Fenretinide induces increased levels of monolysocardiolipin in neuroblastoma

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Abstract

Fenretinide induces apoptosis in various cancers, including neuroblastoma, and mitochondrially-formed ROS is part of the apoptosis-inducing mechanism. Cardiolipin (CL) is a typical phospholipid of the mitochondrial membrane and is prone to oxidation. Therefore the effect of fenretinide on CL was investigated. However, no oxidation of CL was observed in neuroblastoma cell lines (NASS and SJNB10) incubated with fenretinide. Similar results were obtained after enrichment of CL with the highly unsaturated linoleic acid (18:2). Surprisingly, fenretinide increased the monolysocardiolipin (MLCL):CL-ratio which might reflect a disturbed mitochondrial homeostasis and the induction of apoptosis.
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Introduction

Fenretinide (4HPR) is a reactive oxygen species (ROS)-inducing agent that, as a result, induces apoptosis in various cancers, including neuroblastoma. In neuroblastoma, fenretinide inhibits the respiratory chain in the mitochondria, which results in ROS generation. The mitochondrial respiratory chain is the major site of intracellular ROS generation. Oxidative stress can cause changes in phospholipids and membrane integrity, followed by apoptotic response. Cardiolipin (CL) is an unique phospholipid of the mitochondrial membrane and CL is involved in electron transport and adenosine triphosphate (ATP) synthesis, apoptotic signaling and mitochondrial protein import. CL is well known for its high content of unsaturated fatty acids and is therefore, a major target of oxidative modification. For example, linoleic acid (C18:2), one of the most common unsaturated fatty acids, is often part of the CL molecule and is therefore prone to oxidation. Oxidative modification could remodel the CL molecule by two major mechanisms. ROS could induce lipid peroxidation via free-radical mediated chain reactions. Alternatively, the fatty acid chains are targets of enzyme-mediated addition of oxygens via the action of lipoxygenases (LOX) or peroxidases. Oxidation of CL might be essential for the release of pro-apoptotic factors, including cytochrome c, membrane permeabilization and caspase-9 and caspase-3 activation. Thus, excessive ROS generated in mitochondria act as mediators of the apoptotic-signaling pathway.

Peroxidation of CL has been demonstrated after incubation of HL60 cells with H₂O₂ and in heart of a mouse model after ischemia. Selective oxidation of CL was also found in the lungs of mice exposed to hyperoxia. The mass spectrometry (MS) analysis of CL oxidation products identified CL molecular species containing hydroperoxylinoleic acid (C18:2-OOH) along with palmitic C16:0, linoleic C18:2, and stearic C18:0 fatty acids. The degradation of CL can generate monolysocardiolipin (MLCL) via the action of phospholipases and contribute to both the delocalization of cytochrome c and the subsequent attraction of pro-apoptotic peptides to the outer mitochondrial membrane. We hypothesized that fenretinide-induced mitochondrial ROS might trigger apoptosis via cardiolipin oxidation and remodelling. In this study, we describe an increase of MLCL as a result of fenretinide incubation in neuroblastoma together with a decrease of the total CL amount. No oxidation of CL was observed as a consequence of fenretinide incubation in neuroblastoma cells nor were any oxidized CL-products measured after incubation with fenretinide in linoleic acid enriched neuroblastoma cells. The increase of MLCL might reflect a disturbed mitochondrial homeostasis and contribute to both the delocalization of cytochrome c and induction of apoptosis.
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Materials and methods

Chemicals
Fenretinide (Sigma), was dissolved in 100 % ethanol, stored at 4° C and protected from light. Chemicals were purchased from Sigma (St Louis, MO, USA), unless stated otherwise.

Cell culture and incubation
Two neuroblastoma cell lines (NASS, SJNB10) and one leukemia cell line (HL60) were cultured in RPMI-1640 culture medium supplemented with 10 % heat inactivated fetal bovine serum, 50 U/ml penicillin/streptomycin, 4 mM glutamine (Gibco, Invitrogen, CA, USA) and 5 mg/l plasmocin (Invivogen, San Diego, USA). Cells were grown at 37 °C, 5 % CO2 in 95 % humidified air, all culture flasks were obtained from Corning (Corning, NY, USA). All neuroblastoma cell lines were a generous gift of Prof. R. Versteeg (Dept. of Oncogenomics, Academic Medical Centre, Amsterdam). HL60 cells were obtained from ATCC (Manassas, Virginia, USA). All experiments were performed in triplicate unless stated otherwise.

NASS and SJNB10 cells were allowed to adhere overnight, after which the medium was replaced by medium containing different concentrations fenretinide (0-50 µM), depending on the cell line's sensitivity for fenretinide or H2O2 (100 µM). After 4 h, the cells were harvested with trypsin and washed with PBS and the pellets were stored until use at -80 °C. HL-60 cells were incubated with 100 µM H2O2 for 1 h

Enrichment with linoleic acid
In order to increase the amount of unsaturated fatty acids in cellular CL, SJNB10 cells, were cultured for six passages with culture medium supplemented with 10 µM linoleic acid (18:2) (Acros Organics, New Jersey, USA). After six passages, the cells were incubated with 10 µM fenretinide for 4h and harvested, as described above.

In vitro incubation with H2O2
Cardiolipin was oxidized in vitro using a combination of H2O2 and cytochrome c, as described before. Briefly, 2 nmol of bovine heart cardiolipin was incubated with 100 µM H2O2 and 10 µM cytochrome c in a 10 mM Tris-HCl buffer at pH7.4. After 1h incubation at 37 °C, the reaction was stopped by the addition of chloroform:methanol 1:1 (v/v), followed by cardiolipin extraction and measurement, as described in detail below.

Cardiolipin extraction
Neuroblastoma cells were resuspended in PBS and sonicated for 20 s. Protein concentration was determined as described before, using the BCA assay (Thermo Scientific).
Protein (1 mg) was transferred to a glass tube in order to perform a single-phase extraction. 3 ml of chloroform:methanol 1:1 (v/v) was added to the cells. After addition of the internal standard (0.4 nmol of CL(14:0) dissolved in 50 µl chloroform) the mixture was shaken vigorously for 2 min and placed on ice for 15 min, followed by centrifugation at 1000 x g. The supernatant was transferred to another tube, and the protein pellet was extracted another time with 3 ml of chloroform:methanol 2:1 (v/v). The organic layers were combined and evaporated under a stream of nitrogen at 45 °C. The residue was dissolved in 150 µl of chloroform:methanol:water 50:45:5 (v/v/v) containing 0.01% NH₄OH. Subsequently, 10 µl of the dissolved residue was injected into the High-performance Liquid Chromatography (HPLC)-MS system.

**HPLC - Mass spectrometry of cardiolipin**

The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller, and an autosampler (Thermo Electron, Waltham, MA, USA). The column temperature was maintained at 25 °C. The lipid extract was injected onto a LiChrospher 2 250-mm silica-60 column, 5 µm particle diameter (Merck, Darmstadt, Germany). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform/methanol, 97:3, v/v) and solution A (methanol/water, 85:15, v/v). Solutions A and B contained 1 and 0.1 ml of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 ml/min) was as follows: 0–10 min, 20% A–100% A; 10–12 min, 100% A; 12–12.1 min, 100% A–0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A splitter between the HPLC column and the mass spectrometer was used, and 75 µl/min eluent was introduced into the mass spectrometer. A TSQ Quantum AM (Thermo Electron) was used in the negative electrospray ionization mode. Nitrogen was used as the nebulizing gas. The source collision-induced dissociation collision energy was set at 10 V. The spray voltage used was 3600 V, and the capillary temperature was 300 °C. Mass spectra of CL and MLCL molecular species were obtained by continuous scanning from m/z 380 to m/z 1100 with a scan time of 2 s.

**Data analysis**

Quantification of CL and MLCL levels was performed using Xcalibur MS quantification software. For quantification of CL and MLCL levels, the area under the curve of the HPLC profile corresponding to the complete mass spectra of CL (m/z 699–742) and MLCL (m/z 565–610) species was integrated, as was that of the internal standard (m/z 619–622). The MLCL:CL-ratio was calculated from these results, this ratio is based on the complete spectrum of MLCL and CL.
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Statistics
Differences in MLCL:CL-ratio between two groups were analyzed with the independent samples t-test (IBM SPSS Statistics 20).

Results
In an in vitro model we incubated CL(18:2)₄ for 1 h with 100 µM H₂O₂, and observed a mixture of oxidized products. Non-oxidized CL(18:2)₄ with m/z of 723.5 m/z was found, together with peroxidation products of linoleic acid with the following m/z: 731.5, 740, 748.5, 757 and 765 (Fig. 1). To evaluate in vivo peroxidation of CL, SJNB10 and NASS cells were treated with fenretinide. SJNB10 cells are known to generate large amounts of ROS after fenretinide incubation whereas NASS cells hardly produce any ROS after fenretinide incubation. Cells were incubated with fenretinide, after which CL was extracted and the mass spectrum of CL molecules was measured. No oxidized CL-products were observed after fenretinide incubation (Fig. 2A), nor after increasing the concentrations of fenretinide. Surprisingly, increasing the fenretinide concentrations resulted in an increase of the MLCL/CL ratio in NASS (p-value 0.043) cells but not in SJNB10 (p-value 0.308) as compared to untreated cells (Fig. 2B). In SJNB10 cells, the MLCL/CL ratio increased with 23% in cells treated with fenretinide compared to untreated cells, which was due to a decrease in CL concentration and a minimal increase in MLCL. However, in NASS a profound increase of 416%, of the MLCL/CL ratio was

Figure 1. In vitro CL oxidation. In an in vitro model, CL(18:2) was incubated with 100 µM H₂O₂ and 10 µM cytochrome c for 1 h (panel B) or without H₂O₂ (panel A). Oxidized products of CL with higher m/z were present. CL, unoxidized native linoleic acid, i.s = internal standard.
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observed, which was a combination of an increase of MLCL in addition to a decrease of CL. The decrease of the total amount of CL that was observed in both cell lines, as a result of fenretinide incubation, was not significant (data not shown).

SJNB10 cells and HL60 cells were incubated with H$_2$O$_2$ (100 µM) for 1 h, after which CL was extracted and the CL spectrum was studied. No oxidized CL products were identified (data not shown). To increase the susceptibility of CL to oxidation, the amount of unsaturated fatty acids in CL was increased by culturing the cells in the presence of

Figure 2a. MLCL formation. No oxidized products were observed in NASS and SJNB10 cells after incubation with 20 and 10 µM fenretinide respectively for 4 h. Indicated by the same CL profiles in control versus fenretinide-treated cells.
linoleic acid (18:2). After 6 passages of cell culturing in the presence of 18:2, incorporation of 18:2 was observed by a general increase of m/z of the total CL-spectrum, indicated by the arrow in Fig. 3. However, this enrichment with unsaturated fatty acid chains did not result in peroxidation of CL after incubation with fenretinide (Fig. 3).

Discussion

Cardiolipin (CL) is a specific mitochondrial phospholipid and as such, able to bind cytochrome c, which is a pro-apoptotic factor. Previously, it has been shown that cytochrome c, bound to CL liposomes, was released after peroxidation of CL by ROS.18 This liberation of cytochrome c was followed by the release of proapoptotic factors.16,28 Due to its double bonds and its existence in the mitochondrial membrane cardiolipin is prone to oxidation.28,29 As fenretinide is known to induce mitochondrial ROS and inhibit the mitochondrial respiratory chain followed by apoptosis, we hypothesized that alterations in the cardiolipin structure might be a result of fenretinide treatment in neuroblastoma.3

Kim and Kagan reported that CL-oxidation occurred, in pure CL and in CL extracted from HL60 cells respectively, after incubation with ROS-inducing agents.16,30 Asumendi et al., reported CL-peroxidation in leukemia after fenretinide incubation, using the 10-n-nonyl-acridin-orange assay.31,32 In this study, we showed that treatment of neuroblastoma and leukemia cells with the mitochondrial ROS-inducing agent fenretinide or H2O2 did not result in detectable levels of oxidation of CL. Enrichment of cardiolipin with poly-
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Figure 3. Linoleic acid enrichment. Neuroblastoma cells (SJNB10) were enriched with linoleic acid (18:2) and treated with 10 µM fenretinide for 4 h (18:2 + 4HPR). The arrow indicates the shift of the CL profile as a result of 18:2 incubation. No oxidized cardiolipin was observed, as a result of fenretinide treatment.
unsaturated fatty acid acyl chains may lead to increased peroxidation.\textsuperscript{33,34} Although the enrichment of the neuroblastoma cells with the polyunsaturated fatty acid acyl chain, linoleic acid (18:2), was successful, this enrichment did not lead to any detectable levels of CL-oxidation. Our results are in apparent contrast with those reported by others since no CL-OOH (CL-hydroperoxide) was identified in our neuroblastoma cells and leukemia cell line following H\textsubscript{2}O\textsubscript{2} treatment.\textsuperscript{13,16,23,31} Even though no oxidized CL products were identified after incubation with fenretinide or H\textsubscript{2}O\textsubscript{2} in cells, CL oxidation was observed \textit{in vitro} in CL(18:2)\textsubscript{4} incubated with H\textsubscript{2}O\textsubscript{2}. Phospholipid hydroperoxide glutathione peroxidase (PH-GPx) is one of the antioxidant enzymes of the mitochondria that protects cells from lipid peroxidation; it metabolizes phospholipids hydroperoxides in membranes utilizing glutathione (GSH) as reducing agent.\textsuperscript{35} Although, GPx levels were not upregulated as a result of fenretinide incubation in neuroblastoma cells,\textsuperscript{36} it has been established that overexpression of PH-GPx in general suppressed the generation of CL-OOH and the release of cytochrome \textit{c} from mitochondrial inner membrane.\textsuperscript{18} Surprisingly, MLCL (monolyso-CL) was observed in the CL-spectrum of NASS cells after incubation with high concentrations fenretinide. The increase of MLCL might indicate that mitochondrial homeostasis has been disturbed. MLCL may appear when the capacity to repair CL via enzymatic remodeling has been exceeded.\textsuperscript{25} This phenomenon has only been observed in NASS treated with high concentrations of fenretinide and the amount of ROS production after fenretinide incubation in this cell line is low compared to other cell lines (i.e SJNB10). Strengthened by the fact that no CL-OOH formation was observed, this indicates that MLCL formation is a non-oxidative effect. The exact mechanism underlying this MLCL formation remains to be elucidated. In addition, a slight decrease of the total levels of CL after fenretinide incubation was observed, which might be the result of apoptosis and/or may reflect the degradation of CL to MLCL.\textsuperscript{8,37} Formation of MLCL leads to permeabilization of the outer membrane which might result in the release of pro-apoptotic factors\textsuperscript{38} such as cytochrome \textit{c}.\textsuperscript{25,39,40} From our data, we conclude that fenretinide treatment did not result in the production of CL-OOH in neuroblastoma. In one out of two cell lines, formation of MLCL was observed which might indicate destruction of the mitochondrial membrane. The underlying mechanism of this action was not the result of oxidative stress and has to be further elucidated.

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