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# Peroxidative permeabilization of liposomes induced by cytochrome *c*/cardiolipin complex

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ABSTRACT

Interaction of cytochrome *c* with mitochondrial cardiolipin converting this electron transfer protein into peroxidase is accepted to play an essential role in apoptosis. Cytochrome *c*/cardiolipin peroxidase activity was found here to cause leakage of carboxyfluorescein, sulforhodamine B and 3-kDa (but not 10-kDa) fluorescent dextran from liposomes. A marked decrease in the amplitude of the autocorrelation function was detected with a fluorescence correlation spectroscopy setup upon incubation of dye-loaded cardiolipin-containing liposomes with cytochrome *c* and $H_2O_2$, thereby showing release of fluorescent markers from liposomes. The cytochrome *c*/$H_2O_2$-induced liposome leakage was suppressed upon increasing the ionic strength, in contrast to the leakage provoked by Fe/ascorbate, suggesting that the binding of cyt *c* to negatively-charged membranes was required for the permeabilization process. The cyt *c*/$H_2O_2$-induced liposome leakage was abolished by cyanide presumably competing with $H_2O_2$ for coordination with the central iron atom of the heme in cyt *c*. The cytochrome *c*/$H_2O_2$ permeabilization activity was substantially diminished by antioxidants (trolox, butylhydroxytoluene and quercetin) and was precluded if fully saturated tetramyristoyl-cardiolipin was substituted for bovine heart cardiolipin. These data favor the involvement of oxidized cardiolipin molecules in membrane permeabilization resulting from cytochrome *c*/cardiolipin peroxidase activity. In agreement with previous observations, high concentrations of cyt *c* induced liposome leakage in the absence of $H_2O_2$, however this process was not sensitive to antioxidants and cyanide suggesting direct membrane poration by the protein without the involvement of lipid peroxidation.
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## 1. Introduction

Peroxidation of membrane lipids [1–3] has long been considered as one of the harmful consequences of oxidative stress in living cells, manifesting itself, in particular, in perturbing the barrier function of cell membranes. In support of this common idea, experiments with model lipid membranes – planar bilayers and liposomes – have shown that lipid peroxidation results in augmented membrane permeability [4–17]. On the other hand, mechanistic studies of apoptosis have pointed to a key role of cardiolipin peroxidation in the process of cytochrome *c* (cyt *c*) release from mitochondria into cytosol [18–21]. Intriguingly, cardiolipin peroxidation has appeared to be catalyzed by cyt *c* [19, 22–24], the peroxidase activity of which [25–32] is dramatically enhanced upon binding to cardiolipin [19,33–35], partial proteolysis [26,36], denaturation [31], dimerization [37], tyrosine nitration [38], methionine ($met_{80}$) oxidation [39,40] and residues 26 or 41 mutation [41–43]. Induction of lipid peroxidation by cyt *c* has also been reported for other unsaturated lipids [44–49].

In view of the above relationship between lipid peroxidation and membrane permeability, peroxidase activity of cyt *c*/cardiolipin complex may imply its propensity to permeabilize lipid membrane in the presence of hydrogen peroxide. Of note, cyt *c* by itself is also able to induce ion permeability of liposomal [50] and planar bilayer [51] membranes and even leakage of fluorescent dextran and carboxyfluorescein from liposomes, the latter being more pronounced with cardiolipin-containing vesicles [52]. Of relevance to the issue are the data on the cyt *c*-induced formation of non-bilayer structures [53] and morphological transitions [54] in cardiolipin-containing model membranes, as well as permeation of apocytochrome *c* across lipid bilayers [55] along with its ability to induce dye leakage from liposomes [56]. Previously, induction of electrical current across planar bilayer lipid membrane formed from the mixture of soybean phosphatidylcholine and tetraoleoyl-cardiolipin was reported upon the addition of hydrogen peroxide in the presence of cyt *c* [57,58]. To study in detail the membrane-permeabilizing activity of cyt *c* promoted by $H_2O_2$, we applied here a fluorescence dequenching assay and a fluorescence

*Abbreviations:* cyt *c*, cytochrome *c*; CF, 5(6)-carboxyfluorescein; SRB, sulforhodamine B; PC, soybean phosphatidylcholine; CL, bovine heart cardiolipin; TMCL, tetramyristoyl-cardiolipin; DPhPC, diphytanoylphosphatidylcholine; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; FCS, fluorescence correlation spectroscopy; G(τ), autocorrelation function

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correlation spectroscopy (FCS) approach using dye-loaded large unilamellar vesicles of different lipid compositions. A combination of cyt *c* with hydrogen peroxide was found to induce dye release from liposomes, provided that both cardiolipin and bulk phosphatidylcholine components of liposomal membranes contained unsaturated acyl tails. Together with sensitivity to antioxidants, these results allowed to relate the cyt *c*/$H_2O_2$-induced dye leakage to formation of membrane structural defects involving oxidized cardiolipin molecules.

## 2. Materials and methods

### 2.1. Materials

Most chemicals including equine heart cytochrome *c*, bovine heart cardiolipin (CL), sulforhodamine B (SRB), 5(6)-carboxyfluorescein (CF), butylated hydroxytoluene (BHT), trolox and quercetin were from Sigma; 1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine (DPhPC) and tetramyristoyl-cardiolipin (TMCL) were from Avanti Polar Lipids (Alabaster, AL).

### 2.2. Preparation of liposomes

Dye-loaded liposomes were prepared by evaporation under a stream of nitrogen of a 2% solution of a mixture of lipids in chloroform followed by hydration with a buffer solution containing appropriate fluorescent marker. Four different lipid mixtures were used: 1) PC, 5 mg soybean phosphatidylcholine (Sigma, Type II-S), 2) PC/CL, 4 mg soybean phosphatidylcholine and 1 mg bovine heart cardiolipin, 3) PC/TMCL, 4 mg soybean phosphatidylcholine and 1 mg tetramyristoyl-cardiolipin, 4) DPhPC/CL, 4 mg diphytanoylphosphatidylcholine and 1 mg bovine heart cardiolipin. Four different marker solutions (0.5 ml each) were used: 1) 100 mM CF adjusted to pH about 8 by Tris, 2) 1 mM SRB in 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.4, 3) 3 mg/ml rhodamine-labeled 3-kDa dextran in the same buffer, and 4) 10 mg/ml rhodamine-labeled 10-kDa dextran in the same buffer. The mixture was vortexed, passed through several cycles of freezing and thawing, and extruded through 0.1-μm pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound marker was then removed by passage through a Sephadex G-50 coarse column (for SRB) or through a Sephadex G-150 coarse column with a buffer solution containing 100 mM KCl, 10 mM Tris, 10 mM MES, and pH 7.4.

### 2.3. Fluorescence dequenching assay

Fluorescence of liposomes loaded with 100 mM CF was monitored at 520 nm (excitation at 490 nm) with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). The extent of CF efflux was calculated as $(F_t - F_0) / (F_{100} - F_0)$, where $F_0$ and $F_t$ represent the initial fluorescence intensity and the fluorescence intensity at the time *t*, and $F_{100}$ is the fluorescence intensity after complete disruption of liposomes by addition of the detergent Triton-X100 (final concentration, 0.1% w/w).

### 2.4. Fluorescence correlation spectroscopy

The home-made setup was described previously [59]. Briefly, fluorescence excitation and detection utilized a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescent inverted microscope equipped with a 40×, NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence light passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μm core fiber coupled to an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). The signal from an output was sent to a PC using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The data acquisition time was 30 s. The fluorescence was recorded from the confocal volume located at about 50 μm above the coverslip surface with 50 μl of the buffer solution added. Most of the data were collected under the conditions of stirring a suspension by a paddle-shaped 3-mm plastic bar rotated at 600 rpm. To calibrate the setup, we recorded the fluorescence autocorrelation function of Rhodamine 6G solution. Assuming the diffusion coefficient of the dye to be $2.5 \times 10^{-6}$ cm²/s, the value of the confocal radius $\omega = 0.42$ μm was obtained. The correlated fluorescence emission signals were fitted to the three-dimensional autocorrelation function [60,61]:

$$G(\tau) = \frac{1}{N}\left(\frac{1}{1+\frac{\tau}{\tau_D}}\right)\left(\frac{1}{\sqrt{1+\frac{\omega^2\tau}{z_0^2\tau_D}}}\right) \quad (1)$$

with $\tau_D$ being the characteristic correlation time during which a molecule resides in the observation volume of radius $\omega$ and length $z_0$, given by $\tau_D = \omega^2/4D$, where *D* is the diffusion coefficient, N is the mean number of fluorescent particles in the confocal volume. The amplitude of the autocorrelation function is inversely proportional to the number of fluorescent particles ($N = 1/G(\tau \to 0)$), but is independent of the fluorescence intensity of a single particle (in a system of identical particles) and therefore does not depend on the number of fluorophores per vesicle. Particles can be any fluorescent "point objects" in comparison to the dimension of the observation volume (i.e. about 1 μm). Therefore, particles can be single molecules of dye (i.e. SRB), as well as liposomes carrying different numbers of dye molecules. Initially (before the leakage induction) the system has a limited number of particles per observation volume comprising predominantly several liposomes loaded with the dye. After the leakage, the number of particles increases tremendously, because every liposomal particle produces thousands of particles of free dye leading to a significant decrease in the parameter $G(\tau \to 0)$.

## 3. Results and discussion

### 3.1. Cyt c/$H_2O_2$-induced leakage of carboxyfluorescein from liposomes

Fig. 1A illustrates liposome leakage induced by cyt *c* and $H_2O_2$, as monitored by release of the fluorophore carboxyfluorescein encapsulated in liposomes at a self-quenching concentration [62], which manifested itself in an increase of CF fluorescence. It is seen that 100 nM cyt *c* in combination with 1.5 mM $H_2O_2$ caused leakage of CF entrapped in cardiolipin-containing liposomes at low (curve 2), but not at high (100 mM KCl) ionic strength (curve 6). Of note, the $H_2O_2$-induced change in CF fluorescence depended non-monotonically on cyt *c* concentration (Fig. 1B): the increase in fluorescence became smaller at 1 μM cyt *c* (curve 5), whereas at higher cyt *c* concentrations the addition of $H_2O_2$ elicited even a decrease in CF fluorescence (curve 6). Earlier the cyt *c*-dependent quenching of fluorescence and the corresponding absorbance loss associated with oxidative damage were observed with the cyanine dye diS-$c_3$-(5) [63]. Therefore, the cyt *c*/$H_2O_2$-induced reduction of CF fluorescence was most likely due to destruction of CF caused by cyt *c*-mediated lipid peroxidation. This assumption is supported by the fact that CF itself has been shown to serve as a substrate of a peroxidase [64]. Oxidative instability of CF was also observed previously upon photodynamically induced leakage from liposomes [16].

Thus, we found significant stimulation of cyt *c*-induced carboxyfluorescein leakage from liposomes by hydrogen peroxide. However, a quantitative study of cyt *c*/cardiolipin peroxidase effect on the fluorescence of liposomes loaded with CF is hampered by oxidative instability of this dye.

### 3.2. FCS study of cyt c/$H_2O_2$-induced liposome leakage

In recent publications [16,65–67], permeability of vesicle membranes to fluorescent dyes has been studied by FCS, the approach which does not require loading of liposomes with dyes at very high,

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