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Degradation of phospholipids by oxidative stress—Exceptional significance of cardiolipin

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Abstract

The aim of this study was to investigate the effect of oxidative stress on mitochondrial phospholipids. In this context, this study investigated (i) the content of phosphatidylethanolamine (PE), phosphatidylcholine (PC) and cardiolipin (CL), (ii) the correlation of CL degradation with mitochondrial function and (iii) the correlation of CL degradation and CL oxidation. Oxidative stress induced by iron/ascorbate caused a dramatic decrease of these phospholipids, in which CL was the most sensitive phospholipid. Even moderate oxidative stress by hypoxia/reoxygenation caused a decrease in CL that was parallelled by a decrease in active respiration of isolated rat heart mitochondria. The relation between oxidative stress, CL degradation and CL oxidation was studied by *in vitro* treatment of commercially available CL with superoxide anion radicals and H_2O_2. The degradation of CL was mediated by H_2O_2 and required the presence of cytochrome *c*. Other peroxidases such as horse radish peroxidase and glutathione peroxidase had no effect. Cytochrome *c* in the presence of H_2O_2 caused CL oxidation. The data demonstrate that oxidative stress may cause degradation of phospholipids by oxidation, in particular CL; resulting in mitochondrial dysfunction.

Keywords: *Phospholipids, cardiolipin, oxidative stress, mitochondria, cytochrome c.*

Introduction

Imbalance between generation of reactive oxygen species (ROS) and their elimination that results in high ROS concentrations is the reason for oxidative modification of proteins, DNA molecules and lipids [1]. This oxidative stress may lead to structural and functional impairment of cells and even to necrotic or apoptotic cell death [2]. In particular, phospholipids are considered to be vulnerable to oxidative stress due to their unsaturated fatty acid residues [3,4]. PC, PE and CL are the most abundant lipid components of mitochondrial membranes [5]. As constituents of the mitochondrial membrane system, PC and PE are important for the physiologically intact membrane structure and additionally they support mitochondrial function. CL is a mitochondrial phospholipid mainly located in the mitochondrial inner membrane that is required for the function of several mitochondrial enzymes such as respiratory chain complexes and adenine nucleotide translocase [6–10]. It had been demonstrated that abnormalities in CL content and composition are associated with mitochondrial dysfunction [11,12].

This study aims to elucidate the effect of oxidative stress on the phospholipids PE, PC and CL in isolated mitochondria. Strong oxidative stress was induced by iron/ascorbate. Mild oxidative stress was initiated by hypoxia/reoxygenation. Under these conditions the phospholipid content was determined. Additionally, the relation between phospholipid degradation and mitochondrial function was investigated in isolated heart mitochondria exposed to hypoxia/reoxygenation. To study CL oxidation, commercial CL was exposed to H_2O_2 or to superoxide anion radicals *in vitro*.

The content of PE, PC and CL was determined by using HPLC-analysis. In the *in vitro* experiments

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with commercially available CL, the content of main CL molecular species and the amount of oxidized CL were determined by LC-mass spectroscopy.

Materials and methods

Isolation of mitochondria

Rat brain mitochondria were prepared from the brains of 220–240 g male Wistar rats in ice-cold medium containing 250 mM mannitol, 20 mM Tris, 1 mM EGTA, 1 mM EDTA and 0.3% (w/v) bovine serum albumin (BSA) at pH 7.4 (isolation medium) according to Lay and Clark [13]. The mitochondria were well-coupled, as indicated by a respiratory control index greater than 4 with glutamate plus malate as substrates. Protein content was measured according to the method of Lowry et al. [14].

Rat heart mitochondria were prepared in a medium containing 150 mM KCl and 10 mM EDTA at pH 7.4, in detail described by Schaller et al. [15]. Protein content was measured according to the method of Bradford [16] by using BSA as the standard.

Incubation of mitochondria

Iron/ascorbate-induced peroxidation. Rat brain mitochondria (1 mg/ml) were incubated up to 75 min in air atmosphere in a medium containing 0.1 M KCl, 0.01 M TRIS, 50 μM iron II-sulphate and 500 μM ascorbate (pH 7.4) at 25°C. Aliquots were withdrawn after distinct time intervals for the analysis of thiobarbituric acid-reactive substances (TBARS) and phospholipids. The content of TBARS was determined by HPLC/UV detection in principle according to Jentzsch et al. [17]. The rest of the sample was stored at –80°C until phospholipid analysis by HPLC.

Hypoxia/reoxygenation. Rat heart mitochondria (2.6 mg protein/ml) were incubated in a medium containing 10 mM KH_2PO_4, 0.5 mM EGTA, 60 mM KCl, 60 mM TRIS, 110 mM mannitol, and 1 mM free Mg^{2+} (pH 7.4) at 30°C.

Extramitochondrial Ca^{2+} concentration was adjusted by using Ca^{2+}/EGTA buffers. Hypoxia was achieved by bubbling 2 ml of the incubation medium with N_2 until oxygen was not detectable by means of a Clark-electrode. A volume of 2 ml of air-saturated incubation medium was added for reoxygenation.

Measurement of respiration

Oxygen uptake of mitochondria was measured at 30°C in a thermostat-controlled chamber equipped with a Clark-type electrode. For the calibration of the oxygen electrode, the oxygen content of the air-saturated incubation medium was taken to be 217 nmol O_2/ml [18].

Phospholipid extraction and HPLC analysis

Phospholipids were extracted from the incubations with iron/ascorbate-induced lipid peroxidation in rat brain mitochondria by a modified method of Folch et al. [19]. To a sample of 1 ml volume, 14 ml chloroform and 7 ml methanol (v/v) containing 0.05% butylated hydroxytoluene (BHT) were added and intensively mixed. For phase separation, 4.4 ml 0.01 M HCl were added, followed by intensive shaking. Phase separation was completed by centrifugation at 500 x g for 5 min. The upper aqueous phase and the protein layer below were carefully removed. The phospholipid containing lower phase was collected, dried under argon atmosphere and dissolved in chloroform/methanol 2:1 (v/v). HPLC analysis of phospolipids was performed as described by Shafiq-ur-Rehman [20]. Samples of 90 μl volume were injected in an HPLC system (Fa. Merck, Darmstadt, Germany) consisting of an autosampler AS-2000A, a column LiChrospher Si60 (250 × 4 mm, particle diameter 5 μm), a column oven L-5025 (30°C), an HPLC pump L 6200 and a detector L-4250 UV-VIS. An isocratic elution (acetonitrile/methanol/85% phosphoric acid: 90:36:0.36 (v/v/v)) and a flow rate of 1.5 ml/min were used for separation. Absorption was analysed at 204 nm.

Quantification of cardiolipin species by HPLC with fluorescence detection

CL species were analysed as described by Schlame et al. [21]. Tetra-stearoyl-CL, the internal standard, was generated by complete hydrogenation of commercial heart bovine CL (Sigma-Aldrich, St. Louis, MO). In a first step, lipids were extracted from cells according to Bligh and Dyer [22]. Afterwards, the lipid samples were treated with diazomethane for derivatization and then labelled with 1-naphtylacetic anhydride (dissolved in anhydrous pyridine) for HPLC fluorescence detection. After solid phase extraction with Supelclean columns (Supelco LC/Si SPE; Bellefonte, PA, USA), CL-containing samples were dried, re-dissolved in n-hexane-ethanol 1:1 (v/v) and separated by HPLC using a LiChroCart Supersphere column (250 × 4 mm, 4 μm; Darmstadt, Germany). A solvent gradient was used starting from acetonitrile-2-propanol 8:2 (v/v) to acetonitrile-2-propanol 5:5 (v/v) to run separation within 50 min. The HPLC device was a Waters system (Waters Chromatography Division, Milford, MA) consisting of a 616 pump (total flow rate 1.0 ml/min), a 600 S controller, a 717plus Autosampler and a 2475 Multi λ fluorescence detector (excitation wavelength 280 nm,

emission wavelength 360 nm). Data were collected and processed by the Waters Millennium 32 Software. Three cardiolipin peaks representing three different molecular species $(C18:2)_4$-CL, $(C18:2)_3$C18:1-CL and $(C18:2)_2(C18:1)_2$-CL were analysed. The sum of the areas of the three CL peaks was used for CL quantification. The fourth peak under consideration was created by the internal standard, a hydrogenated CL that does not occur in nature. The CL concentrations were calculated in reference to the internal standard. The recovery of CL after sample preparation was 78% (n = 6), as estimated from the phosphate content of the samples [23].

Incubations of commercially available CL

Cardiolipin (5 µg/ml, Sigma-Aldrich, St. Louis, MO) was incubated either in 1 ml PBS or in 1 ml xanthine-buffer in the presence of various additional constituents for a time interval of 15 min at 30°C. Afterwards, samples were shock-frozen in liquid nitrogen and stored at –80°C until analysis. The xanthine-buffer contained 50 mM KH_2PO_4 (pH 7.8), 0.1 mM EDTA and 50 µM xanthine. The additions were: 500 µM H_2O_2, 500 µM H_2O_2 plus 100 mU horse radish peroxidase (Sigma/Aldrich, Taufkirchen, Germany), 500 µM H_2O_2 plus 100 mU glutathione peroxidase (Sigma/Aldrich, Taufkirchen, Germany) and 500 µM H_2O_2 plus 5 mM cytochrome *c* (Boehringer, Ingelheim, Germany). In another set of incubations, 10 mU xanthine oxidase (Calbiochem, Schwalbach, Germany) was added to the xanthine-buffer.

Generation of CL-liposomes

A stock suspension of CL liposomes was prepared by sonification of 50 µg dried CL in 9250 µl PBS. Sonification was carried out in three time intervals of 10 s with 10% of maximal energy (Sonoplus MS72, Badelin, Berlin, Germany). In the incubations 5 µg CL/ml was used.

Quantification of cardiolipin species and oxidized cardiolipin by ESI-MS/MS

Extraction

CL was extracted from the samples by a modified Folch extraction procedure [19]. Briefly, 50 ng of tetra-myristoyl-CL [(C14:0)4-CL; Avanti Polar Lipids, Inc., alabaster, AL] were added as the internal standard to 10 µl incubation mixture containing bovine heart CL in $CHCl_3/CH_3OH$ (2:1, v/v), 0.05% BHT as antioxidant and further additions outlined in the legends of the figures and tables. Aqueous and lipid phases were separated by adding 0.01 M HCL followed by intensive shaking and subsequent centrifugation. The lower lipid phase was collected, dried under nitrogen atmosphere and acidified as described by Schlame et al. [21]. Ice-cold methanol (1 ml), chloroform (0.5 ml) and 0.1 M HCl (0.5 ml) were added to the sample. The solution was intensively mixed and incubated for 5 min on ice. Phase separation was achieved by addition of 0.5 ml of $CHCl_3$ and 0.5 ml of 0.1 M HCl. The chloroform phase was recovered as CL-containing sample. Finally, the sample was dried under nitrogen, dissolved in 1.5 ml $CHCl_3/CH_3OH/H_2O$ (50/45/5, v/v/v), mixed and filtered over 0.2 µm PTFE membranes. This sample was ready for immediate ESI-MS/MS analysis according to Valianpour et al. [24].

HPLC-MS/MS. A TSQ Quantum Discovery Max (Thermo Fisher Scientific GmbH, Dreieich, Germany) was used in the negative electrospray ionization (ESI) mode. The HPLC system consisted of a Surveyor MS quaternary narrowbore pump with integrated vacuum degasser and a Surveyor autosampler. The autosampler tray temperature was maintained at 8°C; 10 µl of the lipid extract dissolved in chloroform/methanol/water (50/45/5) was injected (in partial loop mode) and CL was separated by using a LiChroCart column (125 × 2 mm), LiChrospher Si60 (5 µm particle diameter; Merck, Darmstadt, Germany) and a linear gradient between solution A (chloroform) and solution B (methanol/water, 9:1 (v/v)). Both solutions additionally contained 0.1 ml/l of 25% aqueous ammonia. The gradient was as follows: 0–0.2 min, 92% A, 8% B, 0.2–4.5 min, 92% A to 30% A and 8% B to 70% B, 4.5–6 min, 30% A, 70% B, 6–6.5 min 30% A to 92% A, 70% B to 8% B, 6.5–11 min, 92% A, 8% B. The flow rate was 300 µl/min.

The total time of analysis was 11 min. The eluate between 0.3–6 min was introduced into the mass spectrometer. Nitrogen was used as the nebulizing gas and argon was used as the collision gas at a pressure of 1.5 mTorr. The spray voltage was 3.5 kV, the ion source capillary temperature was set at 375°C and the cone-voltage was 30 V. Daughter fragments from the doubly charged parents derived from $(C18:2)_4$-CL with *m/z* (mass to charge ratio) 723.6 $((M–2H)^{2-}/2)$, $(C18:2)_3$C18:1-CL with *m/z* 724.6 and $(C18:2)_2(C18:1)_2$-CL with *m/z* 725.6 were obtained using a collision energy of 36 eV. These three molecular CL species and the internal standard (*m/z* 619.6) were analysed by mass transfer reaction monitoring their doubly charged ions and their respective fatty acids linoleic acid *m/z* 279.2 and myristic acid *m/z* 227.2 using the selected reaction monitoring (SRM) mode. In Figure 1A a parent scan is shown of *m/z* 279.2, representing linoleate derived from CL, as proofed by the corresponding daughter scan of *m/z* 723.5. Figure 1B shows the daughter scan

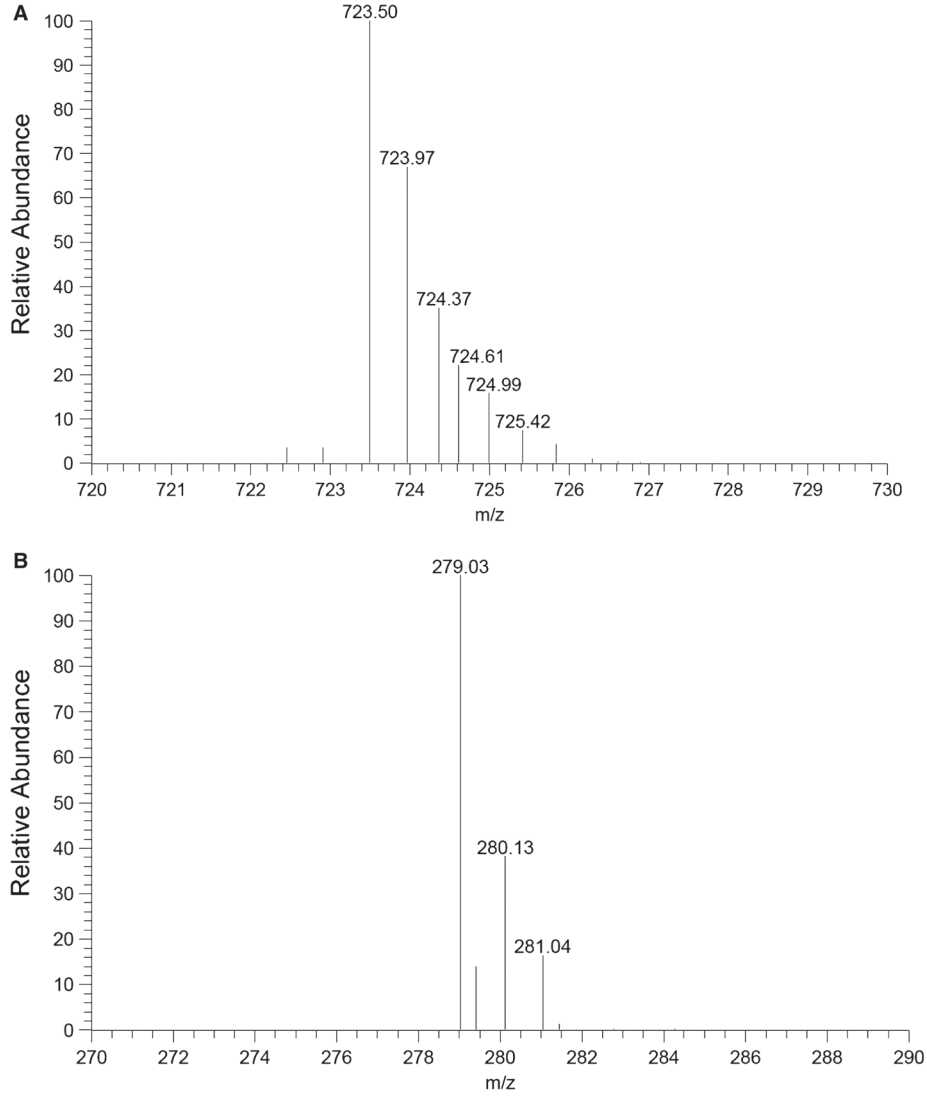

Figure 1. Mass spectrum of commercially available CL. The mass spectrum was obtained following loop injection of CL. Different molecular species are shown. (A) parent scan for *m/z* 279.2. The peak at *m/z* 723.5 belongs to $(C18:2)_4$-CL. The peak at *m/z* 724.61 corresponds to $(C18:2)_3C18:1$-CL and to the second isotope peak of $(C18:2)_4$-CL with *m/z* 723.5. The peak at *m/z* 725.42 corresponds to $(C18:2)_2(C18:1)_2$-CL and to the second isotope peak of $(C18:2)_3C18:1$-CL with *m/z* 724.61. The peak at *m/z* 723.97 belongs to the first isotope of $(C18:2)_4$-CL and the peak at *m/z* 724.99 represents the first isotope of $(C18:2)_3C18:1$-CL. (B) The daughter scan of *m/z* 724.61 $(C18:2)_3C18:1$-CL is shown. The peak at *m/z* 279.03 belongs to C18:2 (linoleate) and the peak at *m/z* 281.04 to C18:1 (oleate).

of *m/z* 724.6 corresponding to $(C18:2)_3C18:1$-CL of the commercially available CL.

[$(C18:2)_3$-monohydroxylinoleic acid-CL] as oxidized CL species was measured in the SRM mode as a transition from *m/z* 731.6 to *m/z* 279.2 (linoleic acid) according to Pope et al. [25]. In Figure 2 the chromatograms of non-oxidized (A) and oxidized CL (B) containing the mass areas (MA) for the comparison of content are shown.

Statistics

Data are presented as mean ± SEM. Details of the statistical analysis are outlined in the legends of the table and the figures.

Results

Effect of iron/ascorbate-induced lipid peroxidation on the content of phospholipids in isolated mitochondria

To study the effect of oxidative stress within a biological system on the content of native phospholipids, we subjected isolated rat brain mitochondria to iron and ascorbate. In the presence of oxygen, iron and ascorbate, mitochondria generate reactive oxygen species, as iron-oxygen intermediates or the reactive hydroxyl radical which oxidize mitochondrial lipids. Under this condition a lag phase is followed by a massive increase in products of lipid peroxidation (usually measured as thiobarbituric acid reactive substances). Finally, plateau levels of thiobarbituric acid reactive

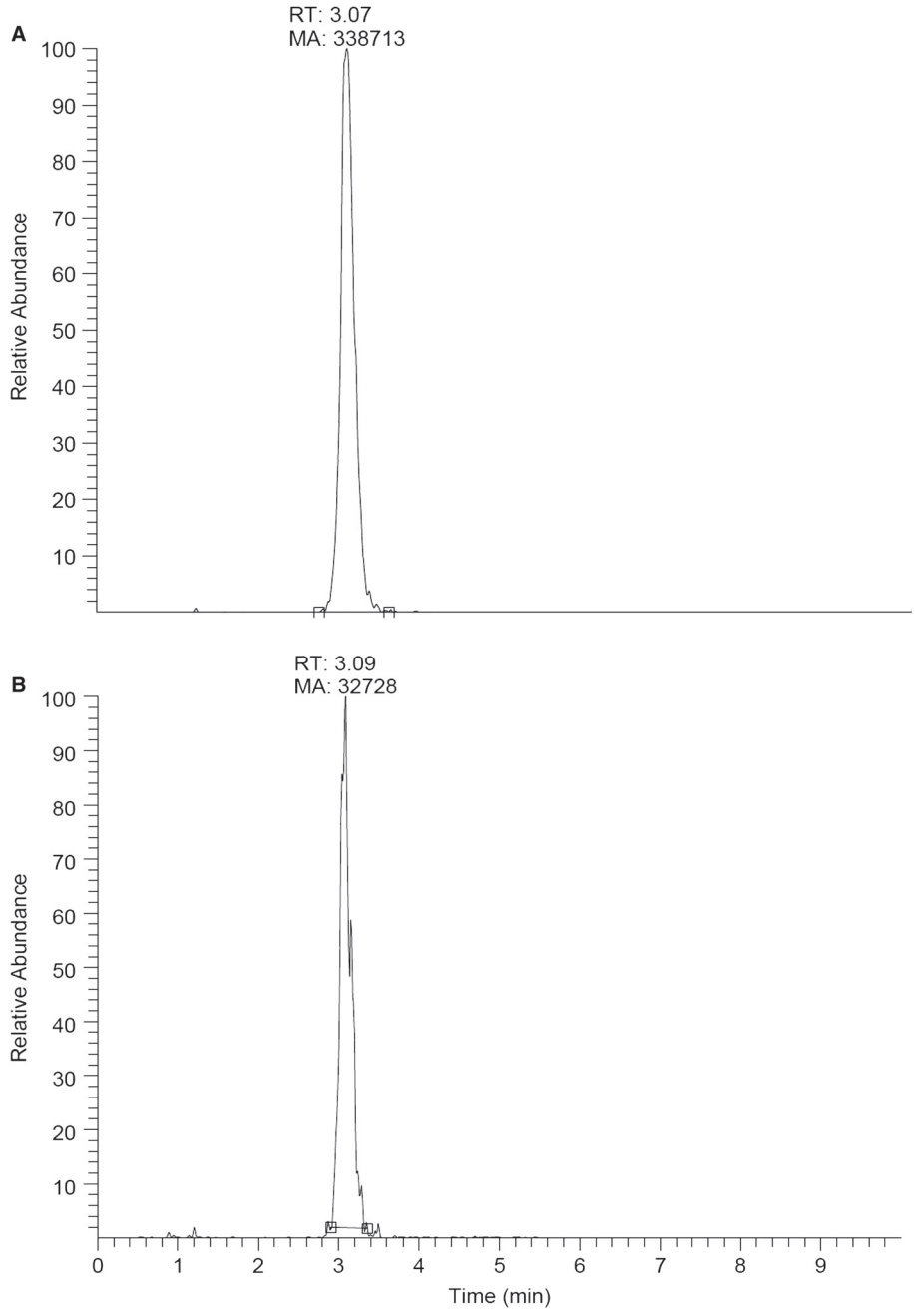

Figure 2. ESI-MS/MS chromatogram of non-oxidized and oxidized CL following HPLC elution. CL (5 μg/ml) was incubated for 15 min at 30°C with no additions (non-oxidized control) or with 500 μM H_2O_2 plus 5 mM cytochrome *c*. $(C18:2)_4$-CL was measured in the SRM mode as the transition from *m/z* 723.5 to *m/z* 279.2 (linoleic acid) (A). $(C18:2)_3$-monohydroxylinoleic acid-CL as oxidized CL species was measured in the SRM mode as the transition from *m/z* 731.6 to *m/z* 279.2 (linoleic acid) (B). For the comparison of the content the peak area (MA) is given.

substances are reached [26]. Here, we analysed the content of CL, PE and PC in isolated mitochondria incubated in the presence of iron and ascorbate in dependence on the incubation time. Data of phospholipid concentrations represented in percentage of initial values from four mitochondrial preparations are depicted as mean ± SEM in Figure 3A. The initial concentrations in nmol/mg mitochondrial protein were 4.51 ± 0.75, 42.37 ± 1.83 and 39.59 ± 4.40 (n = 4) for CL, PE and PC, respectively. It can be seen that CL was faster degraded than PE and PC. The individual values of phospolipid concentrations at distinct incubation times considerably varied between the preparations. However, each experiment revealed that CL was degraded much faster than PE and PC. Therefore, we determined the time at which half of the respective phospholipid had disappeared as a parameter of degradation kinetics. Corresponding data of the four experiments are summarized in Figure 3B. The graph shows that CL was significantly

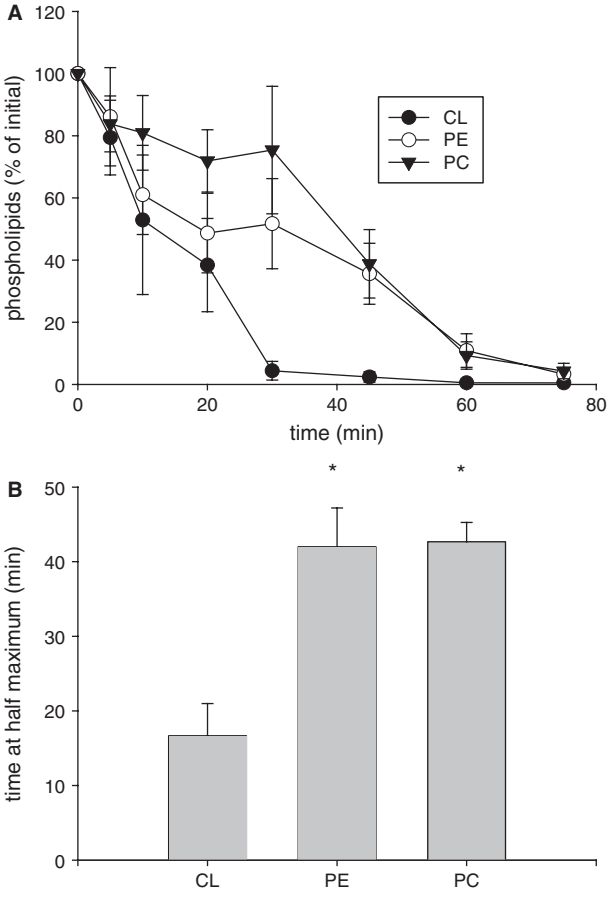

Figure 3. Effect of oxidative stress on the content of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin (CL) in isolated rat brain mitochondria. Brain mitochondria (1 mg mitochondrial protein/ml) were incubated in the corresponding incubation medium in the additional presence of 50 μM iron II-sulphate and 500 μM ascorbate at 25°C. At the indicated times samples were withdrawn for phospholipid analysis. The initial values were in nmol/mg mitochondrial protein 4.51 ± 0.75, 42.37 ± 1.83 and 39.59 ± 4.40 (n = 4) for CL, PE and PC, respectively. (A): time dependency of the phospholipid contents of four mitochondrial preparations. Data are presented as mean ± SEM. (B): time at half maximal phospholipid content of PC, PE and CL. Data are presented as mean ± SEM (n = 4). *Difference to CL was significant with $p < 0.025$ in matched-pair t-tests (Bonferoni correction for two group comparison).

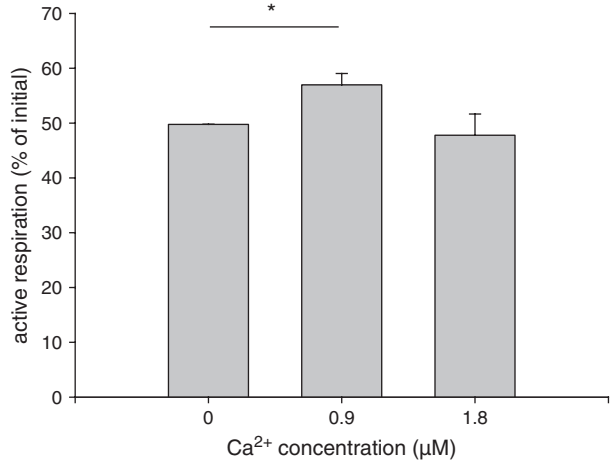

Figure 4. Effect of hypoxia/reoxygenation and Ca^{2+} on active respiration of isolated heart mitochondria. Functionally intact rat heart mitochondria were incubated in the absence or presence of exogenous low micromolecular Ca^{2+} concentrations and exposed to 10 min hypoxia plus 5 min reoxygenation. Oxygen consumption was determined after addition of 5 mM glutamate plus 5 mM malate as substrates and 200 μM ADP to stimulate active respiration. Data are given as percentage of initial (36.9 ± 1.3 nmol O_2/min/mg mitochondrial protein). They are presented as mean ± SEM (n = 7). *Difference was significant with $p < 0.025$ in matched-pair t-tests (Bonferoni correction for two group comparison).

faster degraded compared to PE and PC, which behaved similarly.

Effect of hypoxia/reoxygenation and elevated extramitochodrial Ca^{2+} concentration on mitochondrial function and CL content

Since CL was the most sensitive phospholipid, we focused our attention on this species. CL is an exclusive mitochondrial phospholipid that is closely linked to oxidative phosphorylation. It had been shown that decrease in CL content is associated with mitochondrial dysfunction [12]. To test whether mild oxidative stress affects mitochondrial CL content, we exposed isolated rat heart mitochondria to 10 min hypoxia and 5 min reoxygenation in the absence or in the presence of low micromolar Ca^{2+} concentration. This approach mimics main components of ischemia/reperfusion that occur for instance during heart attack. Mitochondrial function was quantified by determining the active respiration. For this purpose, oxygen consumption was determined after 5 mM glutamate plus 5 mM malate had been added to the incubation medium as substrates and 200 μM ADP to stimulate active respiration. In the absence of hypoxia/reoxygenation and Ca^{2+} in the incubation medium the rate of respiration was 36.9 ± 1.3 nmol O_2/min/mg mitochondrial protein (initial respiration). The data of active respiration determined after 10 min hypoxia and 5 min reoxygenation are presented in Figure 4. Hypoxia/reoxygenation caused a drastic decrease in active respiration down to 49.8 ± 0.1% of initial. At the extramitochondrial Ca^{2+} concentration of 0.9 μM, mitochondria were partially protected from hypoxia/reoxygenation-induced injury (56.97 ± 2.07% of initial). This protective effect disappeared at the higher Ca^{2+} concentration of 1.8 μM.

In parallel, we determined the mitochondrial content of cardiolipin by HPLC analysis. The corresponding data are shown in Figure 5. The loss of mitochondrial function that was caused by 10 min hypoxia and 5 min reoxygenation was parallelled by a dramatic decrease in mitochondrial cardiolipin content down to 63% of the normoxic control (from 1.82 to 1.15 arbitrary fluorescence units).

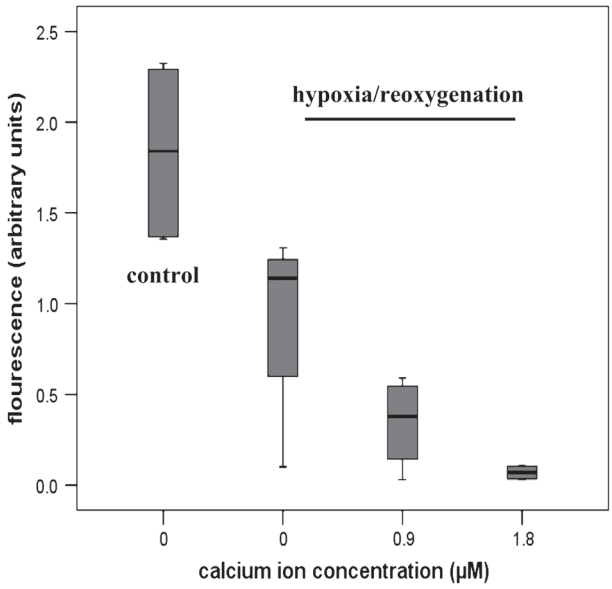

Figure 5. Effect of hypoxia/reoxygenation and Ca^{2+} on CL content in isolated heart mitochondria. Isolated rat heart mitochondria (2.6 mg/ml) were exposed to 10 min hypoxia followed by 5 min reoxygenation in the presence of 0 µM, 0.9 µM and 1.8 µM Ca^{2+}. Afterwards, samples were withdrawn for HPLC-CL analysis. For illustration, peak areas of three CL species were summarized. The CL concentration of the control was 57.1 ± 6.3 nmol/mg mitochondrial protein. Fluorescence intensities of labelled CL are depicted. The difference between all combinations were significant with $p < 0.05$ according to a two-way ANOVA with the animals as independent variable and the condition as fixed effect and two-sided Dunnet *t*-test for the group effect.

The cardiolipin content of untreated controls was determined to 57.1 ± 6.3 nmol/mg mitochondrial protein. When mitochondria were subjected to hypoxia/reoxygenation in the presence of extramitochondrial Ca^{2+}, a further decrease in cardiolipin content was provoked in a dose-dependent manner. At 0.9 µM Ca^{2+}, cardiolipin fell to 24.3% and at 1.8 µM Ca^{2+} to 6.6% of control. This decrease in CL content was not accompanied by an adequate decrease in mitochondrial active respiration. In the presence of both Ca^{2+} concentrations 0.9 and 1.8 µM, active respiration did not fall below 45% of untreated mitochondria (Figure 4).

Effect of H_2O_2 and superoxide anion radicals on commercially available CL—Role of peroxidases

In the next series of experiments we tested the possibility that under oxidative conditions changes in the content of CL might be linked to the oxidation of CL. Therefore, CL extracted from bovine heart was exposed for 15 min at 30°C to either H_2O_2 or superoxide anion radicals. We chose this period of time since significant degradation was observed in intact mitochondria under oxidative conditions at this time span (see Figure 3). According to manufacturer information, the fatty acid distribution was as follows: C16:0 (1%), C16:1 (2%), C18:1 (8%), C18:2 (87%), C18:3 (1%) and other (1%). Therefore, the main part of fatty acid residues comes from C18:2 and C18:1. In consequence, we limited our investigation to $(C18:2)_4$-CL, $(C18:2)_3$C18:1-CL and $(C18:2)_2(C18:1)_2$-CL, covering ~ 95% of the total CL. The experiments repeated with CL liposomes were not different from those described here. In Table I the concentrations of the sum of $(C18:2)_4$-CL, $(C18:2)_3$C18:1-CL and $(C18:2)_2(C18:1)_2$-CL are presented in dependence on the treatment. CL was exposed to 500 µM H_2O_2 (H_2O_2), 5 mM cytochrome *c* (cyt *c*) and to the combination of both (H_2O_2 + cyt *c*). Additionally, we tested the possibility that CL can act as electron donor of other peroxidases. In this context we used horse radish peroxidase (PO, 100 mU), which contains like cytochrome *c* heme in the catalytic centre. Glutathione peroxidase (GPx, 100 mU) was also tested as an enzyme which contains selenocysteine in the active site mainly using glutathione as an electron donor. Hydrogen peroxide alone or in combination with PO (H_2O_2 + PO) and GPx (H_2O_2 + GPx) had no significant effect on the sum of the investigated CL species. However, in the presence of cyt *c* (H_2O_2 + cyt *c*), H_2O_2 caused a dramatic reduction, down to 19.3% of the untreated control. The presence of cyt *c* caused a decrease in the sum of the studied molecular CL species, down to 67% of the untreated control incubation. In

Table I. Effect of H_2O_2 and superoxide anion radicals on CL concentration. CL (5 µg/ml) was exposed to 500 µM H_2O_2 (H_2O_2), 5 mM cytochrome *c* (cyt *c*) or the combination of 500 µM H_2O_2 and 5 mM cytochrome *c* (H_2O_2 + cyt *c*), 500 µM H_2O_2 plus 100 mU horse radish peroxidase (H_2O_2 + PO) and 500 µM H_2O_2 plus 100 mU glutathione peroxidase (H_2O_2 + GPx). Superoxide anion radicals were generated in the presence of 50 µM xanthine and 10 mU xanthine oxidase alone (X/XO) and in combination with cytochrome *c* (X/XO + cyt *c*). The CL concentration (sum of the three molecular species [$(C18:2)_4$-CL, $(C18:2)_3$C18:1-CL, $(C18:2)_2(C18:1)_2$-CL] of the control incubation was 5.12 ± 0.30 µg/ml (no treatment).

Treatment	CL concentration (µg/ml)
no	5.12 ± 0.30 (n = 13)
H_2O_2	5.35 ± 0.26 (n = 7)
Cyt c	3.42 ± 0.16 (n = 10)*
H_2O_2 + cyt c	0.991 ± 0.052 (n = 9)*
H_2O_2 + PO	5.20 ± 0.15 (n = 4)
H_2O_2 + GPx	4.58 ± 0.39 (n = 5)
X/XO	5.49 ± 0.38 (n = 7)
X/XO + cyt c	3.05 ± 0.16 (n = 7)*

Data are presented as mean ± SEM.
*Differences to the CL concentration in the control incubation was significant with $p < 0.05$ (one-way ANOVA with Tukey's HSD test for post-hoc pairwise comparisons). The test further revealed that the differences between the CL concentration in the presence of H_2O_2 and cytochrome *c* and the CL concentrations of all the other incubations were significant with $p < 0.05$. The difference of the CL concentrations in the presence of cytochrome *c* and X/XO plus cytochrome *c* was not significant.

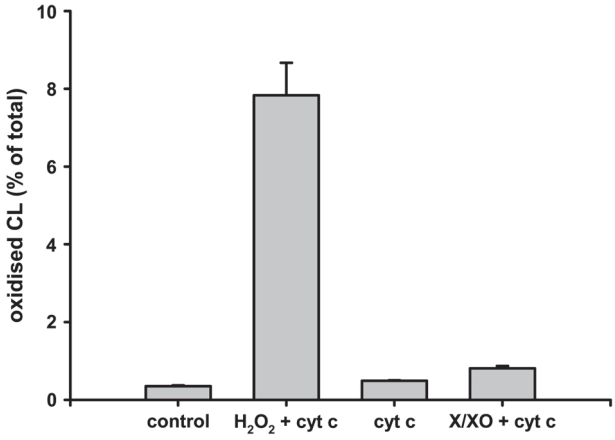

Figure 6. Oxidation of CL by H_2O_2. CL (5 μg/ml) was incubated for 15 min at 30°C with no additions (control), 500 μM H_2O_2 plus 5 mM cytochrome *c* (H_2O_2 + cyt *c*), 5 mM cytochrome *c* (cyt *c*) and 50 μM xanthine plus 10 mU xanthine oxidase plus 5 mM cyt *c* (X/XO + cyt *c*). Oxidized and non-oxidized CL was determined by using LC-MS/MS. $(C18:2)_3$-mono-hydroxylinoleic acid-CL was determined as oxidation product of $(C18:2)_4$-CL. Shown are values as a percentage of the sum of oxidized and non-oxidized $(C18:2)_4$-CL. Data are presented as mean ± SEM (n = 4–6). The differences between all pairs were significant with $p < 0.05$ in a one-way ANOVA with Tukey's HSD test for post-hoc pairwise comparisons based on the logarithms of the oxidized CL in order to compensate for inhomogeneous variances in the four groups.

addition, CL was subjected to superoxide anion radicals by using xanthine/xanthine oxidase as a superoxide anion radical generating system. Superoxide anion radicals alone (X/XO) had no effect on the CL species. In contrast, in the additional presence of cytochrome *c* (X/XO + cyt *c*), the content of the CL species was reduced to 60% of the untreated control. Under these experimental conditions, the relative portion of the three CL species in relation to their sum did not change by more than 3% (not shown).

To elucidate the possibility that decrease in CL content is associated with CL oxidation, we determined the formation of $(C18:2)_3$-monohydroxylinoleic acid-CL as oxidation product of $(C18:2)_4$-CL. Data of this oxidized CL are presented in Figure 6 as a percentage of the sum of oxidized and non-oxidized $(C18:2)_4$-CL. Even under control conditions (control) a basic oxidation of CL of 0.35% was found. The addition of cyt c (cyt c) caused an increase in oxidized CL to 0.49% of total. The combination of xanthine/xanthine oxidase and cytochrome *c* (X/XO + cyt *c*) resulted in CL oxidation to 0.81% of total. However, the combination of H_2O_2 and cyt *c* (H_2O_2 + cyt *c*) led to a 20-fold increase in oxidized CL to 7.83% of total.

Discussion

It had been shown in several studies that strong oxidative stress causes lipid peroxidation, usually demonstrated by measuring thiobarbituric acid reactive substances as products of lipid peroxidation [26]. The aim of our study was to investigate the effect of oxidative stress on the structurally different membrane phospholipids PC, PE and CL in biological systems.

Among the reactive oxygen species that are formed by transfer of electrons to oxygen, the hydroxyl radical is the most reactive molecule [27]. It has a life time of only 1 ns [27]. In contrast to $O_2^{-\cdot}$ and H_2O_2, ·OH does not penetrate through biological membranes since it immediately reacts with surrounding molecules at the place of generation. In the presence of iron and ascorbate, isolated mitochondria generate high quantities of hydroxyl radicals via perferryl radicals and/or Fenton reaction [28]. We found that this kind of oxidative stress caused loss of the membrane phospholipids PC, PE and CL. This loss points towards a radical-mediated oxidation of these phospholipids via the generation of lipid alkoxyl and peroxyl radicals. Unsaturated bounds are favoured places for the attack of radicals. The content of unsaturated bonds might be the reason for the different vulnerability of the investigated phospholipids against oxidative stress. The phospholipids PC, PE and CL contain different amounts of unsaturated bounds in their fatty acid residues, but the degree of 'unsaturation' (calculated as double bond or peroxidizability index [4]), does not differ substantially [29]. CL contains the highest part of linoleic acid (18:2) in brain and in particular in heart mitochondria [29,30], but highly unsaturated fatty acids, as 20:4, 22:4, 22:5 and 22:6, are more enriched in PE of neural mitochondria than in CL [29]. In the particular situation of iron/ascorbate induced oxidative stress, additional targets within the phospholipid molecules should be considered. PC and PE at the one site and CL at the other site considerably differ in their structure. CL is a dimeric phospholipid with an unusual structure of 4 acyl side chains in comparison to PC and PE. It is negatively charged and has a polar OH group in the middle of the molecule. This gives rise for the preferred attack of reactive oxygen species in the polar part of CL (hydroxyl group in *β*-position to the phosphoester bond) and free-radical mediated fragmentation under formation of phosphatidic acid and phospatidylhydroxyacetone [31,32]. This particular structure of CL may be, at least in part, responsible for the high vulnerability of CL in comparison to PE and PC. Another reason for the different vulnerability of PE, PC and CL to oxidative stress may be the requirement of additional factors that selectively mediate the oxidation of certain places within phospholipid molecules. Such activity had been demonstrated for cytochrome *c* that exhibits peroxidase activity [33].

There is a body of evidence that CL is linked to mitochondrial function [34]. CL is physiologically

bound to several membrane proteins. A decrease in the content of natural CL had been shown to be associated with a decrease in the activity of these proteins, resulting in mitochondrial dysfunction [35]. CL-mediated impairment of oxidative phosphorylation is indicated by restriction of mitochondrial respiration and ATP production [36]. It is known that hypoxia/reoxygenation induces mild oxidative stress in isolated mitochondria [37]. We here report that 10 min hypoxia followed by 5 min reoxygenation resulted in decreased active respiration (50% of initial) parallelled by a dramatic decrease in CL concentration in isolated heart mitochondria. However, further decrease in CL content provoked by increasing extramitochondrial Ca^{2+} concentration did not cause further decrease in active respiration. Based on this observation we postulate that only a part of the CL pool is associated with the machinery of oxidative phosphorylation. Apparently, this part of the CL pool is especially sensitive to oxidative stress. Another part of the CL pool seems not to be associated with electron transfer within the respiratory chain but interacts with Ca^{2+}. Compartmentation of CL in mitochondria had been reported [38]. For example, CL can be located, at least, at the inner and also at the outer site of the mitochondrial inner membrane [38]. Under certain conditions it can translocate between different locations [39].

Oxidized CL had been demonstrated in muscle homogenate treated with peroxynitrite [25]. As a main oxidized CL species $(C18:2)_3$-mono-hydroxylinoleic acid-CL had been found. This probably results from the decay of CL hydroperoxide. Thus, these authors provided clear experimental evidence for the oxidation of CL. We also detected oxidized CL after exposure to H_2O_2. This data support the suggestion that loss of CL is, at least partially, mediated by an oxidation process. However, our *in vitro* experiments demonstrate that CL is relatively resistant to the direct attack of hydrogen peroxide and superoxide anion radicals. Even 500 μM H_2O_2 caused neither loss nor oxidation of CL. Moreover, superoxide anion radicals at concentrations that are sufficient to reduce cytochrome *c* also did not cause CL loss or CL oxidation. Oxidation of CL by H_2O_2 required specific peroxidase activity. In our experiments, cytochrome *c* effectively mediated CL oxidation. Peroxidase activity of cytochrome *c* had been described earlier by several authors [40,41]. CL mediates the binding of cytochrome *c* to complex III of the mitochondrial electron transport chain and consequently electron transport. Under oxidative stress, especially at high local H_2O_2 concentrations, cytochrome *c* turns to peroxidase activity allowing CL oxidation [42,43]. Under the control of cytochrome *c*, CL is very sensitive to H_2O_2. It seems that relatively low H_2O_2 concentrations, either present in oxygenated incubation media or generated by spontaneous dismutation of superoxide anion radicals, are sufficient to oxidize CL. We observed even in the incubation medium, always containing small amounts of H_2O_2, moderate CL oxidation. In the presence of xanthine/xanthine oxidase used as superoxide anion radical generating system CL oxidation was also measured.

The generation of $(C18:2)_3$-monohydroxylinoleic acid-CL measured as oxidation product of $(C18:2)_4$- CL only partially accounts for the observed degradation of the three investigated CL species. In the presence of 500 μM H_2O_2 and cytochrome *c* only ~ 8% of the total $(C18:2)_4$-CL were found to be oxidized, whereas the sum of $(C18:2)_4$-CL, $(C18:2)_3C18:1$-CL and $(C18:2)_2(C18:1)_2$-CL were decreased by ~ 80%. ROS exposure of CL may initiate a sequence of steps generating alkyl radicals, peroxyl radicals and hydroperoxides, finally entering in a continuous chain reaction involving other acyl chains of the CL molecule [44]. Several instable intermediates are generated during CL peroxidation. Hence, $(C18:2)_3$-monohydroxylinoleic acid-CL covers only a small part of them. However, changes in the amount of $(C18:2)_3$-monohydroxylinoleic acid-CL were accompanied by parallel changes in CL degradation (CL plus H_2O_2 plus cytochrome *c* vs control, CL plus cytochrome *c* vs control and CL plus X/XO plus cytochrome *c* vs control). Thus, our data demonstrate that CL peroxidation takes place, which is an essential part of CL degradation.

It had been speculated that oxidation of CL triggers the detachment of cytochrome *c* from the mitochondrial membrane and release into the cytosolic compartment [45]. This process can induce the intrinsic pathway of apoptosis. Our data show that H_2O_2-dependent oxidation of CL is specifically mediated by cytochrome *c*. This might be an essential part of the mechanism of apoptosis induction under conditions of oxidative stress. Among the phospolipids CL is especially sensitive to H_2O_2 and mediates cytochrome *c* release, since CL is co-localized with cytochrome *c*.

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