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Published on Web 07/23/2009

Heterolytic Reduction of Fatty Acid Hydroperoxides by Cytochrome *c*/Cardiolipin Complexes: Antioxidant Function in Mitochondria

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More than a century of studies of a small hemoprotein, cytochrome *c* (cyt *c*), have yielded rich information detailing its molecular interactions with mitochondrial complexes III and IV and its role in respiration.[1,2] Another recently established function of cyt *c* is the recruitment and activation of caspases in apoptosis.[3] Lately, discovery of the oxygenase activity of cyt *c* toward the anionic phospholipids cardiolipin (CL) and phosphatidylserine revealed its participation in apoptosis via the release of pro-apoptotic factors[4] and in recognition and clearance of apoptotic cells by phagocytes.[5] The two latter functions are based on cyt *c*'s ability to undergo partial unfolding upon binding with anionic phospholipids, leading to peroxidase activation and oxidation of phospholipids. Hydrogen peroxide (H_2O_2), an effective source of oxidizing equivalents for heme peroxidases, can feed the peroxidase cycle of cyt *c* complexes with anionic phospholipids.[6] Thus, cyt *c* not only produces peroxidized CL but also eliminates H_2O_2, contributing to the maintenance of antioxidant balance.[7] In mitochondria, nonheme glutathione (GSH) peroxidases, particularly GPX4, have been identified as important antioxidant mechanisms regulating oxidative stress via the reduction of (phospho)lipid hydroperoxides.[8] The importance and reported uniqueness of this GPX4 function is emphasized by the lethal phenotype of GPX4 k/o mice. Since heme peroxidases can utilize organic hydroperoxides such as *tert*-butyl hydroperoxide (*t*-BuOOH), cumene hydroperoxide, and peroxyacetic acid (PAA) as sources of oxidizing equivalents,[9] albeit less effectively than H_2O_2, we reasoned that control of lipid hydroperoxides may be yet another important regulatory antioxidant function of cyt *c* in mitochondria.

As reported previously, cyt *c*/CL complex (but not cyt *c* alone) readily reduced H_2O_2 and oxidized CL and phenolic substrates in the peroxidase cycle.[6,10] We found that different free fatty acid hydroperoxides (FFA−OOHs) were better substrates for cyt *c*/CL than H_2O_2 (Figure 1A). While there were some differences for various FFA−OOHs (Figure 1B, Table 1), overall the rates of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) oxidation by cyt *c*/CL complex were about 3 orders of magnitude higher in the presence of FFA−OOHs than H_2O_2. In murine liver mitochondria, the peroxidase activity (possibly associated with endogenous cyt *c* and CL) was also much higher with FFA−OOHs than with *t*-BuOOH (Figure 1C). Notably, cyt *c*/CL complexes acting as peroxidases were specific toward FFA−OOH, whereas horseradish peroxidase (HRP) and myeloperoxidase (MPO) showed greater ability to utilize H_2O_2 (Figure 1A).

The large difference between FFA−OOHs and H_2O_2 may be associated with a distinct reaction mechanism. Cyt *c* is believed to split H_2O_2 homolytically,[11,12] whereas heterolytic cleavage of H_2O_2 has been proposed for HRP, cyt *c* peroxidase, and MPO.[13] For these oxidoreductases, oxidation of the enzyme mediated by H_2O_2 is extremely fast (10^6−10^7 M^{-1} s^{-1}) with highly conserved distal His and Arg supporting heterolytic cleavage of H_2O_2.[9,14,15] To elucidate the reaction mechanism for cyt *c*/CL, we used spin trapping of radical intermediates with subsequent EPR spectroscopy. The homolytic mechanism involves one-electron reduction of hydroperoxide and yields an O-centered radical from peroxide. Two-electron reduction of peroxide via the heterolytic pathway produces alcohol and no free-radical intermediates. Two spin traps, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (POBN), were employed. To trap primary alkoxyl radicals, DMPO (at a high concentration of 0.5 M) was added to cyt *c* and cyt *c*/CL complexes in the presence of *t*-BuOOH, 4-hydroxy-2-nonenal (4HpNE), or (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid [13(S)HpODE]. The concentrations of hydroperoxides were adjusted to obtain equivalent amounts of the oxidized substrate (Figure 2A,B). Neither 4HpNE nor 13(S)HpODE

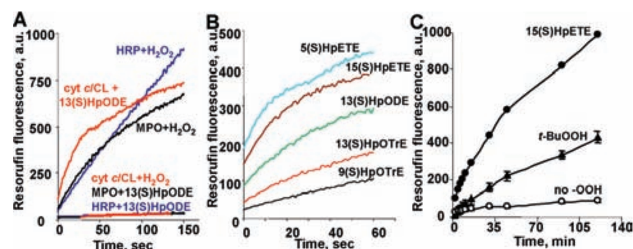

Figure 1. Amplex Red oxidation in vitro (A) by cyt *c*/CL complexes (0.1 μM cyt *c*, 2.5/2.5 μM CL/PC), HRP (5 milliunits/sample), and MPO (5 milliunits/sample) in the presence of 13(S)HpODE (5 μM) or H_2O_2 (5 μM), with 10 μM Amplex Red; (B) by cyt *c*/CL complexes (0.1 μM cyt *c*, 2.5/2.5 μM CL/PC) in the presence of FFA−OOHs (1 μM), with 10 μM AmplexRed; and (C) by murine liver mitochondria (10 μg/sample) in the presence of FFA−OOH or *t*-BuOOH (50 μM), with 50 μM Amplex Red.

Table 1. Rate Constants (k_1) for Cyt *c*/CL Complex Oxidation by −OOH[a]

R−OOH	k_1 (M^{-1} s^{-1})
5(S)HpETE	9.44×10^4
15(S)HpETE	7.52×10^4
13(S)HpODE	5.20×10^4
13(S)HpOTrE	2.43×10^4
9(S)HpOTrE	1.23×10^4
4HpNE	5.79×10^3
PAA	3.42×10^3
H_2O_2	4.64×10^1
t-BuOOH	3.03×10^1

[a] The calculation is described in detail in the Supporting Information.

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10.1021/ja904343c CCC: $40.75 © 2009 American Chemical Society

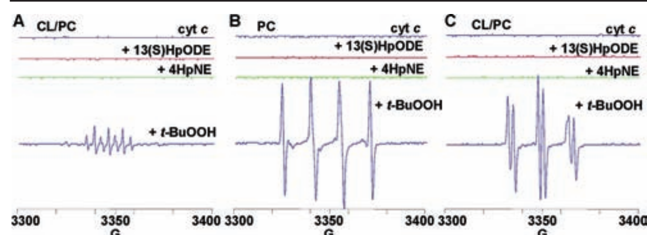

Figure 2. Spin trapping of 13(S)HpODE-, 4HpNE- and *t*-BuOOH-derived radicals generated by cyt *c*/CL under conditions of equal consumption of oxidizing equivalents when cyt *c* was preincubated with (A) CL/PC liposomes and DMPO, (B) PC liposomes and DMPO, and (C) CL/PC liposomes and POBN.

induced the formation of DMPO adducts. In contrast, *t*-BuOOH produced strong EPR signals when CL/PC or PC liposomes were incubated with cyt *c*. DMPO/$\cdot OC(CH_3)_3$ radical adduct ($a^N = 14.90$ G, $a^H_\beta = 16.04$ G, and $a^H_\gamma = 0.63$ G) was detected with PC liposomes. The major signal detected in the presence of cyt *c*/CL complexes was represented by the decomposition product of DMPO adducts [DMPOX, $a^N = 7.25$ G, $a^H_\beta(2H) = 4.05$ G].

Further, hydroperoxide-derived radicals were analyzed using another spin trap, POBN, which has been successfully utilized for the detection of lipid-centered radicals.[16] Again, a strong EPR signal was detected when cyt *c*/CL complexes were incubated with *t*-BuOOH (10 mM) (Figure 2C). The signal was represented by a sum of signals, the major of which had splitting constants of $a^N = 15.7$ G and $a^H = 2.7$ G characteristic of adducts with carbon-centered radicals.[16] No discernible signals were observed with either 4HpNE or 13(S)HpODE. The results of the spin-trapping experiments suggest that cyt *c*/CL peroxidase complexes split FFA−OOHs (in contrast to *t*-BuOOH) predominantly via the heterolytic mechanism.

The major products of heterolytic reduction of hydroperoxides are hydroxy compounds, while a broad spectrum of oxygenated decomposition products may be formed via the free-radical-mediated homolytic pathway. Electrospray ionization mass spectrometry (ESI-MS) analysis demonstrated that 13-hydroxy-(9*Z*,11*E*)-octadecadienoic acid (13-HODE) and other possible products of the heterolytic pathway (*m*/*z* 295, 311, and 327; Figure 3) were the major products generated by cyt *c*/CL from 13(S)HpODE (65 ± 3% of total products), thus confirming the predominance of the heterolytic reaction mechanism.

Heterolytic peroxidase catalysis commonly involves participation of His and Arg residues.[13−15] We predicted the binding sites of FFA−OOHs in cyt *c* (Figure 2S in the Supporting Information) using AutoDock software.[17] The top-ranked conformations with the lowest energy (potential binding sites) for both 4HpNE and 13(S)HpODE were located at a similar site, with the hydroperoxy group in both cases

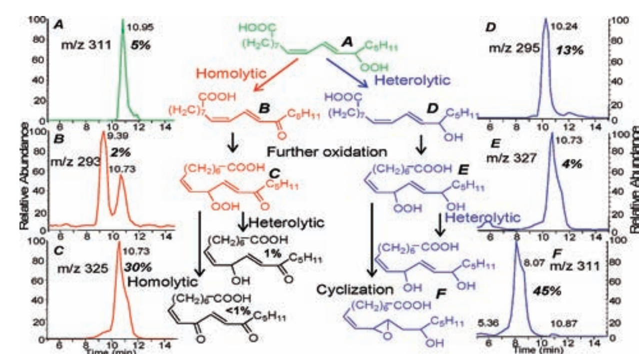

Figure 3. Homo- and heterolytic enzymatic conversion of 13(S)HpODE induced by cyt *c*/CL complex: (A) 13(S)HpODE; products of (B, C) homolytic and (D−F) heterolytic splitting and further oxidation. Amounts of final products are indicated as % of total products.

in proximity to Arg_{38} and His_{33}. In contrast, *t*-BuOOH was found to occupy a different site on cyt *c* in proximity to His_{18}. These results imply that FFA−OOHs undergo a heterolytic reaction as opposed to the homolytic reaction mechanism exhibited by organic hydroperoxides, further corroborating the results obtained experimentally.

Overall, the reaction of cyt *c* with hydroperoxides may proceed via both homo- and heterolytic cleavage of an O−O bond. However, the contribution of the heterolytic mechanism to the reduction of FFA−OOH is overwhelmingly greater than that of the homolytic pathway. Simultaneous involvement of two- and one-electron −OOH reductions is not unique for cyt *c*/CL complexes; peroxidase catalysis by cyclooxygenase-2 utilizes both pathways in a 60:40 ratio.[18] Utilization of FFA−OOHs by cyt *c*/CL complexes in vivo may be an interesting new pathway in the synthesis of oxygenated fatty acids. Our previous studies identified these complexes and oxidation of polyunsaturated species of CL as a required step in execution of the apoptotic program.[4] The source(s) of oxidizing equivalents feeding the peroxidase cycle, however, have not been identified. We further established that cyt *c* also catalyzes phospholipase A-like reaction to hydrolyze peroxidized CLs and release oxygenated FFAs.[19] The current results identify the FFA−OOHs as potent sources of oxidizing equivalents that maintain high levels of peroxidase activity and production of sufficient amounts of oxidatively modified CLs. Physiological role(s) of hydroxy derivatives of FFA warrant further studies.

Acknowledgment. This work was supported by grants from the NIH (U19-AI068021, HL70755, R03TW007320, 2RO1LM007994-05), the NSF (CC0449117), the PittGrid, and la Junta de Extremadura, Orden 2008050288 (A.K.S.A.).

Supporting Information Available: Experimental details, calculations of peroxidase reaction rate constants, prediction of *t*-BuOOH, 4HpNE, and 13(S)-HpODE binding sites, and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA904343C