Mitochondria targeting of non-peroxidizable triphenylphosphonium conjugated oleic acid protects mouse embryonic cells against apoptosis: Role of cardiolipin remodeling.

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ABSTRACT

The early stage of intrinsic apoptosis is characterized by the formation of cardiolipin (CL) /cytochrome c complexes in mitochondria that exhibit a potent peroxidase activity towards polyunsaturated CL. Accumulation of CL oxidation products in mitochondria of apoptotic cells has been found essential for the release of pro-apoptotic factors into the cytosol. We suggested that integration of mono-unsaturated octadecaenoic acid (C18:1) into CL – via its remodeling pathways in mitochondria - will generate non-oxidizable CL species hence protect cells against apoptosis. We synthesized a non-peroxidizable triphenylphosphonium (TPP) C18:1 ester (TPP-C18:1) and used it for targeted delivery into mitochondria of mouse embryonic cells (MEC). Using oxidative lipidomics analysis we established that pro-apoptotic stimulation with actinomycin D (AcD) was accompanied by selective oxidative consumption of CL molecular species containing polyunsaturated octadecadienoic, eicosatetraenoic, eicosatrienoic, docosahexaenoic and docosapentaenoic acids. Pretreatment of MEC with TPP-C18:1 resulted in: i) significant decrease of CL polyunsaturated molecular species and simultaneous elevation of non-oxidizable CL molecular species containing C18:1 and ii) suppression of AcD induced apoptosis. An inhibitor of long chain acyl-CoA synthase, triacsin C, blocked integration of C18:1 into CL molecules and restored MEC's sensitivity to AcD-induced apoptosis. Thus, metabolic remodeling of CL can be a new strategy in regulation of apoptotic cell death pathway and lead to the development of new preventive and therapeutic approaches against pathological conditions where apoptosis is a major contributor, eg, acute radiation syndrome. Supported by NIH U19 AI068021, HL70755, HL094488, ES020693, NIOSH OH008282.

METHODS

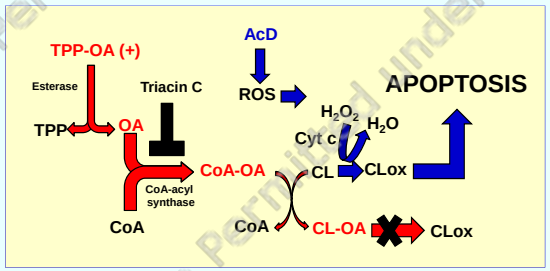
Synthesis of TPP-$C_{18:1}$. 3-[(Z)-octadec-9-enoyl]oxypropyl-triphenyl-phosphonium chloride: A suspension of $C_{18:1}$ (1 mmol) and silver nitrate (2 mmol) was stirred at 25 °C for 2 hrs. (3-bromopropyl)triphenylphosphonium bromide (1 mmol) was added and the reaction mixture was further stirred at 25 °C for 12 hrs. Thereafter, the mixture was filtered and the filtrate evaporated to dryness under reduced pressure. The remaining residue was re-dissolved in 50% methanol containing 1% $NaHCO_3$ and 1% NaCl. The TPP ester was extracted with ethyl acetate and the extract dried over Na_2SO_4. Evaporation of the organic solvent afforded 0.55 mmol of 3-[(Z)-octadec-9-enoyl]oxypropyl-triphenyl-phosphonium chloride (ESI-MS analysis revealed a single peak with m/z = 585.4).

Cell culture. Mouse embryonic cells were grown in Dulbecco's Modified Eagle Medium containing 15% fetal bovine serum, 25 mM HEPES, 0.05 mg/ml uridine, 0.05mM 2-mercaptoethanol, 1x MEM (Invitrogen, Carlsbad, CA) and 100 U/ml penicillin/streptomycin in a humidified atmosphere (5% CO_2 plus 95% air). Cells were pretreated with TPP-$C_{18:1}$ (1-50μM) at 37°C for 2 hrs and after that exposed to AcD (100 ng/ml) at 37°C for 16 hrs. To block ASCL cells were treated with triacsin C (10 μM) at 37°C for 30 min. Cell viability was measured using AlamarBlue assay (Invitrogen, Carlsbad, CA). 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).

Analysis of CL. Lipids were extracted using the Folch procedure. Lipid phosphorus was determined by a micro-method. LC/MS was performed using a Dionex Ultimate™ 3000 HPLC coupled on-line to a linear ion trap mass spectrometer (LXQ Thermo-Fisher). CL was separated by 2D-HPTLC and fatty acids were analyzed by LC/MS after hydrolysis of CL with porcine pancreatic phospholipase A_2 (PLA_2) as described.

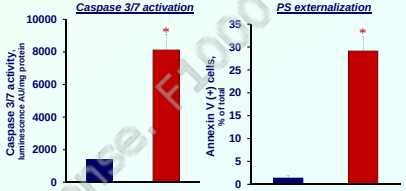
Analysis of TPP-$C_{18:1}$. Mitochondria were isolated from MEC treated with TPP-$C_{18:1}$ (50 μM, for 2h at 37°C). TPP-$C_{18:1}$ was extracted from mitochondria by Folch procedure and LC/MS in positive mode was performed using a Dionex Ultimate™ 3000 HPLC coupled on-line to a linear ion trap mass spectrometer (LXQ Thermo-Fisher). TPP-$C_{18:1}$ and TPP were separated on a normal phase column (Luna 3 μm Silica 100A, 150x2 mm, (Phenomenex, Torrance CA)) with flow rate 0.2 mL/min using gradient solvents containing 5 mM CH_3COONH_4 (A – n-hexane:2-propanol:water, 43:57:1 (v/v/v) and B - n-hexane:2-propanol:water, 43:57:10 (v/v/v). At these conditions the retention times for TPP-$C_{18:1}$ and TPP were 27.9 and 50.6 min, respectively.

Statistics. The results are presented as mean ± S.E.M. 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.



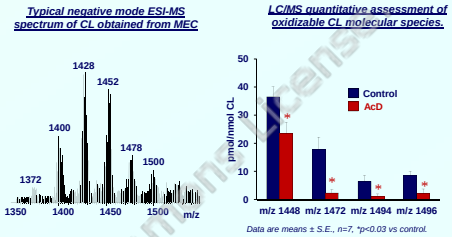
RESULTS

Figure 1. Apoptosis induced by AcD (100 ng/mL, for 16 hrs at 37°C) in mouse embryonic cells.



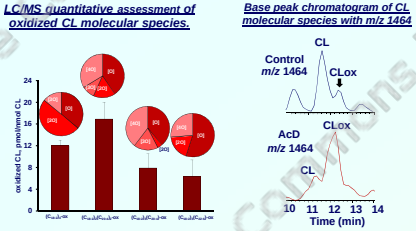
Data are means ± S.E., n=10, *p<0.05 vs control.

Figure 2. AcD-induced oxidation of cardiolipin in mouse embryonic cells.



Data are means ± S.E., n=7, *p<0.03 vs control.

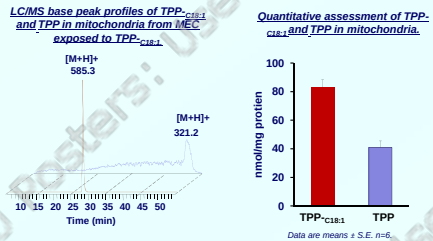
Figure 3. AcD-induced oxidation of cardiolipin in mouse embryonic cells.



Data are means ± S.E., n=7.

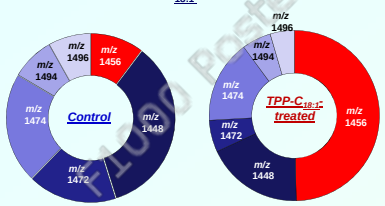
CL oxidation products with 1-4 oxygens in each oxidized CL molecular species were detected and shown on inserts. A higher intensity of the peak with m/z 1464 corresponding to oxygenated CL molecular species ($(_{18:2})_3(_{18:2}$-OH), retention time 12.2 min) was detected in AcD treated cells.

Figure 4. LC/MS analysis of TPP-$C_{18:1}$ and its hydrolysis product, TPP, in mitochondria of mouse embrionic cells.



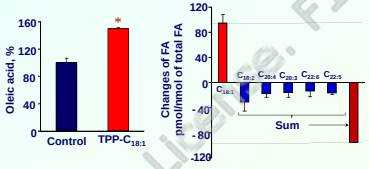
Data are means ± S.E. n=6

Figure 5. Content of cardiolipin molecular species in control and TPP-$C_{18:1}$ treated cells.



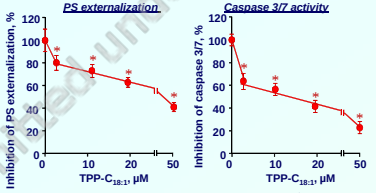
The amounts of CL molecular species with m/z 1448, m/z 1472, m/z 1494 and m/z 1496 in TPP-$C_{18:1}$ treated cells were dropped to 21.8 ± 3.3, 7.2 ± 2.0, 6.5 ± 2.3 and 5.4 ± 1.8 pmol/nmol of total CL from 36.5 ± 3.6, 17.9 ± 4.3, 8.7 ± 1.6 and 8.6 ± 1.4 pmol/nmol of total CL in control cells, respectively. Consequently, the amount of CL molecular species with m/z 1456 was increased from 10.8 ± 2.7 to 58.6 ± 7.2 pmol/nmol of total CL. Data are means ± S.E., n= 3-10.

Figure 6. Content of $C_{18:1}$ and changes in cardiolipin fatty acid composition in mouse embryonic cells treated with TPP-$C_{18:1}$



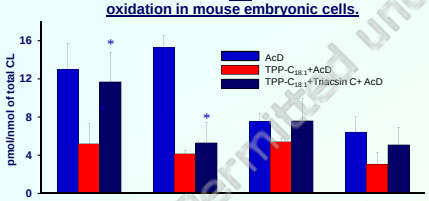
Data are means ± S.E., n=8, *p<0.05 vs control.

Figure 7. Effect of TPP-$C_{18:1}$ on AcD-induced apoptosis in mouse embryonic cells.



Data are means ± S.E., n=8, *p<0.05 vs control.

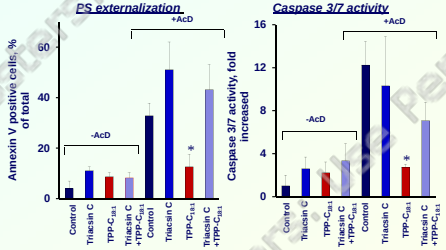
Figure 8. Effect of AcD, TPP-$C_{18:1}$ and triacsin C on cardiolipin oxidation in mouse embryonic cells.



Data are presented as increase of oxidized CL molecular species.

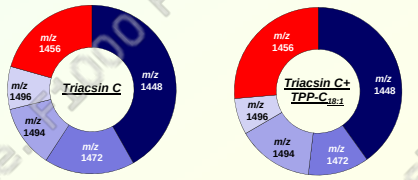
Data are means ± S.E., n=3-7, *p<0.05 vs control.

Figure 9. Effect of triacsin C on apoptosis induced by AcD in mouse embryonic cells treated with TPP-$C_{18:1}$



Data are means ± S.E., n=3-10, *p<0.05 vs control.

Figure 10. Content of cardiolipin molecular species in triacsin C treated and triacsin C treated TPP-$C_{18:1}$ exposed cells.



The amounts of CL molecular species with m/z 1448, m/z 1472, m/z 1494 and m/z 1496 were estimated as 33.3 ± 1.9, 13.8 ± 2.0, 9.6 ± 1.8 and 6.5 ± 1.6 pmol/nmol of total CL in triacsin C pre-treated and 37.6 ± 3.9, 11.0 ± 3.1, 6.6 ± 4.3 and 3.9 ± 3.1 pmol/nmol of total CL in triacsin pre-treated/TPP-$C_{18:1}$ exposed cells, respectively. Consequently, the amount of CL molecular species with m/z 1456 was 16.4 ± 3.4 to 24.7 ± 4.2 pmol/nmol of total CL. Data are means ± S.E., n=3-5.

CONCLUSIONS

- Pro-apoptotic stimulation with actinomycin D (AcD) was accompanied by selective oxidative consumption of CL molecular species containing polyunsaturated octadecadienoic ($C_{18:2}$), eicosatetraenoic ($C_{20:4}$), and docosahexaenoic ($C_{22:6}$) acids.
- Pretreatment of MEC with TPP-$C_{18:1}$ resulted in: i) significant decrease of CL polyunsaturated molecular species and simultaneous elevation of poorly-oxidizable CL molecular species containing $C_{18:1}$ and ii) suppression of AcD induced apoptosis. An inhibitor of long chain acyl-CoA synthase (ACSL), triacsin C, blocked integration of $C_{18:1}$ into CL molecules and restored cell's sensitivity to AcD-induced apoptosis.