cyt *c*-dependent peroxidation of TLCL. Thus NBD-CL formed a strong complex with cyt *c*, which, however, had no oxidative potency toward endogenous polyunsaturated CLs. Importantly, NBD-CL was by far a more potent inhibitor of the peroxidase activity than C₁₂-NBD-PC, thus clearly demonstrating selectivity of NBD-CL. Given that C₆-NBD-PC had no effect on the peroxidase activity of the cyt *c*/CL complex at all, it is tempting to speculate that the presence of a long acyl chain is required for the positioning of the NBD moiety

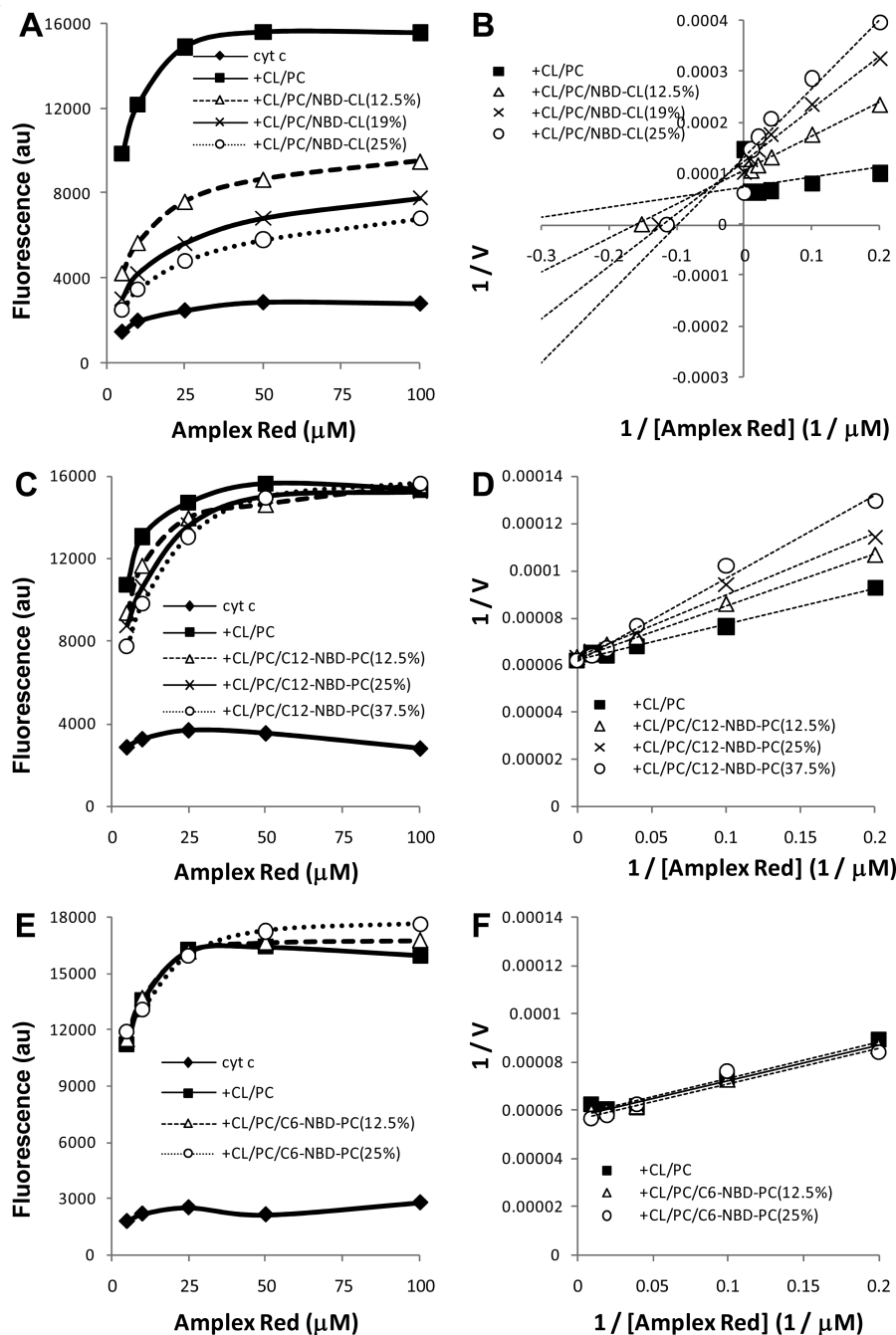


FIGURE 7: Effects of NBD-CL on oxidation of Amplex Red by cyt *c*/H₂O₂. Cyt *c* (0.5 μM) was preincubated for 10 min with DOPC/TOCL liposomes (1:1) at a ratio of 1:25. Mol % of NBD-TOCL, C₁₂-NBD-PC, and C₆-NBD-PC in liposomes was as indicated. Inhibition of the peroxidase activity of cyt *c*/CL complexes by NBD-CL (A, B), C₁₂-NBD-PC (C, D), and C₆-NBD-PC (E, F) was assessed by fluorescence in 20 mM HEPES buffer (pH 7.4) containing H₂O₂ (100 μM), DTPA (100 μM), and Amplex Red in the range of 5–100 μM . (A, C, E) Dependence of the reaction rate on the substrate concentration; (B, D, F) Lineweaver–Burk plots for peroxidase activity.

within the protein environment and for its interaction with the active site.

Additional insights into the mechanisms underlying the inhibition of cyt *c*/CL peroxidase activity by NBD-CL were gained from spectral analysis of NBD moiety. H₂O₂-induced decomposition of NBD manifested itself by fluorescence decay in the presence of the cyt *c*/CL complex. Moreover, MS data revealed that NBD-CL was predominantly converted into a product lacking the NBD moiety, thus suggesting that cleavage of NBD occurred at the amine–benzene bond. The amino group participates in the formation of the intramolecular charge transfer state responsible for the fluorescence

of NBD. Thus, the loss of amine by NBD should lead to the fluorescence loss as observed in our experiments.

Four major mechanisms of NBD metabolism by cytochromes P450 and peroxidases have been proposed including heteroatom oxidation, carbon hydroxylation, reductive ring opening, and reduction of the nitro group. Heteroatom oxidation is one of the most widespread reactions catalyzed by oxoferryl species of both cytochromes P450 and peroxidases. Various peroxidases including horseradish peroxidase, cytochrome *c* peroxidase, lactoperoxidase, and prostaglandin synthetase are able to catalyze oxidation of primary, secondary, and tertiary aromatic and heterocyclic amines (51–55).

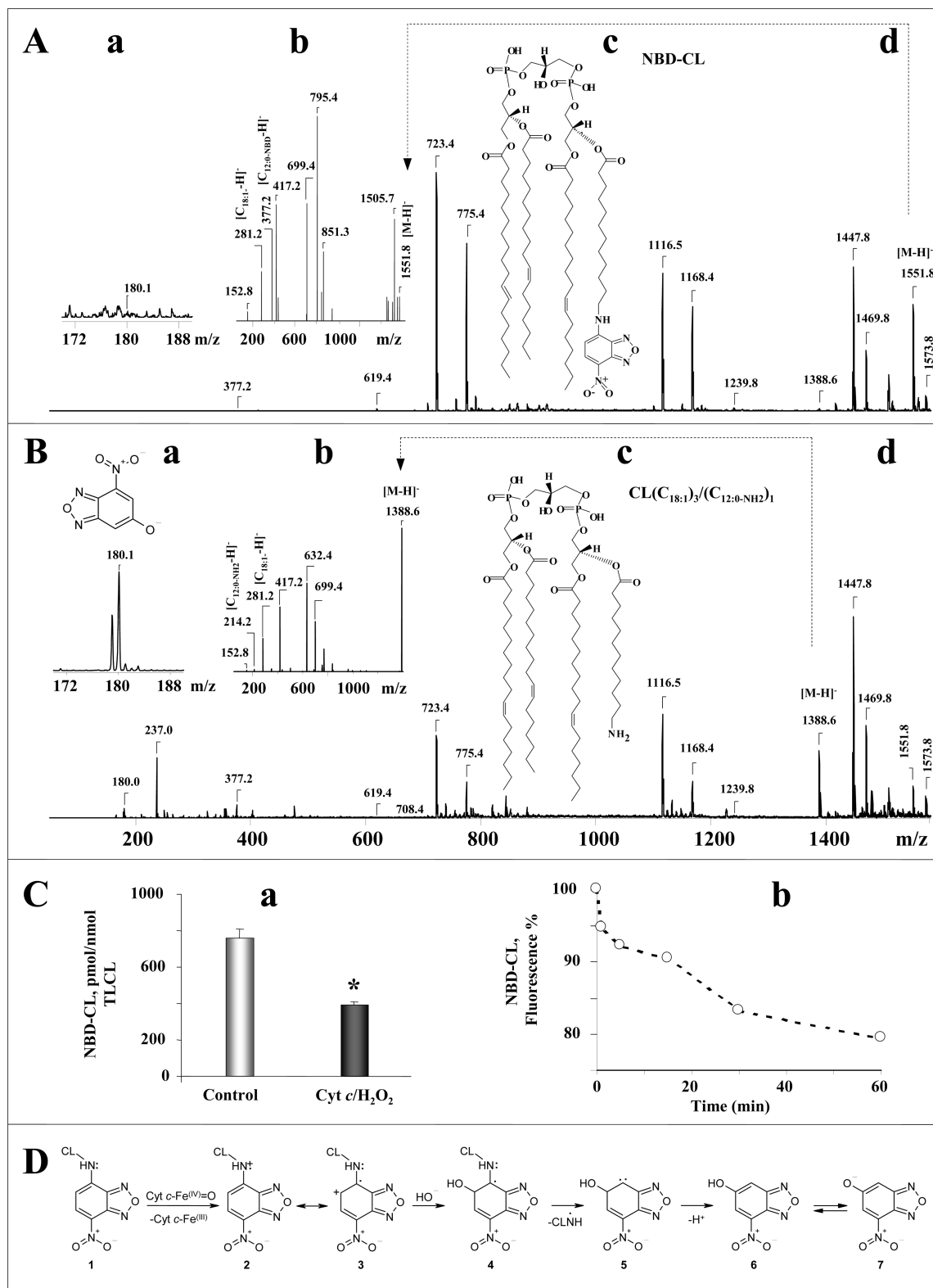


FIGURE 8: Mass spectrometry and fluorescence analysis of NBD-CL and its oxidation products produced by the cyt *c*/CL complex. (A, d, B, d) Typical mass spectra of TLCL, NBD-CL, and its H₂O₂-induced oxidation product in the presence of cyt *c* were acquired in negative ion mode using full-range zoom (m/z 50–1600) scans. Typical MS/MS fragmentation profiles of singly charged molecular species of NBD-CL before and after its incubation with cyt *c*/H₂O₂ are presented on panels A, b and B, b, respectively. Panels A, c and B, c show chemical structures of parent ions that were fragmented. Panels A, a and B, a show the formation of the NBD-CL degradation fragment with m/z 180 (insert) and its structure after incubation of liposomes with cyt *c*/H₂O₂. (C, a) Quantitative analysis of NBD-CL decomposition. Cyt *c* (5 μ M) was incubated with DOPC/TLCL/NBD-CL (2:1:1) liposomes (total concentration of phospholipids was 500 μ M) in the presence of H₂O₂ (100 μ M) for 30 min. The concentration of NBD-CL in the samples was confirmed in independent experiments which included ESI-MS protocol and lipid phosphorus determination. TMCL was used as internal standard, and NBD-CL was used as a reference standard. *, $p < 0.05$ versus control, $n = 6$ independent experiments. (C, b) Typical time course of NBD-CL fluorescence decay induced by cyt *c* via peroxidase mechanism. Cyt *c* (1 μ M) was preincubated for 10 min with DOPC/TOCL/NBD-CL liposomes (50:49:1) at the cyt *c*/CL ratio of 1:33. NBD-CL fluorescence was measured during 1 h incubation with H₂O₂ (50 μ M). (D) Scheme of reaction intermediates illustrating the proposed mechanism of NBD-CL degradation by the peroxidase activity of cyt *c* in the presence of H₂O₂.

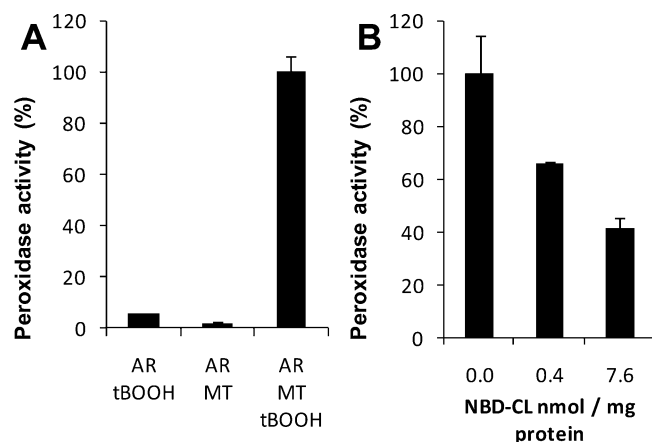


FIGURE 9: Effect of NBD-CL on peroxidase activity of mouse liver mitochondria. (A, B) Mitochondrial peroxidase activity assayed with Amplex Red (AR) as a substrate. Incubation conditions: mitochondria (MT) (0.25 mg protein/mL), AR (50 μ M), tBOOH (2 mM), HEPES (25 mM, pH 7.4), and incubation time 10 min.

These reactions are usually initiated by electron transfer from a nitrogen atom to a reactive heme intermediate and result in N-dealkylation of amines, radical dimerization, or adduct formation (52, 54, 55). Generation of the nitrogen-centered cation radical, the first intermediate of amine oxidation, has been reported during peroxidase-dependent oxidation of several aromatic amines (52, 53). Similarly, P450-catalyzed oxidation of nitrogen atoms proceeds via electron abstraction from nitrogen and cation radical formation and leads to N-dealkylation (reviewed in ref 56). Hydrocarbon hydroxylation involves oxygen incorporation at the carbon atoms of the benzene ring or at the adjacent carbon atom to the amino group. This classical reaction for P450 enzymes has been suggested to be catalyzed by oxoferryl species (57, 58). NBD contains an oxadiazole ring, which may undergo a reductive N–O bond cleavage to generate the ring-opened products as has been reported for oxadiazoles and their close analogue, isoxazole (59, 60). In addition, NBD's nitro group, which is essential for fluorescence properties of the molecule, could be reduced by P450 enzymes to amine (61). The latter mechanism is the least likely to occur, since there were no efficient electron donors for nitro reduction (like NADPH) in our enzymatic system. Hydroxylation of carbon atoms in the benzene ring has not been observed by MS analysis after *cyt c*/H₂O₂-dependent NBD-CL oxidation. Instead, MS data revealed cleavage of the bond between the benzene ring and amine. This suggests that the secondary amine that links the acyl chain and NBD is a likely oxidation site. The only difference with a common heteroatom oxidation mechanism is that NBD-CL oxidation resulted in the bond cleavage between nitrogen and the ring system rather than in N-dealkylation. This can be explained, at least in part, by a reported lower probability of N-dealkylation with increase of acyl chain length (i.e., N-demethylation is favored over N-deethylation by a factor of 16) (62); in our case, the acyl moiety was represented by the C₁₂ chain. Overall, the proposed mechanism for the regiospecific oxidation of the NBD moiety in the modified CL is illustrated by the scheme shown in Figure 8D.

Finally, we demonstrated that NBD-CL is incorporated into isolated mitochondria, where it inhibits tBuOOH-dependent peroxidase activity toward Amplex Red. Most of the current approaches aimed at protection of mitochondria

against oxidative insults are based on the use of conjugates with mitochondria-targeted moieties (e.g., triphenylphosphonium) and active cargoes represented by antioxidants, spin traps and nitroxides, quinones, vitamins, and peptides (9–11, 13–16). A combination of the nonoxidizable trioleoyl moiety with the C₁₂-NBD fragment within one CL molecule is a promising novel approach to create inhibitors of peroxidase activity of *cyt c*/CL complexes by exploiting high-affinity binding of NBD-CL to *cyt c* and regiospecific catabolism of the NBD moiety. Thus, NBD-CL functions not as a random chain-breaking lipid antioxidant/free radical scavenger but specifically inhibits enzymatic activity of *cyt c* involved in the execution of apoptotic program in mitochondria. By effectively preventing peroxidation of endogenous polyunsaturated molecular species of CL, NBD-CL may act as a promising regulator of apoptosis.

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