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LC/MS characterization of rotenone induced cardiolipin oxidation in human lymphocytes: Implications for mitochondrial dysfunction associated with Parkinson's disease

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Abstract

Scope—Rotenone is a toxicant believed to contribute to the development of Parkinson's disease.

Methods and results—Using human peripheral blood lymphocytes we demonstrated that exposure to rotenone resulted in disruption of electron transport accompanied by the production of reactive oxygen species, development of apoptosis and elevation of peroxidase activity of mitochondria. Employing LC/MS based lipidomics/oxidative lipidomics we characterized molecular species of cardiolipin (CL) and its oxidation/hydrolysis products formed early in apoptosis and associated with the rotenone-induced mitochondrial dysfunction.

Conclusions—The major oxidized CL species - tetra-linoleoyl-CL – underwent oxidation to yield epoxy-C_{18:2} and dihydroxy-C_{18:2} derivatives predominantly localized in *sn*-1 and *sn*-2 positions, respectively. In addition, accumulation of mono-lyso-CL species and oxygenated free C_{18:2} were detected in rotenone-treated lymphocytes. These oxidation/hydrolysis products may be useful for the development of new biomarkers of mitochondrial dysfunction.

Keywords

Cardiolipin peroxidation; lymphocytes; apoptosis; mitochondrial dysfunction; Parkinson's disease biomarkers

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder in the elderly resulting in the damage and death of dopaminergic neurons in the brain [1]. Oxidative stress and mitochondrial dysfunction have been implicated as important contributors to neuronal death induced in *substantia nigra* of patients with PD [2–4]. In particular, increased lipid peroxidation products have been found in PD brains [5]. During the last three decades,

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epidemiological and toxicological studies provided data that pesticides are potential toxicants for dopamine-producing neurons and contribute to the development of PD [6]. Accordingly, exposure to pesticides has been associated with increased incidence of PD [7, 8]. Mitochondria are targets for a number of environmental compounds including pesticides [6, 7] such as rotenone [9, 10]. Rotenone is highly lipophilic, easily crosses the blood-brain barrier and accumulates in mitochondria where it binds to complex I, inhibits the flow of electrons and results in generation of superoxide [11]. However, the mechanistic links between the oxidative stress, lipid peroxidation, neuronal death, mitochondrial impairments induced by pesticides have not been identified.

Blood cells and circulating lymphocytes have been often used to study the pathogenic mechanisms of neurodegenerative diseases, including PD. Mitochondrial complex deficit [12, 13] and up-regulation of the α -synuclein gene [14] that render these cells susceptible to apoptosis were detected in lymphocytes from PD patients [15, 16]. Recently, we demonstrated that oxidation of a mitochondria-specific phospholipid, cardiolipin (CL), is essential for the execution of apoptosis (release of proapoptotic factors from mitochondria into the cytosol) in primary rat cortical neurons *in vitro* [17, 18] and in rat brain *in vivo* [19, 20]. Intermembrane space hemoprotein, cytochrome *c* (cyt *c*) has been identified as a catalyst of CL peroxidation [21]. CL is a negatively charged phospholipid with four fatty acid residues [21]. It is found exclusively in the inner-mitochondrial membrane where it accounts for 25% of all phospholipids [22] and plays a significant role in mitochondria bioenergetics [23–26]. Taking into account that lymphocytes express the catecholaminergic system and, similar to neuronal cells, molecular death machinery, including the release of mitochondrial pro-apoptotic factors into the cytosol, leading to the typical morphological and biochemical characteristics of apoptosis [27–29], we suggested that cyt *c* can be involved in the generation of specifically oxygenated molecular species of CL in circulating lymphocytes exposed to rotenone.

In this paper, by using lipidomics/oxidative lipidomics approaches we identified and characterized molecular species of CL in human peripheral blood lymphocytes, evaluated specific profiles of rotenone-induced CL oxidation products and established their association with the production of H_2O_2 , impairments of mitochondrial dysfunction and apoptosis in human lymphocytes exposed to rotenone.

Material and Methods

Reagents

Tetra-linoleyl-cardiolipin (TLCL) and tetra-myristoyl-cardiolipin (TMCL) were purchased from Avanti Polar Lipids Inc (Alabaster, AL). Cytochrome *c* (cyt *c*), diethylenetriaminepentaacetic acid (DTPA), PLA_1 from *Thermomyces lanuginosus*, PLA_2 from porcine pancreas, H_2O_2 and all organic solvents (HPLC grade), Hystopaque 1077, PBS (Ca^{2+} , Mg^{2+} free), rotenone, DMSO were purchased from Sigma-Aldrich (St. Louis, MO). HPTLC silica G plates were purchased from Whatman (Schleicher & Schuell, England). Fetal bovine serum, RPMI 1640 medium Penicillin/Streptomycin were from Life Technologies (Grand Island, NY). Heptadecanoic acid ($C_{17:0}$) was obtained from Matreya LLC (Pleasant Gap, PA). 9S-hydroperoxy-10E,12Z-octadecadienoic acid, 9S-hydroxy-10E,

12Z-octadecadienoic acid, 13-oxo-9Z,11E-octadecadienoic acid, 13S-hydroxy-9Z,11E-octadecadienoic acid, 13S-hydroperoxy-9Z,11E-octadecadienoic acid, 9S-hydroxy-10E, 12Z-octadecadienoic-9,10,12,13-d₄ acid, 9(10)epoxy-12Z-octadecadienoic acid, 12(13)epoxy-9Z-octadecadienoic acid were purchased from Cayman Chemical Co (Ann Arbor, Michigan, USA).

Isolation of human peripheral blood lymphocytes—Lymphocytes were isolated from buffy coat obtained from Central Blood Bank by differential centrifugation using Hystopaque 1077 as described [30].

Production of reactive oxygen species—*Superoxide and hydrogen peroxide* were detected by using dihydroethidium (DHE) and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) assays, respectively, as previously described [31]. The data are presented as fold change of the mean intensity of either ethidium or DCF fluorescence compared with DHE or DCFH-DA loaded controls for superoxide and hydrogen peroxide, respectively.

Detection of apoptosis—Apoptosis was evaluated by phosphatidylserine (PS) externalization using Annexin V–FITC apoptosis detection kit (Biovision, Mountain View, CA) and caspase 3/7 with a luminescence Caspase–Glo™ 3/7 assay kit (Promega, Madison, WI).

Assessments of mitochondrial functional state—*Mitochondrial membrane potential (MMP)* was determined by JC-1 staining. Briefly, lymphocytes were stained with 10 µg/ml of JC-1 at 37 °C for 15 min and then washed twice with PBS. The samples were analyzed immediately by using RF-5301 PC spectrofluorometer (Shimadzu, Japan) (excitation 485 nm, slits 5 nm). The ratio of red (aggregates, 590 nm) and green (monomer, 529 nm) fluorescence was used as a relative measure of MMP. In addition the lymphocytes were examined under a Nikon ECLIPSE TE 200 fluorescence microscope (Tokyo, Japan) equipped with a digital Hamamatsu CCD camera (C4742-95-12NBR) and analyzed using the MetaImaging Series™ software version 4.6 (Universal Imaging Corp., Downingtown, PA). *Cellular ATP* content was measured by using an adenosine 5'-triphosphate bioluminescence somatic cell assay kit (Sigma) according to the manufacturer's instructions. *Complex I activity* was measured by consumption of NADH at 340nm as described [32].

Isolation of mitochondria and detection of peroxidase activity—Mitochondria were isolated from human peripheral blood lymphocytes as described [33]. Peroxidase activity was detected as previously described [34]. Briefly mitochondria (2 mg of protein/ml) were incubated with alamethicin (0.1 mg/ml) on ice for 15 min in 150 mM KCl, 0.5 mM EGTA, 25 mM KH₂PO₄ (pH 7.0). After that mitochondria were centrifuged (15,000g for 15 min) and re-suspended in 25 mM HEPES (pH 7.4) containing 100 µM DTPA, Amplex Red (50 µM) and *tert*-BOOH (2 mM). Fluorescence of resorufin, a product of Amplex Red oxidation, was measured using Shimadzu RF5301–PC spectrofluorometer (λ_{ex} and λ_{em} – 575 and 585 nm, respectively).

Analysis of CL and its oxygenated molecular species—Lipids were extracted using Folch procedure [35]. Lipid phosphorus was determined by a micro-method [36].

LC/MS was performed using a Dionex Ultimate™ 3000 HPLC system coupled on-line to a linear ion trap mass spectrometer (LXQ Thermo-Fisher) as described [37]. To fully characterize oxygenated free fatty acids and diversified CL oxidation products we chose to pre-separate them from other phospholipids by 2D-HPTLC as previously described [38]. Corresponding spots were scraped-off and lipids extracted [35]. Thus obtained CLs were treated either with phospholipase A₁ (PLA₁) from *Thermomyces lanuginosus* or PLA₂ from porcine pancreatic phospholipase A₂ (PLA₂) to liberate fatty acids from *sn*-1 and *sn*-2 positions and analyzed by LC-MS. Briefly, CLs were treated with PLA₁ (10 µl/µmol CL) or PLA₂ (10U/µmol of CL) in 0.5 M borate buffer, pH 9.0 containing 20 mM cholic acid, 2 mM CaCl₂ and 100 µM DTPA for 30 min. Under these conditions, almost 99% of CLs were hydrolyzed. At the end of incubation, lipids were extracted and fatty acids were analyzed by LC/MS using reverse phase C₁₈ column. The differentiation between isobaric epoxy-C_{18:2} CL species from hydroxy-C_{18:2}-containing species was achieved via i) treatment of CLs by exogenous PLA₁/PLA₂ resulting in the release of LA residues and ii) their subsequent separation and analysis by LC-MS using C₁₈ column and two gradient solvent systems (system A: tetrahydrofuran/methanol/water/CH₃COOH, 25:30:44.9:0.1 (v/v) and System B: methanol/water, 90:10 (v/v)) as previously described [39]. Under these conditions, the retention times for epoxy-C_{18:2} (m/z 295) and hydroxy-C_{18:2} (m/z 295) were 21.16 and 17.04 min, respectively. This was confirmed by comparison with the standards of epoxy-C_{18:2} (m/z 295) and hydroxy-C_{18:2} (m/z 295) available from Cayman Chemicals. Additionally, several major classes of phospholipids, including CLs, were separated and analyzed by LC-MS as described [37]. For quantitative assessments TMCL and oxygenated fatty acids were used as internal standards. Mono-lyso-CL was prepared from TMCL as described [40].

Statistics—The results are presented as mean ± S.D. values from at least three experiments, and statistical analyses were performed by either paired/unpaired Student's t-test or one-way ANOVA. The statistical significance of differences was set at $p < 0.05$.

Results

Rotenone induces mitochondria dysfunction in human lymphocytes—After exposure of human peripheral blood lymphocytes to rotenone (100 and 250 µM, 12 and 18 hrs at 37°C), the mitochondrial functions were assessed by measurements of MMP, determination of complex I activity and ATP levels. Rotenone induced significant inhibition of complex I activity in a dose- and time dependent manners (Fig 1A). Significant reductions of the MMP and ATP contents were detected in lymphocytes treated with rotenone (Fig.1B, C). Thus exposure of human lymphocytes to rotenone resulted in disruption of electron transport and mitochondrial dysfunction.

Generation of reactive oxygen species in human lymphocytes exposed to rotenone—Interrupted mitochondrial electron transport, particularly at the level of complex I, is known to cause a massive production of superoxide (53). To determine whether superoxide, indeed, has been generated in rotenone-treated cells we used DHE assay. We found that rotenone caused concentration-dependent production of superoxide

(Fig. 2A). Given that mitochondrial superoxide is rapidly converted to membrane permeable and relatively stable hydrogen peroxide we examined the intracellular concentration of hydrogen peroxide using DCFH-DA assay. A significant increase of DCF fluorescence was observed in human lymphocytes following rotenone exposure (Fig. 2B). Thus, rotenone-induced mitochondria dysfunction in lymphocytes was accompanied by the production of ROS.

Rotenone induces apoptosis in human lymphocytes—Assuming that generation of ROS is one of the pre-requisites for triggering apoptosis, we further determined whether rotenone-induced production of superoxide and H₂O₂ was accompanied by apoptosis. Indeed, we found that caspase 3/7 activity was significantly increased in lymphocytes exposed to rotenone as compared with control non-exposed lymphocytes (Fig. 3A). A 2.5 and 2.8-fold increase in caspase 3/7 activity was detected either for 12 or 18 hrs after the exposure to 100 and 250 μM of rotenone, respectively. In addition, using Annexin V binding assay we were able to detect a significant number of lymphocytes with externalized PS on the cell surface after their exposure to 100 and 250 μM of rotenone for 12 and 18 hrs (Fig. 3B). Thus, treatment of lymphocytes to rotenone resulted in development of apoptotic cell death pathway.

Rotenone stimulates peroxidase activity in mitochondria—We suggested that in lymphocytes, during rotenone-induced apoptosis, mitochondrial phospholipid CL interacts with cyt *c* to form a complex with peroxidase activity that consequently results in selective oxidation of CL polyunsaturated molecular species. To detect peroxidase activity of cyt *c*/CL complexes we isolated mitochondria from lymphocytes exposed to rotenone. To remove free cyt *c* we treated mitochondria with a channel-forming antibiotic, alamethicin (9). Notably, mitochondria isolated from lymphocytes exposed to rotenone at concentrations of 100 and 250 μM for 18 hrs exhibited significantly elevated levels of peroxidase activity as compared to mitochondria from control, non-treated lymphocytes (Fig. 4).

Identification of CL molecular species in human lymphocytes—As CL oxidation is required for the execution of apoptotic program, we further employed LC/MS to analyze CL molecular species and their oxidation products in lymphocytes. In a typical negative mode MS spectrum of CL, two major clusters were detected (Fig. 5A). MS/MS analysis was performed (Supporting Information Fig. S1) showed that CLs were represented by seven major molecular species predominantly containing readily peroxidizable linoleic acid residues (C_{18:2}) (Table 1).

Identification of CL molecular species in human lymphocytes exposed to rotenone—Next, we performed detailed structural characterization of oxygenated CL molecular species in human peripheral blood lymphocytes. Quantitative assessments of rotenone-induced changes in CL revealed a significant reduction of highly unsaturated species of CL, particularly TLCL (Fig. 5B). The loss of “oxidizable” TLCL was dependent on concentration of rotenone and accompanied by the appearance of its oxygenated species with one, two and three oxygen functionalities whereby mono-oxygenated derivatives were predominant (Fig. 5C). The structure of oxygenated CLs was confirmed by MS/MS analysis

as exemplified by a typical fragmentation pattern of mono-oxygenated TLCL molecular (Supporting Information Fig. S2).

Stereo-specificity of TLCL oxygenation in *sn*-1 and *sn*-2 positions was examined by LC/ESI-MS using hydrolysis of CL with PLA₁ and/or PLA₂, respectively. Quantitative MS analysis of fatty acids liberated from *sn*-1 position revealed a significantly decreased C_{18:2} content (Fig. 6Aa) and elevated levels of its oxygenated product with 1–3 oxygens (Fig. 6Ab) that markedly exceeded oxidative loss of C_{18:2} from the *sn*-2 position (Fig. 6Ba). Notably, the major oxygenated differences between oxygenated products generated in *sn*-1 and *sn*-2 positions were not only quantitative but also qualitative with epoxy-C_{18:2} derivatives (Fig. 6Ab and Supporting Information Fig.S3) and dihydroxy-C_{18:2} derivatives (Fig. 6Bb and Supporting Information Fig. S4) accumulating in *sn*-1 and *sn*-2 positions, respectively.

To test whether cyt *c* could be a candidate catalyst involved in rotenone-induced generation of oxygenated molecular species of CL, we performed a model oxidation experiment using TLCL, the major molecular species of CL present in human peripheral blood lymphocytes (Fig. 4A, Table 1). When TLCL was incubated in the presence of cyt *c* and hydrogen peroxide for 30 min at 37°C we found that a decreased content of TLCL was accompanied by the accumulation of its oxygenated products with 1–3 oxygens with similar composition and stereo-specificity as those detected in rotenone-exposed lymphocytes (Fig. 7A, B). These results are compatible with involvement of cyt *c* in catalysis of TLCL oxidation in lymphocytes exposed to rotenone.

Rotenone induced accumulation of mono-lyso-CL in human lymphocytes—It is possible that the loss of CL in rotenone-challenged lymphocytes may be associated, at least in part, with activation of endogenous PLA₂. The presence of Ca²⁺-independent iPLA₂ in capable of utilizing (oxidatively modified) CL species in mitochondria has been reported [41–43]. Indeed, treatment of lymphocytes with rotenone resulted in increased content of mono-lyso-CL species (Fig. 8A). In line with this accumulation of oxygenated C_{18:2} containing from one to three oxygens in the fraction of free fatty acids was detected (Fig. 8B). After rotenone exposure, the endogenous contents of mono-lyso-CL and free fatty acids – likely released from phospholipids by endogenous PLA₂ - are shown in Figs. 8Ab and 8Bb. We found that the content of free C_{18:2} with two oxygens (m/z 311) was higher than that of mono-oxygenated C_{18:2}. This suggests that endogenous PLA₂ – likely mitochondrial iPLA₂ – hydrolyzed peroxidized TLCLs with two oxygens (m/z 311) more effectively than CLs with mono-oxygenated C_{18:2} (m/z 295). No significant accumulation of other lyso-phospholipids in lymphocytes in response to rotenone was detected (data not shown). This suggests that accumulated mono-lyso-CL molecular species originated from oxygenated TLCL formed in lymphocytes upon rotenone exposure.

Discussion

Although oxygenated fatty acids and phospholipids are critical signaling molecules (and/or biomarkers) in several neurological disorders [44], essential information on molecular targets, particularly specific polyunsaturated molecular species of phospholipids undergoing

oxidation and leading to mitochondrial dysfunction and their association with neurodegenerative disease such as PD, is lacking. While the general association of oxidative stress with PD has been emphasized in numerous studies (reviewed in [45–48], attempts to link the products of oxidative modification of different biomolecules to specific pathogenetic pathways of PD were not victorious [5, 49]. This may be due, at least in part, to insufficient information on the diversity and structure of oxidized biomolecules generated in mitochondria – the major metabolic candidate as a source of impaired and oxidatively modified PD-associated molecular species. Among those, CLs – unique and functionally essential phospholipids of mitochondria – may be of particular importance [50–52]. In this paper by using LC/MS-based oxidative lipidomics, we characterized all major molecular species of CL and its oxidized molecular species formed in rotenone-associated dysfunctional mitochondria in human peripheral blood lymphocytes.

Changes in the CL content as well as its composition have been shown to be responsible for mitochondrial dysfunction associated with several pathological conditions [53–56]. CL profile in mitochondria can be changed due to: i) loss of CL as a result of alteration in CL synthase activity [2, 31, 57], ii) altered fatty acid composition of CL as a result of disruption of CL remodeling process [58–60] and, iii) CL oxidation due to ROS generation [55]. Generation of ROS [8, 61] and activation of the intrinsic apoptotic cell death pathway [62] have been associated with rotenone-induced neuron degeneration *in vitro* and *in vivo* [53–55], dissipation of MMP, release cyt *c* from mitochondria into the cytosol and apoptosis [63]. Chronic and systemic inhibition of complex I leads to selective degeneration of dopaminergic neurons and produces neuro-pathological features of PD [4, 64].

Having in mind potential detection of mitochondrial phospholipid biomarkers of PD, we examined human peripheral blood lymphocytes that are often used to identify the mechanism leading to development of neurodegenerative diseases such as PD and Alzheimer disease [8, 65, 66]. In fact, increased apoptosis of lymphocytes in patients with PD has been documented [15, 16]. Both extrinsic and intrinsic apoptotic pathways were recognized in lymphocytes of PD patients [16, 67, 68]. Notably, rotenone induced apoptosis in lymphocytes has been linked to its ability to generate ROS ($O_2^{\cdot -}/H_2O_2$) leading to mitochondrial damage [10]. Further rotenone-induced inhibition of complex I activity and impairment of electron transport leading to massive production of ROS [69], and possibly protein and lipid peroxidation have been documented in dopaminergic cell line using BODYPI oxidation assays [70]. However, specific features of CL peroxidation and identification of CL oxidation products as essential factors in mitochondrial stages of lymphocyte apoptosis, have not been studied so far.

Here, we demonstrated that exposure of human lymphocytes to rotenone is associated with mitochondria dysfunction, ROS production, development of apoptotic cell death pathway and accumulation of oxygenated species of highly unsaturated CL containing four $C_{18:2}$ residues as well as its metabolite mono-lyso-CL. Detailed structural analysis of CL oxidation products revealed unusual features of rotenone-triggered peroxidation: i) predominant peroxidation of TLCL, CL containing four $C_{18:2}$ residues ii) preferential accumulation of oxygenated $C_{18:2}$ in *sn*-1 rather than *sn*-2 position; iii) quantitative

abundance of mono-oxygenated species vs species with two and three oxygen functionalities. Further studies will determine whether these specific features are uniquely associated with the rotenone-driven inhibition of respiratory complex I or may be common to other oxidative routes leading to the execution of apoptotic program in lymphocytes.

Our previous work has identified cyt *c*, an intermembrane space electron carrier, as a catalyst of the reaction during which it binds CL to yield a complex with CL-specific peroxidase activity [34]. The complex generates oxygenated CL species at the early stage of apoptosis *in vitro* and *in vivo* [17, 18, 20, 37, 71, 72]. Moreover, cyt *c*/CL complexes can interact with α -synuclein to form oligomers with high peroxidase activity [52] thus contributing to the formation of Lewis bodies – a morphological hallmark of PD. These results provided direct evidence for previously suggested conversion of cyt *c* into peroxidase and its possible role in neurodegenerative process [73], including pathogenesis of PD [74, 75]. Notably, while peroxidase activity cyclooxygenase-2 and peroxidation of non-esterified fatty acids have been linked to the pathogenesis of PD [74, 75] the oxidation of esterified lipids has not been yet investigated. Our data indicate that in rotenone treated lymphocytes, cyt *c* utilizes ROS, particularly hydrogen peroxide, formed during apoptotic cell death, to cause peroxidation of C_{18:2}-containing CL species in mitochondria (Fig. 9). In addition, rotenone-induced oxidative stress and ROS production can cause activation of Ca²⁺-independent iPLA₂ [76] resulting in the accumulation of CL hydrolysis products such as mono-lyso-CL and oxygenated fatty acids. iPLA₂ has been identified as the major endogenous type of PLA₂ capable of hydrolyzing peroxidized phospholipids in mitochondria [41, 77].

We found that the most predominant molecular species of CL that underwent oxidative modification after exposure of human lymphocytes to rotenone was tetralinoleoyl-CL with four symmetric C_{18:2}-residues in both *sn*-1 and *sn*-2 positions. In addition we were able to detect endogenously formed TLCLox, non-ox-mono-lyso-CL and C_{18:2}-ox. To determine which of C_{18:2} residues was a preferred substrate of rotenone-induced peroxidation, we treated isolated CL fraction with either PLA₁ or PLA₂ – to produce lyso-CLs and release C_{18:2} from the respective *sn*-1 or *sn*-2 positions. This resulted in liberation of oxygenated C_{18:2} species and di-lyso-CLs whereby higher contents of oxygenated C_{18:2} was produced by PLA₁ (as compared to PLA₂). Because 99% of total CLs were hydrolyzed and converted into di-lyso-CLs and FAs, we further analyzed oxygenated species of C_{18:2}. We found that only one out of four C_{18:2} in TLCL molecule (in either *sn*-1 or *sn*-2 positions) underwent oxidative modification upon rotenone exposure. Assuming that endogenous iPLA₂ cleaves predominantly C_{18:2}-ox in *sn*-2 but not non-ox-C_{18:2}, C_{18:2}-ox plus non-ox-mono-lyso-CL should be expected hydrolysis products – in line with our observations. Thus not only CL peroxidation products but also mono-lyso-CL in lymphocytes may be used as biomarkers of PD-associated metabolic disturbances – similar to recent finding in patients with Barth syndrome, a disease associated with mitochondrial dysfunction [78].

While this study has been focused on CLs as well as their oxidized and hydrolyzed metabolites, we have also analyzed several other major classes of phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin. We found that exposure of lymphocytes to rotenone resulted in

significant accumulation of several oxygenated molecular species of TLCL as well as its hydrolysis products – mono-lyso-CL and oxygenated free C_{18:2}. These rotenone-induced changes were CL-specific: neither oxygenated products nor hydrolysis products were detected in other examined classes of phospholipids. Thus no rotenone-induced remodeling of other phospholipids took place under experimental conditions used.

Apoptotic cell death pathway is activated in lymphocytes of PD patients [15, 16]. Therefore, peripheral blood lymphocytes are considered as potential candidate-biomarkers of mitochondrial dysfunction in PD. Given a recently established role of selective peroxidation of a mitochondria-specific phospholipid, CL, in execution of mitochondrial stages of apoptosis, one can assume that detailed analysis of CL peroxidation products may lead to the development of useful biomarkers. It has been reported that micromolar concentrations of rotenone (10–250 µM) induce apoptosis in isolated human lymphocytes [10]. However, specific role of CL peroxidation as an essential factor in mitochondrial stages of lymphocyte apoptosis, has not been yet studied. In the current work, we found that rotenone (at concentrations of 100 and 250 µM) was effective in stimulating selective accumulation of CL oxidation products and induction of apoptosis in isolated human lymphocytes. While the concentrations of rotenone used may seem to be relatively high, one should consider them in the context of its known toxicity and exposure doses. The estimates of rotenone toxicity for humans are commonly based on animal studies. In rat rotenone PD model with administration of pesticide through I.V. route (3–18 mg/kg/day) [79, 80], its concentration in circulation is within micromolar range. Of note, rotenone is usually sold as 1 – 5% formulation that corresponds to approximately its 30–150 mM solution. Thus agricultural workers can be occupationally exposed to relatively high doses of rotenone.

In conclusion, we demonstrated that exposure of human peripheral blood lymphocytes to a pesticide, rotenone, causes time- and dose-dependent selective oxidation of TLCL, accumulation of its hydrolysis products - mono-lyso-CLs - as well as generation of TLCL oxygenated molecular species associated with mitochondrial dysfunction likely through enzymatic *cyt c* catalyzed reactions triggered early in apoptosis. We believe that characterization of oxidatively modified CL molecular species as well as identification its hydrolysis products are important for better understanding of PD pathogenesis and may lead to the development of new biomarkers of mitochondrial dysfunction associated with PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CL	cardiolipin
<i>cyt c</i>	cytochrome <i>c</i>

DCFH-DA	2',7'-dichlorfluorescein-diacetate
DHE	dihydroethidium
DTPA	diethylenetriaminepentaacetic acid
MMP	mitochondrial membrane potential
PD	Parkinson's disease
PS	phosphatidylserine
PLA₂	phospholipase A ₂
PLA₁	phospholipase A ₁
ROS	reactive oxygen species
TLCL	tetra-linoleyl-cardiolipin
TMCL	tetra-myristoyl-cardiolipin

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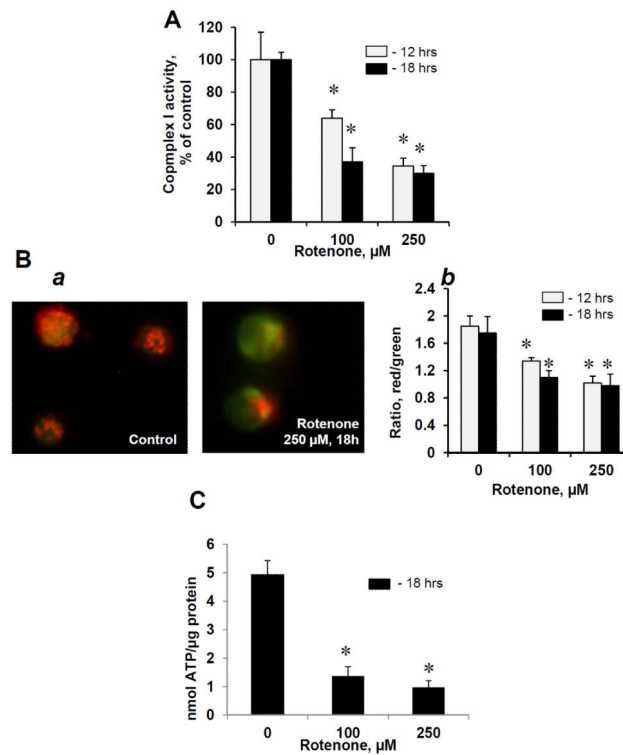


Figure 1. Effects of rotenone on mitochondrial functions in human peripheral blood lymphocytes Complex I activity (A), MMP (B) and content of ATP (C) in human lymphocytes exposed to rotenone (100, 250 μM , 12 and 18 hrs at 37°C). Typical fluorescent images of control and rotenone-treated lymphocytes (B,a) and assessment of MMP by using flow cytometry (B,b). Data are means \pm S.E., n=6, *p<0.05 vs control.

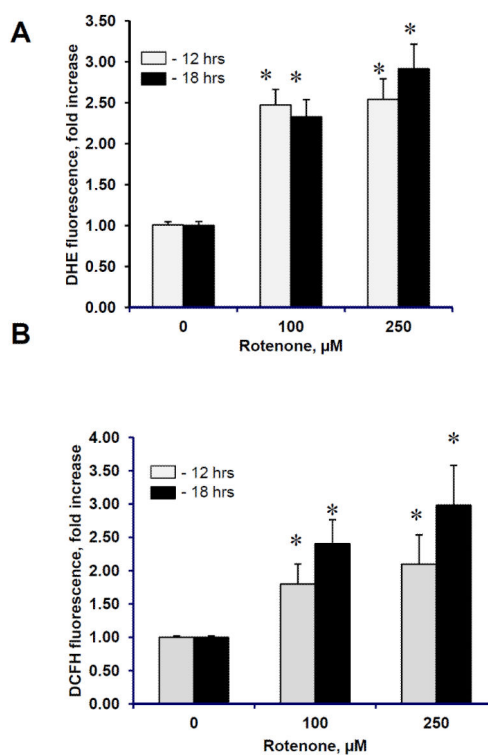


Figure 2. Rotenone induced generation of reactive oxygen species generated in human peripheral blood lymphocytes
Superoxide (A) and hydrogen peroxide (B) formation in lymphocytes exposed to rotenone (100 and 250 μM for 12 and 18 hrs at 37°C). Data are means \pm S.E., $n=6$, $*p<0.05$ vs control.

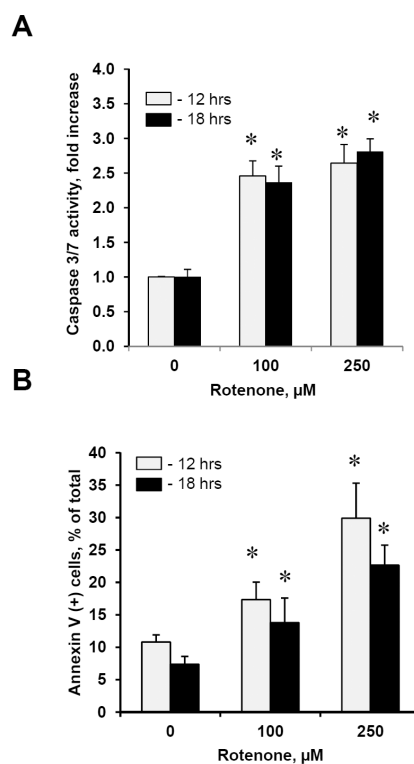


Figure 3. Apoptosis induced by rotenone in human peripheral blood lymphocytes
Caspase 3/7 activation (A) and PS externalization (B) in lymphocytes exposed to rotenone (100 and 250 μM for 12 and 18 hrs, at 37°C). Data are means ± S.E., n=6, *p<0.05 vs control.

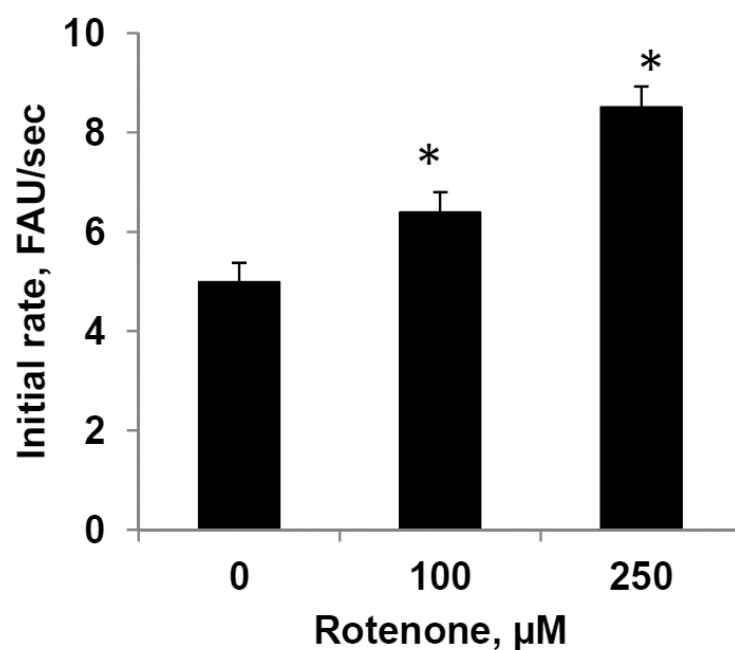


Figure 4. Peroxidase activity of mitochondria isolated from human peripheral blood lymphocytes

Mitochondria were isolated from lymphocytes exposed to rotenone (100 and 250 μM for 18 hrs, at 37°C) treated with alamethicin and peroxidase activity was detected. Data are means \pm S.E., $n=3$, * $p<0.05$ vs control.

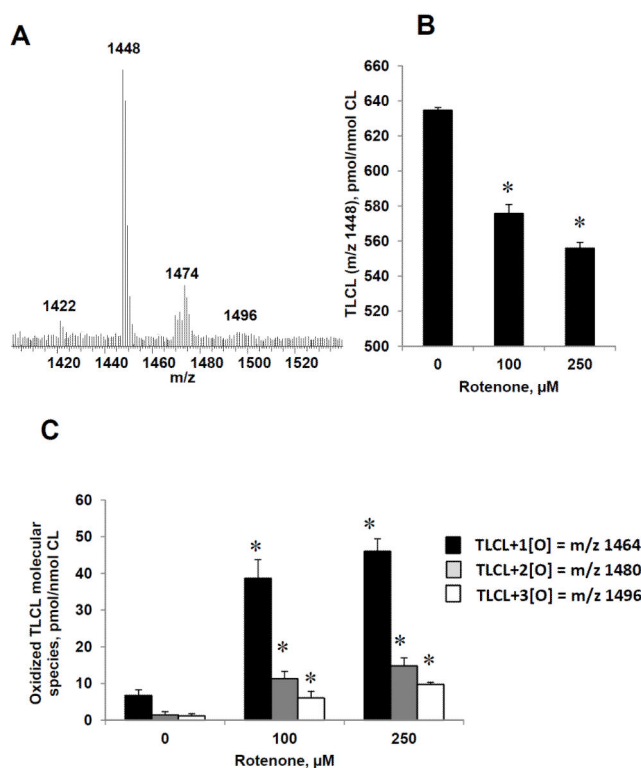


Figure 5. Rotenone induced oxidation of CL in human peripheral blood lymphocytes

Typical negative mode ESI-MS spectrum of CL obtained from human lymphocytes (A), LC/MS quantitative assessment of TLCL (B) and oxidized (C) TLCL molecular species. Note: the decrease of TLCL and accumulation of its oxygenated species were dependent on rotenone concentration. Oxygenated molecular species of TLCL with m/z 1464 (plus 1 oxygen), 1480 (plus two oxygens) and 1496 (plus three oxygens) were detected in rotenone treated lymphocytes (18 hrs). Data are means \pm S.E., n=5, *p<0.03 vs control.

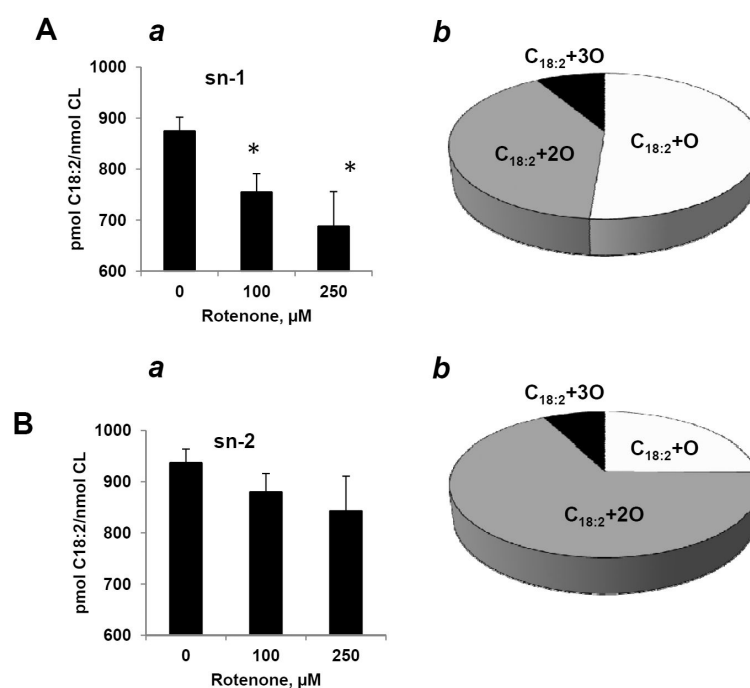


Figure 6. Identification of esterified oxygenated fatty acids in human peripheral blood lymphocytes exposed to rotenone

Quantitative LC/MS assessment of oxygenated C_{18:2} localized and *sn*-1 (A) and *sn*-2 (B) positions of CL from rotenone-exposed lymphocytes (100 and 250 μM , for 18 hrs at 37°C. (a) Decrease of oxidizable C_{18:2} and (b) formation of oxygenated C_{18:2} hydrolyzed from *sn*-1 and *sn*-2 positions of CL, respectively. Data are means \pm S.E., n=5, *p<0.03 vs control.

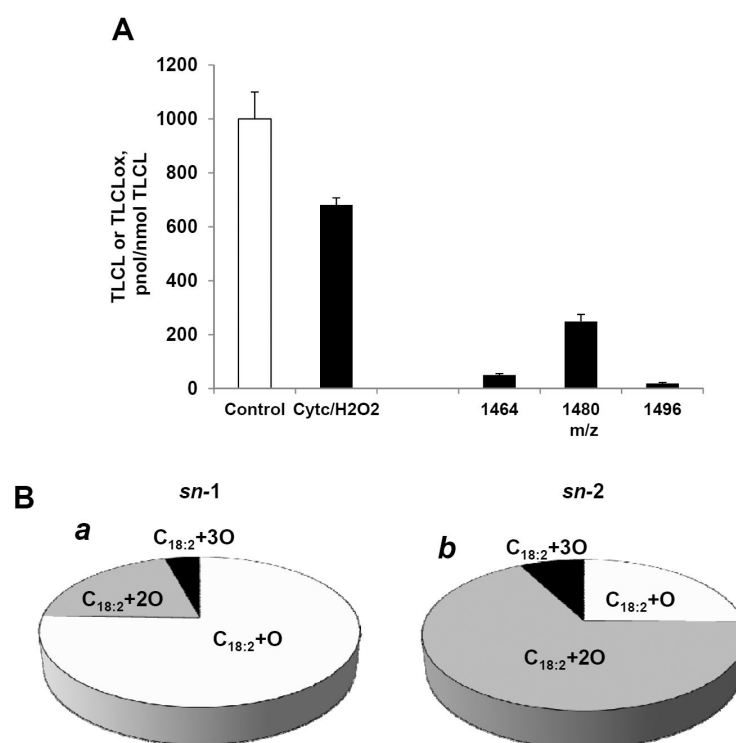


Figure 7. Cyt c induced oxidation of TLCL in the presence of H₂O₂

Quantitative LC/MS assessment of TLCL and its oxygenated species (A) formed in cyt *c* driven reaction. Oxygenated products of C_{18:2} (B) formed in *sn*-1 (a) and *sn*-2 (b) positions of CL upon treatment of TLCL with cyt *c*/H₂O₂. After incubation with cyt *c*/H₂O₂, TLCL was treated either with PLA₁ or PLA₂. Liberated oxygenated and non-oxygenated C_{18:2} were analyzed by reverse phase LC/MS. Data are means ± S.E., n=5, *p<0.05 vs control.

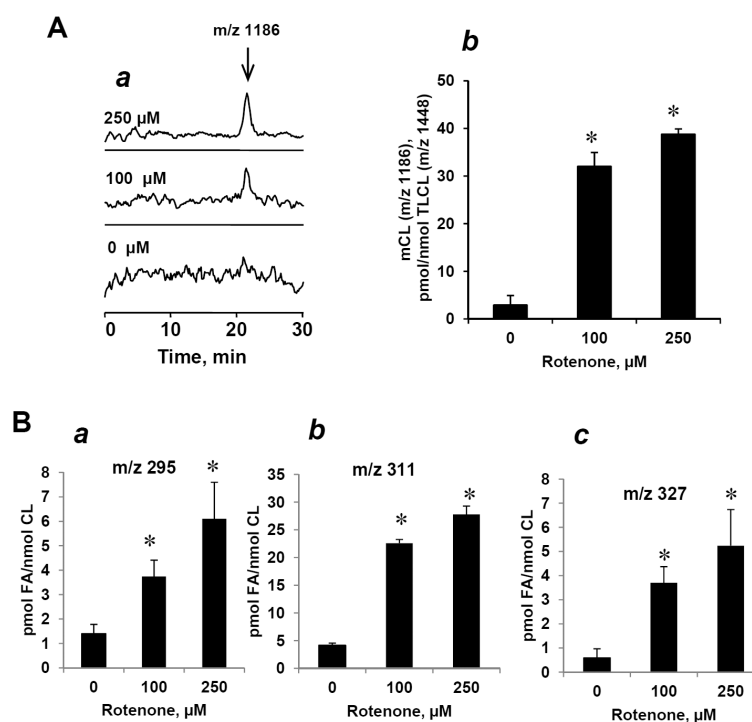


Figure 8. Rotenone induced accumulation of mono-lyso-CL and oxygenated free fatty acids in human peripheral blood lymphocytes

(A) LC/MS base profile (a) and quantitative assessment (b) of mono-lyso-CL. (B) Content of free C_{18:2} containing one (a) two (b) and three (c) oxygens in lymphocytes treated with rotenone (100 and 250 μ M for 18 hrs at 37°C. Note: the increase of mono-lyso-CL was accompanied by accumulation of oxygenated free C_{18:2}. Data are means \pm S.E., n=5, *p<0.05 vs control.

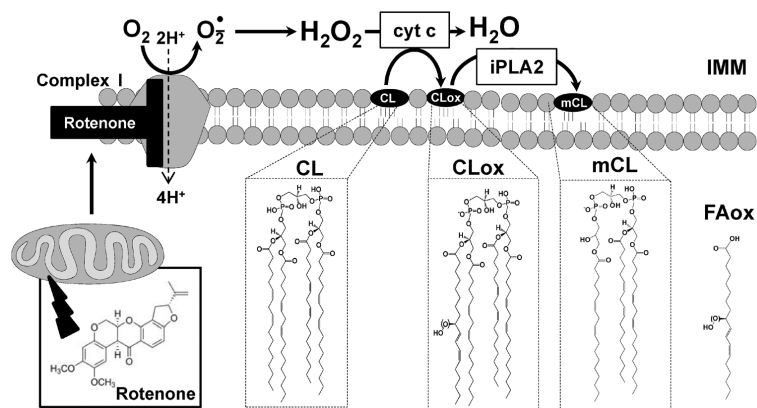


Figure 9. Proposed mechanism of rotenone-induced CL oxidation and its hydrolysis in human lymphocytes mitochondria

IMM-inner mitochondrial membrane; cyt *c*, cytochrome *c*; iPLA₂- Ca²⁺-independent phospholipase A₂; CL, cardiolipin; CLOx, oxygenated cardiolipin; mCL, monolyso-cardiolipin; FAox, oxygenated C_{18:2}.

Table 1

Identification and quantitative assessment of major CL molecular species in human peripheral blood lymphocytes by LC/MS

m/z	Molecular species	pmol/nmol CL
1421.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{16:1}	30.5 ± 7.4
1447.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{18:2}	632.0 ± 24.0
1449.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{18:1}	147.1 ± 6.8
1469.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{20:5}	46.8 ± 3.9
1471.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{20:4}	49.0 ± 7.6
1473.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{20:3}	96.2 ± 11.0
1475.9	C _{18:2} /C _{18:2} /C _{18:2} /C _{20:2}	54.3 ± 5.5

Data are means ± SD, n=5.