Protective Role of Mitochondrial Unsaturated Lipids on the Preservation of the Apoptotic Ability of Cytochrome c Exposed to Singlet Oxygen

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Cytochrome c-mediated apoptosis in cells submitted to photodynamic therapy raises the question about the ability of photodynamically oxidized cytochrome c (cyt c405) to trigger apoptosis as well as the effect of membranes on protein photodamage. Cytochrome c was submitted to irradiation in the presence of MB+ in phosphate buffer and in the presence of four types of phosphatidylcholine/phosphatidylethanolamine/cardioliipin (PCPECL) liposomes (50/30/20%): totally saturated lipids (tPCPECL), totally unsaturated lipids (puPCPECL), partially unsaturated (80%) lipids, with unsaturation in the PC and PE content and puPCPECL80, partially unsaturated (20%) lipids, with unsaturation in the CL content (puPCPECL20). Cyt c405 was formed by irradiation in buffered water and in tPCPECL and puPCPECL20 liposomes. In the presence of tPCPECL and puPCPECL80, cytochrome c was protected from photodynamic damage (lipid-protected cytochrome c). In CL liposomes, 25% unsaturated lipids were enough to protect cytochrome c. The presence of unsaturated lipids, in amounts varying according to the liposome composition, are crucial to protect cytochrome c. Interesting findings corroborating the unsaturated lipids as cytochrome c protectors were obtained from the analysis of the lipid-oxidized derivatives of the samples. Native cytochrome c, lipid-protected cytochrome c, and cyt c405 were microinjected in aortic smooth muscle cells. Apoptosis, characterized by nucleus blebbing and chromatin condensation, was detected in cells loaded with native and lipid protected cytochrome c but not in cells loaded with cyt c405. These results suggest that photodynamic therapy-promoted apoptosis is feasible due to the protective effect of the mitochondrial lipids on the cytochrome c structure and function.

Singlet oxygen (O2 (1Δg))⁵ is an excited state of molecular oxygen that can be generated by an energy transfer mechanism from a triplet excited state of a compound to the ground state of molecular oxygen (1, 2). Singlet oxygen O2 (1Δg) can be produced in biological systems, and, due to its high electrophilicity, reactivity and relatively long lifetime (2–4 μs in H2O and ~700 μs in CCL4) can promote damage in biological molecules (3–5). In mammalian cells, O2 (1Δg) can be generated during oxidative stress, and it is able to attack DNA, protein thiol groups, and membrane lipids (6–15).

The pro-oxidant activity of singlet oxygen, triplet excited species, and free radicals has been used in medicine for the treatment of tumors in a process that is known as photodynamic therapy (PDT) (16). PDT involves in situ generation of reactive species promoting tumor regression (17). The mechanism of tumor destruction involves the direct oxidation (type I mechanism) of biological targets (membranes, proteins, and DNA), as well as oxidation mediated by O2 (1Δg) (type II mechanism) (18). In the type II mechanism, O2 (1Δg) is generated through energy transfer from excited triplet species to molecular oxygen (17).

Proteins are important targets for the pro-oxidant action of O2 (1Δg). In the case of hemoproteins, this excited species can damage both the apoproteins and the prosthetic group (19–21). Among the potential protein targets for O2 (1Δg), cytochrome c deserves special consideration, because it is a mitochondrial protein fundamental to the cell respiration and activation of the apoptosis cascade (22, 23). Therefore, O2 (1Δg)-mediated alterations in cytochrome c structure and reactivity can influence activation of caspases in the apoptosome.

Previously we compared the effect of photogenerated singlet oxygen (O2 (1Δg)) (type II mechanism) and free radicals (type I

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The abbreviations used are: O2 (1Δg), singlet molecular oxygen; CL, cardioliipin; cyt c405, singlet oxygen-oxidized cytochrome c; LOH, lipid-derived alcohol; LOO2, lipid-derived hydroxyl radical; LOOH, lipid-derived peroxyde; MB+, methylene blue; MDA, malondialdehyde; PC, phosphatidylcholine; PDT, photodynamic therapy; PE, phosphatidylethanolamine; puCL25, -50, and -75CL liposomes containing 25, 50, and 75% unsaturated lipids, respectively; puPCPECL20, PCPECL liposomes containing partially unsaturated (20%) lipids, with unsaturation in the CL content; puPCPECL80, PCPECL liposomes containing partially unsaturated (80%) lipids, with unsaturation in the PE content; SMC, smooth muscle cell; tPC, CL liposomes containing totally saturated lipids; tPCPECL, PCPECL liposomes containing totally saturated lipids; tPCPECL, CL liposomes containing totally unsaturated lipids; tPCPECL, PCPECL liposomes containing totally unsaturated lipids.
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Mechanism) on cytochrome c structure and reactivity. Both reactive species were obtained by photoexcitation of methylene blue (MB) in the monomer and dimer forms obtained by varying the micelle/MB ratio. Over a pH range in which cytochrome c is in the native form, \( \text{O}_2 \) (\( \Delta g \)) and free radicals induced the conversion of heme iron from its native low spin state to a high spin state with axial symmetry (g ~ 6.0) and with the Soret band shifted from 409 to 405 nm (cyc405). The direct attack on the heme group was only detected under conditions that favored free radical production (MB dimer in SDS micelles) or in the presence of a less structured form of the protein (above pH 9.3). cyc405 exhibited no alterations in the mass of the heme group but showed oxidative modifications in methionine (Met-65 and Met-80) and tyrosine (Tyr-74) residues. Damage of cyc405 tyrosine residue impaired its reduction by diphenylacetaldehyde, but not by \( \beta \)-mercaptoethanol, which was able to reduce cyc405, generating cytochrome c Fe(II) in the high spin state (spin 2) (24). Considering that PDT can induce cytochrome c detachment from the inner mitochondrial membrane leading to apoptosis, it remained to be ascertained whether the oxidative stress produced by photodynamic therapy also affects cytochrome c ability to trigger apoptosis. In this regard, the well known ability of polyunsaturated fatty acids to trap \( \text{O}_2 \) (25), points out the lipid fraction of the inner mitochondrial membrane, with which cytochrome c is peripherally associated, as a potential protector against the photodynamic protein oxidation. In this case, the attack to the inner mitochondrial membrane and membrane-bound proteins can promote lack of the transmembrane potential (\( \Delta \Psi \)), detachment of cytochrome c from the membrane and triggering of apoptosis in the cytosol (26, 27). In this regard, in a model system, liposomes containing polyunsaturated lipids were able to protect cytochrome c from the attack by free radicals produced in the course of the reaction of the protein with \( t \)-butylhydroperoxide (28). In this study, we investigated the ability of mitochondrial lipids to protect cytochrome c against photodynamic oxidation and the loss of the ability to trigger apoptosis.

**MATERIALS AND METHODS**

**Chemicals**—Cytochrome c (horse heart, type III) was acquired from Sigma. Lipids were acquired from Avanti Polar Lipids, Inc. (Alabaster, AL). MB\(^{+} \) was purchased from Aldrich and twice recrystallized using ethanol.

**Generation of \( \text{O}_2 \) (\( \Delta g \)) by Photosensitization of MB\(^{+} \)—**Samples were irradiated at 35 °C in a glass chamber, in 5 mM phosphate-buffered water or in 5 mM phosphate-buffered liposomes (PCPECL or CL). A 500-watt halogen light bulb, set at a distance of 30 cm, was used with a cutoff glass filter (500 nm ± 50 nm; wavelength mean value ± half bandwidth, Melles Griot, CA). The glass chamber was cooled by circulating water. Other additions are indicated in the figure legends.

**Purification of Cytochrome c Modified by Reactive Species**—The samples containing cytochrome c with Soret band peaking at 405 nm (cyc405) obtained in buffered water were purified with Chelex 100\(^{\circ} \), which has a binding affinity for MB\(^{+} \). Purification of MB\(^{+} \) with Chelex 100\(^{\circ} \) particles was monitored by spectrophotometry, indicating the disappearance of the MB\(^{+} \) absorbance spectrum. The samples containing cytochrome c submitted to MB\(^{+} \) photodynamic action associated with PCPECL liposomes were purified in a fast-protein liquid chromatography system (Amersham Biosciences) equipped with two P-500 pumps, a Superdex HR200 column, and a UV-visible detector. The flow rate was 0.5 ml/min, and the buffer consisted of 0.3 M sodium phosphate, pH 7.5. The high ionic strength buffer was used to promote dissociation of cytochrome c from the liposomes. The liposomes were eluted in the void volume, cytochrome c was included in the resin, and MB\(^{+} \) was eluted in the total volume of the beds. The elution of liposomes, cytochrome c, and MB\(^{+} \) was monitored by turbidity at 280 nm, absorbance at 409 nm (Soret band peak), and absorbance at 660 nm, respectively. The fractions that contained cytochrome c were pooled and dialyzed exhaustively against deionized water. After dialysis the sample was lyophilized.

**Electronic Absorption Spectrometry**—Electronic absorption measurements of cytochrome c were conducted in a photodiode spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD), using quartz cuvettes of 1-cm light path and a slit of 0.5 nm.

**Preparation and Analysis of Liposomes**—Lipids were first dissolved in chloroform, which was evaporated with N\(_2\) gas. The lipid residue was kept under reduced pressure for at least 2 h, after which it was hydrated by adding cold 5 mM phosphate buffer. After the addition of buffer, the mixture was then stirred with a vortex, and sonication was performed in a Ney Ultrasonic sonicator (J. M. Ney Co., Bloomfield, CT) during 30 min. The mean diameter of sonicated unilamellar liposomes was previously determined (27) as being 61.1 ± 0.3 nm by dynamic light scattering using a ZetaPlus-ZetaPotential analyzer (Brookhaven Instruments Corp., Holtsville, NY). When indicated, unilamellar liposomes were also obtained by extrusion of hydrated lipid dispersions in an Avanti Mini-extruder acquired from Avanti Polar Lipids, Inc. (Alabaster, AL). Samples were subjected to 11 passes through two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA) installed in tandem. The mean diameter of both sonicated and extruded liposomes was in agreement with images obtained by atomic force microscopy in an SPM 9600 scanning probe microscope (Shimadzu Corp.). Atomic force microscopy images (Fig. 1, A and B) were obtained by measurement of the interaction forces between the tip and the sample surface (29, 30) containing liposomes. The samples were analyzed in the contact mode at room temperature (20 °C) and atmospheric pressure (760 mmHg). A triangular silicon tip was used for this analysis, and the resonant frequencies of the cantilever were found to be ~200 kHz. Cardiolipin liposome films were obtained by immobilization on silicon-based support. Immediately before the analysis, the samples of fresh liposomes were diluted to 1 mM in 10 mM phosphate buffer, pH 7.4, and a drop of constant volume (10 \( \mu l \)) was deposited onto a small plate of silicon (~0.5 cm\(^2 \)) previously submitted to stringent cleaning (31). 2 min after deposition on the silicon plate, the sample excess was removed by using filter paper, and the drying was completed at 4 °C.

**Lipid Oxidation Assays**—Oxidative damage of liposomes was measured by dosage of the lipid hydroperoxide and malondialdehyde content. Lipid hydroperoxide (LOOH) measurement was done by oxidation of Fe\(^{2+} \) in the presence of xelenol.
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orange. Sonicated unilamellar liposomes (1 mM) were incubated in the presence or in the absence of 4 μM cytochrome c and/or 8 μM MB+ during 30 min, at 4 °C. An aliquot of the sample (50 μl) was mixed, and the mixture was incubated during 30 min at room temperature (25 °C) with 950 μl of hydroperoxide reagent, containing 100 μM xylenol orange, 250 μM (NH4)2Fe(SO4)2, 25 mM H2SO4 and 4 mM butylated hydroxytoluene in 90% methanol. The oxidation of Fe2+ by LOOH generates Fe3+ that reacts with xylenol orange forming the colored compound that absorbs at 560 nm. LOOH concentration was calculated from \( \varepsilon_{560\text{nm}} = 4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \). For the determination of MDA content present in liposomes, samples of lipids (1 mM) were incubated in the presence or absence of 4 μM cytochrome c and/or 8 μM MB+ during 30 min, at 4 °C (final volume, 150 μl). To determine malondialdehyde (MDA), 150 μl of 1% thiobarbituric acid prepared in 50 mM NaOH plus 15 μl of 10 M NaOH and 75 μl of 20% H3PO4 was added to the sample, followed by further incubation during 20 min at 85 °C. The MDA-thiobarbituric acid complex was extracted with 300 μl of n-butanol, and the absorbance was measured at 532 nm. MDA concentration was calculated from \( \varepsilon_{532\text{nm}} = 1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1} \).

Cell Culture and Apoptosis Assay—Rabbit aortic smooth muscle cells (SMCs) were grown in RPMI 1640 containing 10% fetal bovine serum, 200 μg/ml G418, and 100 μg/ml hygromycin. Cells were plated on glass cell locate coverslips (Eppendorf) at a density of 40,000/well in 24-well plates for 17–20 h prior to microinjection. Following microinjection, flat cells (normal) and round/shrunken cells with condensed nuclei (apoptotic) were counted and are presented as percent apoptotic cells. In microinjection experiments, 50 cells were counted for each condition.

Microinjection—Cell microinjection was performed on the stage of a Nikon Diaphot 300 inverted microscope using an Eppendorf pressure injector (model 5246) and micro-manipulator (model 5171). Microinjection needles (0.1-μm inner diameter) were pulled from glass capillaries using a horizontal electrode puller (Sutter Instrument, model P-97) and loaded using Eppendorf microloaders. Cells were plated on glass cell locate coverslips (Eppendorf) 24 h prior to injection. To identify injected cells, the solution for microinjection contained 0.3% dextran-conjugated Texas Red dye (M, 10,000, lysine-fixable, Molecular Probes) in H2O. Dye alone or dye plus cytochrome c (Sigma, C7752 from horse heart, diluted in H2O and freshly prepared for each experiment) was injected into the cytoplasm of cells (pressure, 80–100 hPa; time, 0.3 s). Cells were switched into fresh medium immediately after injection. The intracellular concentrations of microinjected proteins are estimated to represent a 10- to 100-fold dilution of the pipette concentration based on previous calibrations using similar microinjection apparatus and an estimated cellular volume of 4–5 μl (32). Thus, we estimate that the amount of cytochrome c injected is proportional to the pipette concentration and that 40–400 fg are delivered when the pipette concentration is 1 mg/ml. Accordingly, with a pipette concentration of 30 mg/ml (our maximal concentration) we estimate that 1.2–12 pg per cell are delivered. At various times, injected cells were scored for apoptosis as described above. After scoring for apoptosis, Hoechst dye 33342 (Sigma) was added to the medium to a final concentration of 4 μg/ml. Cells were incubated at 37 °C for 120 min and photographed under UV light.
Caspase-3 Fluorometric Assay—SMCs were collected, counted, and centrifuged at 250 × g for 10 min. The supernatant was gently removed and discarded, and the cell pellet was lysed by the addition of Cell Lysis Buffer (R&D Systems) according to the number of cells present in the pellet (25 μl of cold Cell Lysis Buffer per 1 × 10⁶ cells). This number can be estimated from the number of initially cultured cells. The cells were incubated for 10 min, and the amount of protein was determined by BCA protein assay. For the reaction, 100 μl of cell lysate was mixed with an equal volume of 2× Reaction Buffer (R&D Systems) and 3 ml of fresh dithiothreitol stock solution. To each reaction well 25 μM native cytochrome c, lipid-protected cytochrome c, or cytc405 was added prior to the addition of 10 ml of Caspase-3 fluorogenic substrate (DEVD-7-amino-4-trifluoromethylcoumarin). The plate was incubated for 2 h, and the fluorescence was read (excitation at 400 nm and reading at 505 nm).

Statistical Analysis—The statistical analysis for Figs. 4A, 4B, 4C, 6, and 7 data were done by using analysis of variance calculating software (GraphPad). The post-hoc tests (Bonferroni correction) were done by using QuickCalcs (GraphPad).

RESULTS

Effect of the Membrane Composition on O₂ (Δψ)-induced Oxidative Modifications on Cytochrome c—Horse heart cytochrome c (4 μM) was submitted to irradiation in the presence of 10 μM MB⁺ in phosphate-buffered water and in the presence of four types of 2 mM PC/PE/CL liposomes (50/30/20%). The liposome composition differed according to the saturation degree of the lipid acyl chains: totally saturated lipids (tsPCPECL), totally unsaturated lipids (tuPCPECL), partially unsaturated (80%) lipids, with unsaturation in the PC and PE content (puPCPECL80), and partially unsaturated (20%) lipids, with unsaturation in the CL content.
Protection of Cytochrome c by Unsaturated Lipids

**Fig. 2.** Spectra of cytochrome c and MB+ before and after irradiation in homogeneous and heterogeneous media. The samples were irradiated, at most, during 50 min with a 500-watt halogen lamp in: A, 2 mM puPCPECL20 liposomes and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; B, 2 mM tuPCPECL and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; C, experimental conditions identical to that described in B, except 300 mM NaCl was added to the sample after 5 min under irradiation; D, 2 mM puPCPECL80 and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; E, 2 mM tsPCPECL and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; F, 2 mM tsPCPECL and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; G, led to the blue shift of cytochrome c Soret band compatible with the formation of cyt405. Compared with the results obtained in the presence of membranes with high unsaturated acyl chain content, the degree of MB+ bleaching was also decreased in the presence of puPCPECL20 and tuPCPECL.

**Fig. 3.** Spectra of cytochrome c and MB+ before and after irradiation in CL liposomes. The cytochrome c samples were irradiated, at most, during 50 min with a 500-watt halogen lamp in: A, 2 mM puPCPECL20 liposomes and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; B, 2 mM tuPCPECL20 liposomes and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; C, experimental conditions identical to that described in B, except 300 mM NaCl was added to the sample after 5 min under irradiation; D, 2 mM puPCPECL80 and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; E, 2 mM tuPCPECL80 and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; F, 2 mM tsPCPECL and the spectra were run before (dashed line) and after 5 min (thick solid line) under irradiation; G, led to the blue shift of cytochrome c Soret band compatible with the formation of cyt405. Compared with the results obtained in the presence of membranes with high unsaturated acyl chain content, the degree of MB+ bleaching was also decreased in the presence of puPCPECL20 and tuPCPECL.

(0.55% of the incident light). Fig. 2A shows the alterations in the cytochrome c spectra promoted by O_2 (\(^{1}\Delta_g\)), in phosphate buffer at pH 7.4. Singlet oxygen was generated by the irradiation of MB+ with a 500-watt halogen lamp. As previously described, upon irradiation, two phenomena occurred concomitantly: the reduction of cytochrome c by excited MB+ and irreversible conversion of cytochrome c Fe(II) to Fe(III) mediated by O_2 (\(^{1}\Delta_g\)) associated with blue shift of the Soret band compatible with the formation of cyt405 (24). A percentage of reduced cytochrome c can be identified in the spectrum obtained after 10 min of irradiation that exhibited Q bands \(\beta\) and \(\alpha\) (indicated in the figure) overlapped by the MB+ spectrum. Fig. 2B shows the spectra of cytochrome c associated with tuPCPECL and obtained at 0, 5, and 10 min after the irradiation in the same conditions. The presence of tuPCPECL promoted significant differences in cytochrome c and MB+ behavior during the irradiation. In this condition, the cytochrome c Soret band did not exhibit progressive blue shift but only bleaching. On the other hand, the presence of unsaturated lipids drastically increased MB+ bleaching percentage exhibited after 10 min under irradiation (87%) as compared with aqueous buffered medium (10%). Fig. 2C shows the spectra of cytochrome c and MB+ obtained before and after 5- and 10-min exposure to the halogen lamp. In this condition, after 5 min of irradiation, 300 mM NaCl was added to the medium to promote cytochrome c dissociation from the liposome surface. The irradiation of the system after increase of ionic strength did not promote significant cytochrome c bleaching when compared with Fig. 2B. This result suggests that cytochrome c bleaching was promoted by free radicals generated in the membrane. The addition of 300 mM NaCl led to a progressive increase in turbidity of the sample, and thus, it was not possible to get UV-visible spectra at 50 min. Because of high turbidity of the sample containing NaCl, the spectra obtained at 50 min were omitted from Fig. 2 (B and C). Fig. 2D shows the alterations in the cytochrome c and MB+ spectra during the irradiation with visible light in the presence of puPCPECL80. For cytochrome c, the result obtained in this condition was quite similar to that obtained in the presence of tuPCPECL, an expected result due to the high content of unsaturated lipids present in puPCPECL80.

Considering that in this model of membrane the CL content consists exclusively of the saturated form of the lipid, this result suggests that the electrostatic and principally lipid-extended interaction of CL with cytochrome c did not affect the impairment of cytochrome c blue shift observed in the presence of tuPCPECL. However, in the presence of saturated CL, a decrease in the degree of MB+ bleaching (from 87% to 70%) was observed. Fig. 2, E and F, and their insets, show, respectively, the results obtained in the presence of puPCPECL20 and tsPCPECL. In the presence of puPCPECL20 and tsPCPECL, photodynamically generated O_2 (\(^{1}\Delta_g\)) led to the blue shift of cytochrome c Soret band compatible with the formation of cyt405. Compared with the results obtained in the presence of membranes with high unsaturated acyl chain content, the degree of MB+ bleaching was also decreased in the presence of puPCPECL20 and tuPCPECL.
Protection of Cytochrome c by Unsaturation Lipids

A. Lipid-oxidized products from tuPCPECL liposomes; MDA (dense pattern) and lipid hydroperoxide (gray color) were determined in 2 mM tuPCPECL liposomes after 50 min under irradiation in the following conditions: 2 mM tuPCPECL liposomes (control); 2 mM tuPCPECL liposomes plus 4 μM cytochrome c (Cyt c); 2 mM tuPCPECL liposomes plus 10 μM methylene blue (+MB); 2 mM tuPCPECL liposomes plus 4 μM cytochrome c and 10 μM methylene blue (+MB and Cyt c). The asterisk indicates significantly different (p < 0.05) from the control, and the symbol *** indicates significantly different (p < 0.05) from cytochrome c and MB alone. B, lipid-oxidized products of CL liposomes in the presence of cytochrome c; MDA (dense pattern) and lipid hydroperoxide (gray color) determined in liposomes containing 0, 25, 50, 75, and 100% of unsaturated CL. For MDA, statistical analysis considered that the means are significantly different from each other. The symbols *, **, and § indicate significantly different (p < 0.05) from 25%, 50, and 100% puCL, respectively.

C, lipid-oxidized products of CL liposomes in the presence of cytochrome c and MB; MDA (dense pattern) and lipid hydroperoxide (gray color) were determined in liposomes containing 0, 25, 50, 75, and 100% unsaturated CL. For MDA, statistical analysis considered that the means are significantly different from each other. The symbols *, **, and § indicate significantly different (p < 0.05) from 25%, 50, and 100% puCL, respectively. The results are the mean of at least three independent experiments, and the error bars correspond to the standard deviations. Statistical analysis was done by using GraphPad analysis of variance calculating software. The post-hoc tests (Bonferroni correction) were done by using QuickCalcs (GraphPad).

The various compositions of lipids could affect the size or shape of the sonicated liposomes, identical results were obtained with round 100 nm extruded PCPECL liposomes (not shown). To establish whether the lipid composition influences the protection of cytochrome c against O2 (\( \Delta_{\nu} \))-promoted oxidation, the protein was subjected to photodynamic action in the presence of 100 nm-diameter-extruded CL liposomes containing 0, 25, 50, 75, and 100% unsaturated lipid (tsCL, puCL25, puCL50, puCL75, and tuCL, respectively).

Effect of O2 (\( \Delta_{\nu} \)) on Cytochrome c-Containing PCPECL and CL Liposomes—From the above results we concluded that the presence of unsaturated lipids prevented the formation of O2 (\( \Delta_{\nu} \))-promoted cytochrome c405 as well as favored the formation of leuco-MB+. Thus, it was important to determine the oxidative lipid damage that accompanied the photochemically promoted changes in the dye and in the protein (Fig. 4, A–C).
affecting MDA content present in the sample (see control MDA content). In fact, it is well known that the oxidative attack of O$_2$ (1$\Delta_g$) on lipid acyl chain produces lipid peroxides (33) (Scheme 1). The lipid hydroperoxide content was significantly lowered in the presence of cytochrome $c$ that competed with MB$^+$ for the lipid damage. In fact, the MDA content determined in tuPCPECL liposomes containing both cytochrome $c$ and MB$^+$ was almost identical to that obtained when MB$^+$ was absent. Interesting findings were those from the results obtained with CL liposomes (Fig. 4, B and C). Similarly to that observed for tuPCPECL, CL liposomes containing unsaturated lipids did not exhibit an increase of MDA content after irradiation in the presence of MB$^+$ (not shown). However, in this condition, the LOOH present in control sample decreased to zero (not shown). This result is in agreement with the ability of triplet MB$^+$ to react with LOOH (34) and suggests that contrary to PCPECL, MB$^+$ should be bound to the negatively charged CL liposomes. In the absence (Fig. 4B, dense pattern bars) and in the presence of MB$^+$ (Fig. 4C, dense pattern bars), cytochrome c-promoted increase of MDA content was proportional to the unsaturated CL content and exhibited a hyperbolic profile. Otherwise, the LOOH content of CL liposomes assayed with cytochrome c (Fig. 4B, gray bars) exhibited an exponential growth. As expected, although exhibiting similar growing profile, the amount of LOOH was significantly lower when MB$^+$ was also present, because the triplet dye can react with LOOH (34) (Fig. 4C, gray bars). In the presence of cytochrome c, the exponential growth of LOOH content proportional to the unsaturated CL content suggested that secondary oxidative routes contributed to the formation of the lipid-derivative peroxide. Previously, it was demonstrated that the reaction of cytochrome c with aldehydes produces triplet carbonyls prone to generate O$_2$ (1$\Delta_g$) via energy transfer (35–38). In fact the LOOH content in CL liposomes containing unsaturated lipids increased 10-fold in phosphate-buffered D$_2$O (not shown). Despite the generation of O$_2$ (1$\Delta_g$), no significant cytochrome c damage was detected in the conditions of Fig. 4B (not shown). Taken together, the result described above and the results presented in Figs. 2B, 2D, 3A, 4A, and 4B suggest that, whatever the O$_2$ (1$\Delta_g$)
source, unsaturated lipids are actually efficient to protect cytochrome c against the oxidative damages promoted by this excited species.

**Pro-apoptotic Activity of cytC405 and Lipid-protected Cytochrome c**—Previously, the characterization of cytC405 revealed that this oxidized form of cytochrome c exhibited significant structural and functional differences as compared with the native form (24). However, the capacity of cytC405 to trigger apoptosis remains to be investigated. Altogether, considering that the mitochondrial model membranes were able to protect cytochrome c against photochemically generated O$_2^-$ ($^1\Delta_g$), it was important to investigate whether the lipid-protected cytochrome c retained its capacity to trigger apoptosis.

The physiologically relevant dose of cytochrome c for cellular microinjection in SMCs was determined as described by Li et al. (32). In SMCs the onset of apoptosis with half-maximal effects was observed 2 h after the microinjection of 0.4 mg/ml native cytochrome c. Having determined the appropriate cytochrome c dose to trigger apoptosis in SMCs, the effects of the microinjection of cytC405 and lipid-protected cytochrome c (Fig. 5, A and B) were analyzed. Fig. 5A shows that, 2 h after microinjection of cytC405, most cells remained with a round well-defined nucleus border and absence of chromatin condensation. Similar results were obtained with the microinjection of the buffered medium (not shown). Fig. 5B shows that, at the same time interval after lipid-protected cytochrome c microinjection, most cells exhibited condensed and fragmented nuclei typical of apoptosis. This result was similar to that observed after microinjection of native cytochrome c (not shown). The corresponding insets of Fig. 5 (A and B) are zoom images of normal and apoptotic cell nuclei, respectively.

Following microinjection, cells were counted, and the results were presented as percentage of apoptotic cells (Fig. 6). According to Fig. 6, the microinjection of cytC405 did not induce a significant increase in the percentage of apoptosis of SMCs as compared with the control (microinjection of buffered saline) and the microinjection of MB$^+$. However, the microinjection of lipid-protected cytochrome c induced apoptosis of ~85% of SMCs, a percentage very close to that induced by native cytochrome c (~97%).

These results indicate that in homogeneous medium photo-generated O$_2^-$ ($^1\Delta_g$) induced oxidative damage in the cytochrome c structure and promoted the loss of its capacity to activate caspases. The presence of unsaturated lipids protected the cytochrome c structure and the ability to activate caspases, because they were the principal target for O$_2^-$ ($^1\Delta_g$). To confirm that caspase activation was involved in the apoptosis observed in our experimental conditions, we performed caspase-3 fluorometric assays by using native cytochrome c, cytC405, and lipid-protected cytochrome c. Caspase-3, also known as CPP-32, Yama, or Apopain (39), is an intracellular cysteine protease that exists as a proenzyme, becoming activated during caspase events associated with apoptosis. The SMC cell lysate was incu-
degree, e.g. highly alkaline pH. In conditions in which exclusively $O_2$ ($1^{3} \Delta_g$) was generated, only the amino acid residues were the target for the excited species at the expense of alterations in cytochrome $c$ structure and spin state produced by different factors such as the presence of SDS micelles. However, the previously studied model system, *i.e.* cytochrome $c$ in phosphate buffer and in phosphate-buffered SDS micelles, had the objective to identify the types of modifications that $O_2$ ($1^{3} \Delta_g$) could produce in the cytochrome $c$ structure, but it did not reproduce the biological conditions in which the photodynamically generated reactive species could act on cytochrome $c$ during PDT. In mitochondria, cytochrome $c$ is associated with the inner membrane, which contains high amounts of unsaturated phospholipids. Thus, the polyunsaturated lipids present in the inner mitochondrial membrane could protect cytochrome $c$ against the attack of $O_2$ ($1^{3} \Delta_g$). Actually, as demonstrated by Fig. 2 (A–F), the cytochrome $c$ Soret band blue shift, an indication of $O_2$ ($1^{3} \Delta_g$) attack on the Met-80 residue, was observed only in the presence of *ts*PCPECL and *pu*PCPECL20. In the presence of *ts*PCPECL and *pu*PCPECL80, cytochrome $c$ remained in the low spin state during the irradiation. The experiments carried out with *pu*CL25 (Fig. 3A), *pu*CL50 (not shown), *pu*CL75 (not shown), and *ts*CL (Fig. 3B) liposomes showed that, whatever the lipid composition, the presence of unsaturated lipids was crucial for cytochrome $c$ protection against $O_2$ ($1^{3} \Delta_g$)-promoted oxidation. However, the percentage of unsaturated lipids necessary to protect significantly cytochrome $c$ varied according to the composition of the membrane. Thus, in PCPECL liposomes 20% of unsaturated lipids were not enough to protect cytochrome $c$, but CL liposomes containing 25% unsaturated CL were efficient to completely protect cytochrome $c$. However, PCPECL and CL liposomes containing exclusively saturated lipids were both inefficient to protect cytochrome $c$. The partial bleaching of cytochrome $c$ observed in the presence of lipids could be attributed to the attack of lipid- or dye-derived free radicals on the porphyrin ring (24, 28, 40).

In the experimental conditions used in the present study in which oxidized lipid derivatives were detected (presence of cytochrome $c$, and presence of photoexcited MB$^{+}$ and both protein and photoexcited dye) the occurrence of two oxidative routes for lipids can be presumed. Polyunsaturated fatty acids react with $O_2$ ($1^{3} \Delta_g$) to give a mixture of conjugated and non-conjugated isomers of the produced hydroperoxides, whereas a radical attack leads to the production of the conjugated isomers only (25). The reaction mechanism of $O_2$ ($1^{3} \Delta_g$) with polyunsaturated lipids differs from that exhibited by free radicals (Scheme 1, paths 1 and 2, respectively) (42). In a peroxide-free lipid system (Scheme 1, path 2), highly reactive free radicals can abstract a hydrogen atom from a methylene group whose C–H bond is weakened by the presence of an adjacent double bond. The carbon radical tends to be stabilized by a molecular rearrangement to form a conjugated diene. Under aerobic conditions conjugated dienes can react with $O_2$ to give a peroxy radical, LOO$^{\cdot}$. The formation of peroxy radical leads to the propagation of the lipid peroxidation. The termination of the process is achieved by the following processes: (i) formation of lipid peroxide LOOH by a reducing agent; (ii) reaction of L with LOO$^{\cdot}$ to form the stable LOOL dimers; and (iii) combination of

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**FIGURE 7. Caspase activation by different cytochrome $c$ types.** SMC pellet was lysed by the addition of 25 μl of cold Cell Lysis Buffer (R&D Systems) per 1 × 10$^6$ cells. For the reaction, 100 μl of cell lysate was mixed with an equal volume of 2× reaction buffer and 3 ml of fresh diethiothreitol stock solution. To each reaction well 25 μM native cytochrome $c$, lipid-protected cytochrome $c$, or cyt$c$405 were added prior to the addition of 10 ml of Caspase-3 fluorogenic substrate (DEVD-7-amino-4-trifluoromethylcoumarin). The plate was incubated for 2 h, and the fluorescence was read (excitation at 400 nm and reading at 505 nm). The results correspond to the mean of at least two independent experiments, and the error bars correspond to the standard deviation. The asterisk indicates significantly different ($p < 0.05$) from the control, and the symbol $^\ddagger$ indicates significantly different ($p < 0.05$) from cyt$c$405, according to statistical analysis performed by using GraphPad software (an analysis of variance calculating program) and post-hoc tests (Bonferroni correction) that were done by using QuickCalc (GraphPad).
two peroxide molecules to form hydroxylated derivatives (LOH). Some bonds between lipid peroxides and membrane proteins are also possible. However, O2 (Δg) can react and be added to either end carbon of a double bond (Scheme 1, path I), which assumes the trans configuration. As described for alkenes and polyenes, the formed hydroperoxide can react with the triplet MB+ and lead to the bleaching of the dye (34). In fact, the percentage of MB+ bleaching observed during irradiation was increased by changing phosphate buffer by phosphate-buffered ttpCPECL and ptpCPECL80 liposomes (Fig. 2, B–D) and even more exacerbated in the presence of unsaturated CL liposomes (Fig. 3A and not shown results) whose negatively charged surface should exhibit high affinity for the dye.

The reaction of cytochrome c with lipid-oxidized derivatives such as aldehydes and peroxides could lead to the formation of free radical derivatives that initiate a cascade of oxidative processes that terminate with the production of MDA (path 2) (43, 44). In fact, the presence of cytochrome c increased the MDA content both in the presence and in the absence of MB+ (Fig. 4, A–C). Among the lipid-oxidized derivatives able to react with cytochrome c, LOOH seemed to be one of the principal candidates. However, the exponential growth of LOOH observed in CL liposomes assayed with cytochrome c in the absence of MB+ suggests that lipid-derived carbonyl compounds should be the best substrates for cytochrome c in this condition. As mentioned before, the triplet products that are expected from this reaction could generate O2 (Δg) that contributed to the increase of LOOH content (Scheme 1, path I). Thus, the lowering of LOOH content observed in MB+-containing liposomes (Fig. 4, A and C) when cytochrome c was present could be better explained by the competition for the damage between the protein and the dye than by LOOH consumption by cytochrome c. On the other hand, CL-derived aldehydes and peroxides should be the preferential targets for cytochrome c attack (28), because the LOOH content was lower than that of MDA in liposomes with low amounts of unsaturated CL (Fig. 4, A–C). In this case, the LOOH detected should be formed via the reduction of LOO' by a reducing agent. The assignment of CL as the principal target for cytochrome c attack was reinforced by the significant decrease of Soret band bleaching (from 36% to 12% at 10 min) when ttpCPECL (Fig. 2B) was replaced by ptpCPECL80 (Fig. 2D) that, despite the presence of unsaturated PC and PE, contained non-reactive saturated CL. In comparison with Fig. 4A, the drastic decrease of the production of lipid-oxidized derivatives in ptpCPECL80 assayed with cytochrome c (not shown) is also in agreement with the specificity of CL derivatives as cytochrome c substrates.

In liposomes containing high amounts of unsaturated CL, in the presence of cytochrome c, O2 (Δg) produced by energy transfer from triplet carbonyls could also contribute to LOOH production (Scheme 1). In the presence of photoseceted MB+, the content of LOOH produced by oxidant species is expected to always be underestimated. Because it was preferentially partitioned in the lipid bilayer (45), MB+, in the triplet state, could access and react with hydroperoxides (34). In fact, MB+ bleaching degree was particularly higher in the presence of ttpCPECL and ptpCPECL80. This proposal was corroborated by the dramatic increase in MB+ bleaching in the presence of the negatively charged CL liposomes that exhibit high affinity for the positively charged MB+. The above described results suggest that the typical mechanism of O2 (Δg) reaction with polydienes occurred in the model systems used in this study (34). In this condition, besides the presence of lipid traps for O2 (Δg), the relatively rapid dye degradation contributes to the protection of cytochrome c. The dye degradation also terminates the process of lipid peroxidation.

Therefore, in the presence of membranes with high content of unsaturated lipids, chemically and photodynamically generated O2 (Δg) was significantly trapped by the lipid content that prevented the attack on cytochrome c Met-80 and the consequent Soret band blue shift. This proposal was corroborated by the cytochrome c Soret band blue shift observed when O2 (Δg) was generated in the presence of ttpCPECL, ptpCPECL20, and ttpCL liposomes. However, a part of the cytochrome c population had the heme group chemically attacked by the lipid- and/or dye-derived free radicals leading to the observed partial Soret band bleaching.

As mentioned before, the formation of MDA in the presence of cytochrome c was expected to come preferentially from the reaction of heme iron with CL derivatives. The inner mitochondrial membrane exhibits unsaturated CL, but ~45% of the CL content is present at the inner leaflet (26, 47, 48). Thus, in biological conditions, the structural organization of the inner mitochondrial membrane could protect cytochrome c against O2 (Δg) attack. On the other hand, by impairing the contact of cytochrome c with around 40% of the CL content, the membrane organization disfavors the production of free radicals and excited species produced by the attack of cytochrome c on the CL content. Furthermore, in a similar way as shown in Fig. 2C, the detachment of cytochrome c from the inner mitochondrial membrane following the opening of the permeability transition pore could also prevent extensive oxidative damage of the protein.

Based on the data presented in this study it is possible to postulate a sequence of events in mitochondria that culminate in apoptosis. As previously described (46), inside the cell, MB+ exhibits high affinity for the mitochondrial inner membranes that become loaded with the dye. The irradiation of the medium leads to the oxidative damage of the mitochondrial lipid content and the opening of the mitochondrial permeability transition pore (41). Mitochondrial permeability transition pore opening leads to loss of the mitochondrial transmembrane potential and the consequent increase of pH of the intermembrane space. As previously characterized (27) and confirmed by experiments with mitoplasts,6 the pH increase is expected to reduce the affinity of cytochrome c for the inner mitochondrial membrane. Thus, these events culminate in the detachment of cytochrome c from the inner mitochondrial membrane to participate, in the cytosol, of the upstream or downstream processes of caspase activation (26).

6 T. Rodrigues, L. P. de França, C. Kawai, P. A. de Faria, K. C. U. Mugnol, F. M. Braga, I. L. S. Tersariol, S. S. Smailli, and I. L. Nantes, unpublished results.
CONCLUSION

The results presented here allow the following conclusions: (i) Photodynamically generated O₂ (Δε₁) can attack cytochrome c Met-80 both in buffered water and in the presence of PCPECL and CL liposomes. (ii) PCPECL liposomes with high content of unsaturated lipids act as O₂ (Δε₁)-trapping and prevent the conversion of the heme group to the high spin form (cyt c405). (iii) The percentage of unsaturated lipid necessary to protect cytochrome c depends on the composition of the membrane. (iv) In the above condition, if cytochrome c remains attached to the oxidized membrane, the heme iron can react with CL-derived products generating free radicals that attack the heme group and promote Soret band bleaching. The free radical-promoted cytochrome c bleaching can also be prevented by unsaturated lipids. (v) When extensive lipid oxidation occurs, the attack of cytochrome c on lipid-derived aldehydes produces triplet carbonyls that generate O₂ (Δε₁) via energy transfer. These events lead to an exponential growth of the lipid peroxide content as well as the saturation of MDA content. (vi) The inner mitochondrial membrane organization and composition besides the expected detachment of cytochrome c from the membrane as a consequence of the mitochondrial permeability transition pore opening could prevent the oxidative damage of the protein and preserve its capacity to trigger apoptosis.

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