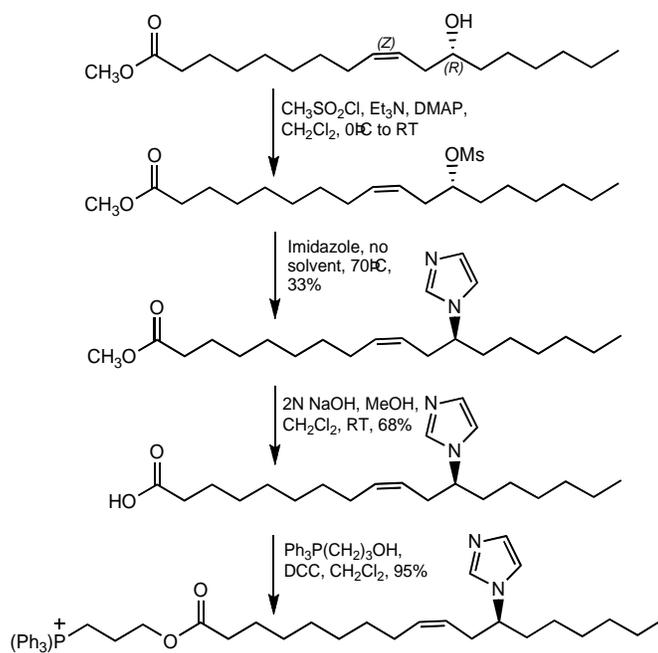
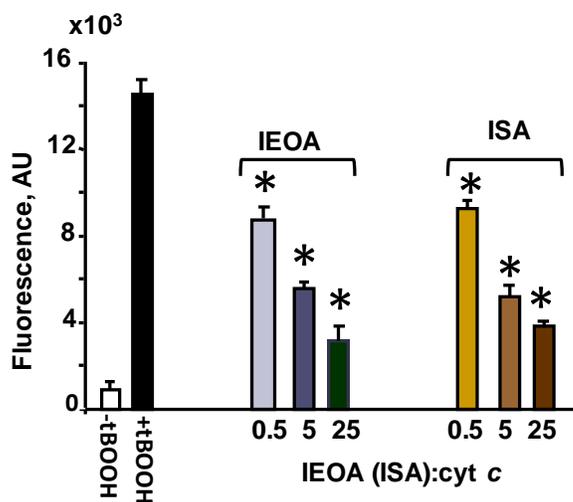


SUPPLEMENTARY INFORMATION

Supplementary Figure S1. A general synthetic method for synthesis of conjugates starting from ricinoleic acid.

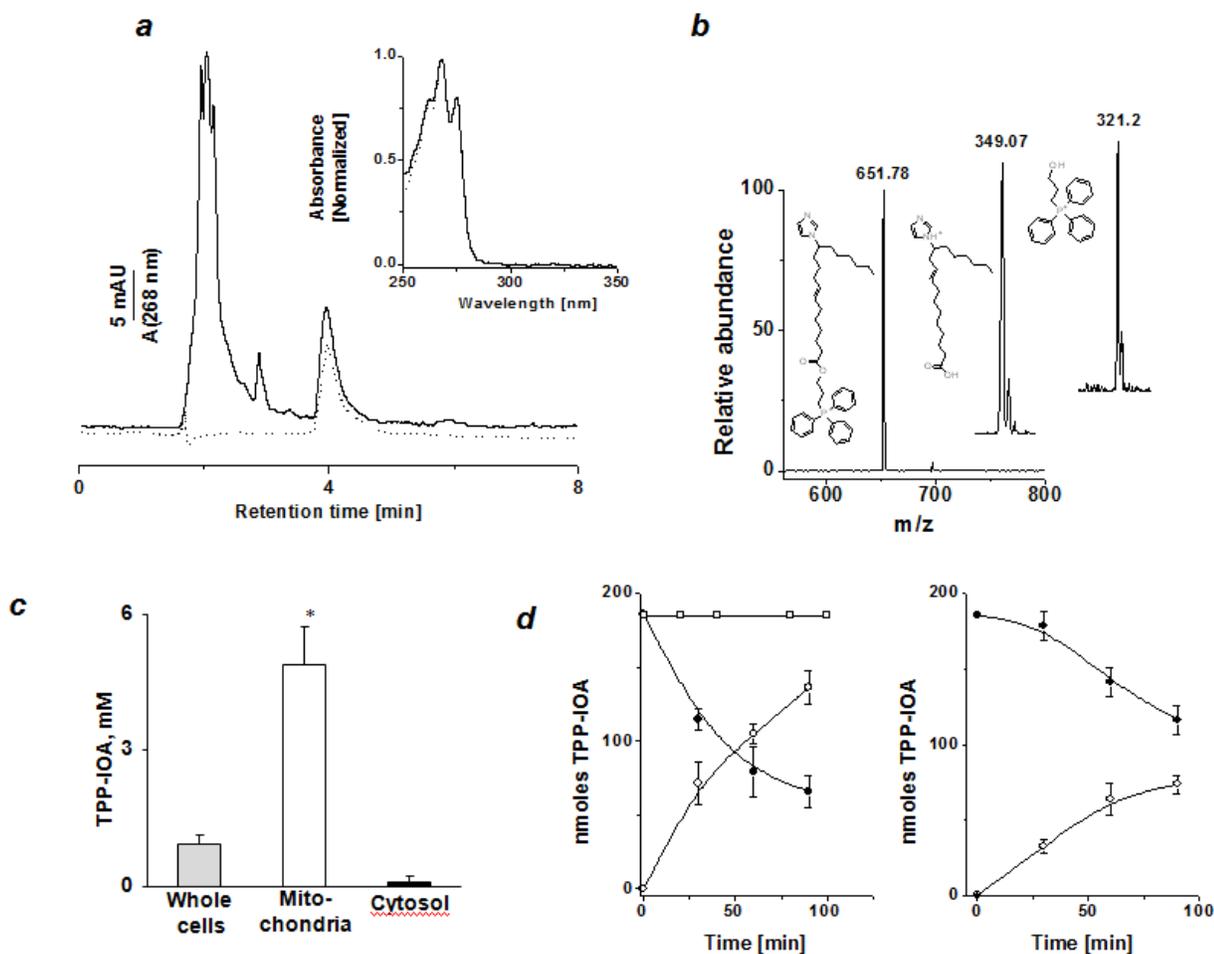


Supplementary Figure S2. Assessments of peroxidase activity of mouse liver mitochondria by oxidation of Amplex Red to resorufin. Data are means \pm S.D., n=3, *p<0.01 vs control (no ISA, IEOA added).



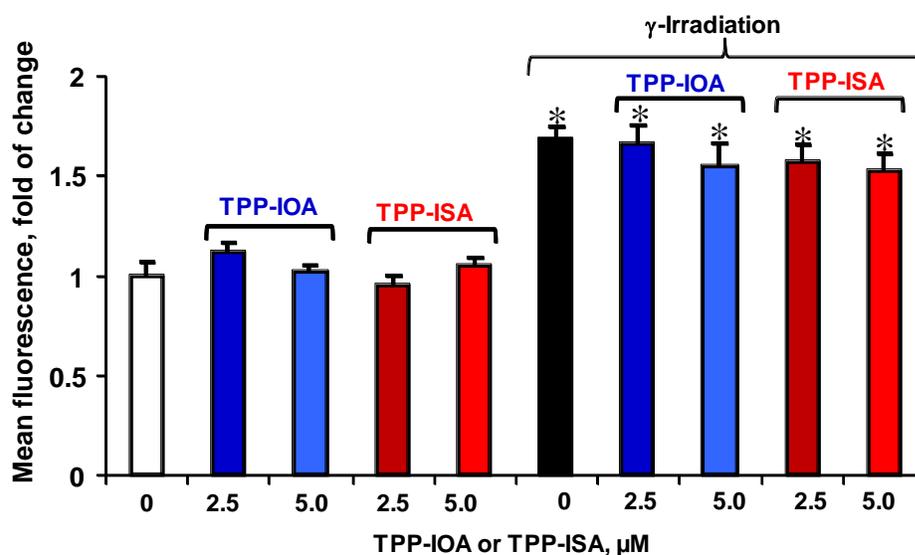
Supplementary Figure S3. HPLC and ESI-MS analysis of hydrolysis of TPP-IOA and its hydrolysis products in mitochondria and cytosol of mouse embryonic cells.

(a) – HPLC-UV profile (solid lines) of mitochondrial extract from cells exposed to TPP-IOA (10 μ M; incubation time, 30 min); dashed lines, standard solution of TPP-IOA (50 μ M). Inset: overlapped UV spectra of the peaks reflecting the elution of a standard solution of TPP-IOA (R_t = 4 min; dashed lines) and mitochondrial extract from mouse embryonic cells (solid lines). (b) – ESI-MS analysis of products formed during the hydrolysis of TPP-IOA in mitochondria. (c) - Compartmentalization of TPP-IOA in mouse embryonic cells. The content of TPP-IOA was assessed by HPLC as described in supplementary methods. Data are means \pm S.E., $n = 3$, * $p < 0.03$ vs whole cells or cytosol. (d) - Time course of TPP-IOA hydrolysis (closed circles) and accumulation of $(\text{Ph})_3\text{P}^+\text{C}_3\text{H}_7\text{OH}$ (open circles) by cytosol (left panel) (1 mg protein/ml) and mitochondrial homogenates (right panel) (1 mg protein/ml). Incubations were carried out in 0.1 M phosphate buffer (pH 7.0) for 25 min at 37 $^\circ\text{C}$; and reaction was stopped with CH_3CN denatured proteins were centrifuged, and the supernatant was subjected to HPLC analysis. The volume of samples was 1 ml.



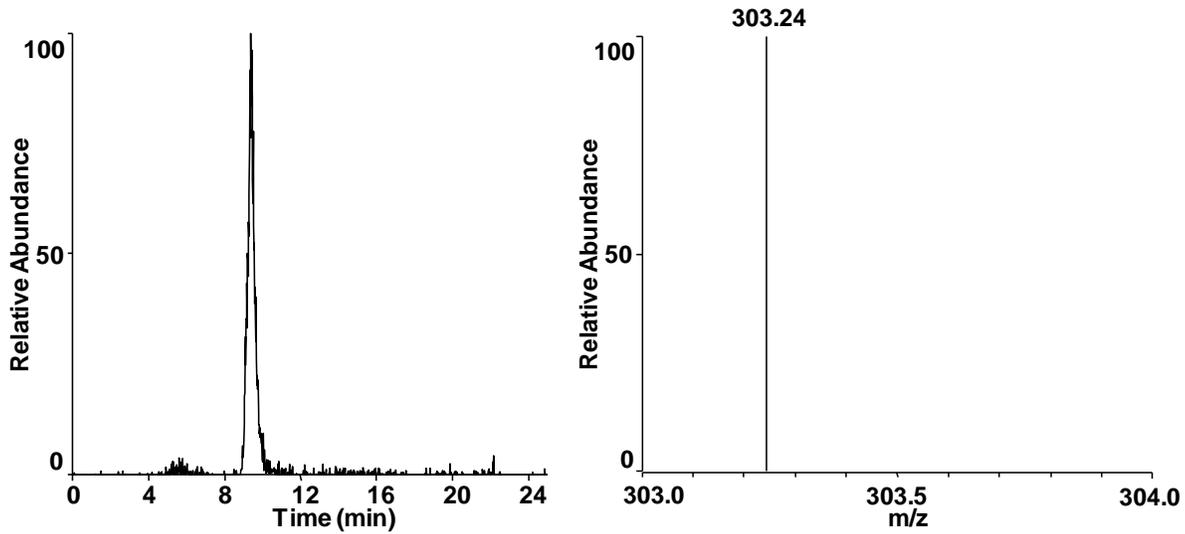
Supplementary Figure S4. Assessments of superoxide generation in mouse embryonic cells after treatment with TPP-IOA and TPP-ISA.

Cells were γ -irradiated at a dose of 10 Gy and then incubated with different concentrations of TPP-IOA or TPP-ISA (2.5 and 5 μ M) for 18 hrs. To estimate superoxide generation, cells were incubated with 5 μ M of DHE for 30 min at 37°C. Collected cells were re-suspended in PBS and fluorescence of 2-hydroxyethidium was analyzed using FACScan supplied with CellQuest software. Data are means \pm S.D., n = 3. *p<0.01 vs control cells (without any treatment).



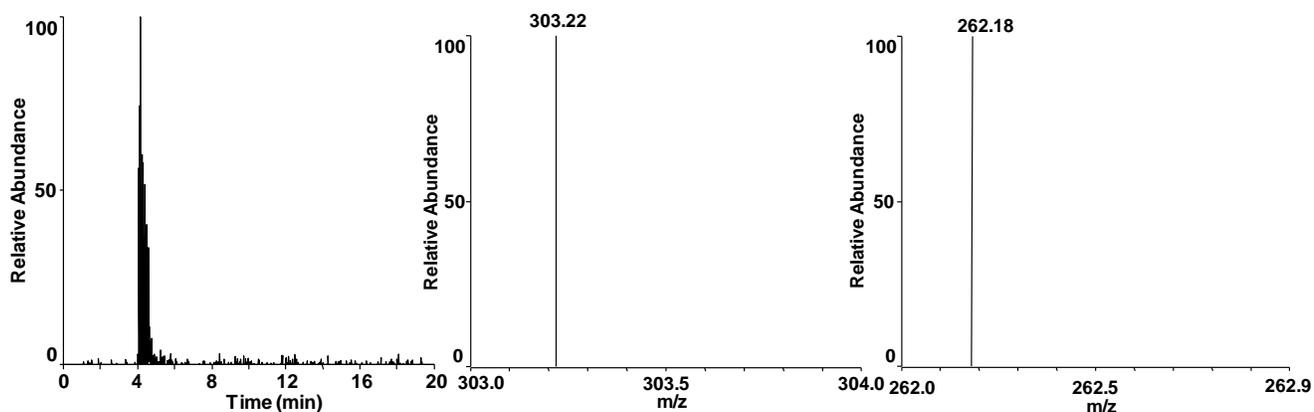
Supplementary Figure S5. Selective reaction monitoring (SRM) analysis of TPP-IOA.

TPP-IOA was quantitated by SRM analysis. A plasma sample (10 min after i.p. injection of TPP-IOA, 5 mg/kg body weight) containing TPP-IOA was chromatographed on a C18 column and exhibited a retention time of 9.4 min (left panel). The transition m/z 651→303 was measured (right panel) corresponding to TPP-IOA to TPP-propyl minus water.



Supplementary Figure S6. Multiple reaction monitoring (MRM) analysis of products formed during TPP-IOA hydrolysis.

TPP-IOA hydrolysis was measured by the presence of the TPP-propyl group by MRM analysis. A small intestine sample (10 min time point) containing TPP-IOA hydrolysis products were chromatographed on a C18 column. The TPP-propyl group exhibited a retention time of 4.17 min (left panel). The transitions m/z 321-->303 corresponding to TPP-propyl to TPP-propyl minus water (middle panel) and m/z 303-->262 corresponding to TPP-propyl minus water to TPP, were measured (right panel).



Supplementary Methods

Materials. Unless indicated, all reagents used were purchased from Sigma (St. Louis, MO).

Synthesis of imidazole-substituted fatty acids. The synthesis of the imidazole fatty acids relied on the ready availability of the naturally occurring fatty acid (9Z,12R)-(+)-ricinoleic acid (Supplementary Fig. S1). The 12-OH group offered a site for heterocycle substitution on a long chain fatty acid without having to prepare new starting materials. Briefly, either methyl ricinoleic acid or methyl 12-hydroxystearate (Pfaltz & Bauer) was mesylated on the 12-hydroxy group ($\text{CH}_3\text{SO}_2\text{Cl}$, Et_3N , 4-dimethylaminopyridine (DMAP); CH_2Cl_2 , 0°C to RT). The crude mesylates were used without purification as they tended to decompose when chromatographed on silica. Substitution of the mesylates by imidazole could be effected in tetrahydrofuran (THF) solution using potassium *tert*-butoxide and 18-crown-6, but almost identical yields could be obtained by simply heating the mesylates with 2-3 equivalents of imidazole at $\sim 80\text{-}90^\circ\text{C}$ in the absence of solvent. Hydrolysis of the methyl esters with NaOH in a mixture of MeOH/ CH_2Cl_2 afforded the free carboxylic acids that could then be esterified with 3-hydroxypropyltriphenylphosphonium bromide (DCC, CH_2Cl_2). Overall yields were about 20% from the starting fatty acid methyl esters. A general synthetic method is shown for synthesis of conjugates starting from ricinoleic acid.

3-Hydroxypropyltriphenylphosphine (192.2 mg, 0.540 mmol), N,N'-dicyclohexylcarbodiimide 231.2 mg (1.12 mmol) and 12-imidazole stearic acid (12-(1*H*-imidazol-1-yl)octadecanoic acid) (171.7 mg, 0.489 mmol) were dissolved in 25 mL dichloromethane. The mixture was allowed to stir at room temperature over night. The mixture was filtered using Celite to remove precipitated urea evaporated to give a crude product that was purified via column chromatography on silica (dichloromethane:methanol, 10:1) to yield 253.6 mg (0.368 mmol, 75.3%) of a slightly yellow, thick oil. $R_f = 0.34$ (dichloromethane : methanol 10:1). ^1H NMR (300 MHz, CDCl_3) δ 7.9-7.6 (m, 15H), 7.45 (s, 1H), 7.04 (s, 1H), 6.87 (s, 1H), 4.35 (t, 2H), 4.01 (m, 2H), 3.86 (m, 1H), 2.21 (t, 2H), 1.99 (m, 2H), 1.69 (m, 4H), 1.50 (t, 2H), 1.25-1.05 (m, 22H), 0.82 (t, 3H); ^{13}C NMR (CDCl_3) δ 173.1, 134.9, 134.9, 133.4, 133.3, 130.4, 130.2, 118.2, 117.0, 116.2, 62.9, 62.6, 58.4, 36.0, 33.8, 31.2, 29.0, 28.8, 28.7, 28.5, 25.7, 25.6, 24.5, 22.2, 21.9, 19.8, 19.1, 13.7; MS (FAB) m/z 653 (M^+ , 100%), 375 (9%), 319 (12%), 275 (12%), 262 (14%), 183 (12%), 69 (55%).

3-Hydroxypropyltriphenylphosphine (876.5 mg, 2.46 mmol), N,N'-dicyclohexylcarbodiimide (485.6 mg, 2.35 mmol) and 12-imidazol-1-yl-(*Z*)-9-octadecaenoate (816.7 mg, 2.34 mmol) were dissolved in 50 mL dichloromethane. The mixture was allowed to stir at room temperature over night. The mixture was filtered using Celite to remove precipitated urea and evaporated to provide a crude product that was purified via column chromatography (dichloromethane : methanol 10:1) to yield 1.54 g (2.23 mmol, 95.3%) of a light yellow, thick oil. $R_f = 0.58$ (dichloromethane : methanol 10:1). ^1H NMR (300 MHz, CDCl_3) δ 7.90-7.65 (m, 15H), 7.54 (s, 1H), 7.03 (s, 1H), 6.93 (s, 1H), 5.37 (m, 1H), 5.14 (m, 1H), 4.34 (t, 2H), 3.98 (m, 3H), 2.43 (m, 2H), 2.22 (t, 2H), 1.98 (m, 2H), 1.85-1.72 (m, 4H), 1.51 (m, 2H), 1.25-1.15 (m, 18H), 0.81 (t, 3H); ^{13}C NMR (CDCl_3) δ 172.9, 134.82, 134.78, 133.2, 133.1, 133.0, 130.2, 130.0, 128.1, 123.4, 117.9, 116.8, 116.4, 62.7, 62.5, 58.4, 49.4, 34.9, 33.7, 33.6, 31.1, 28.8, 28.6, 28.5, 28.3, 26.7, 25.5, 24.3, 22.0, 21.8, 19.6, 18.9, 13.5 ; MS (FAB) m/z 651 (M^+ , 100%), 375 (11%), 319 (15%), 303 (12%), 289 (11%).

Exposure of mouse embryonic cells to γ -Irradiation: Mouse embryonic cells were seeded on 35 mm dish at a cell density of 5×10^4 /dish, and allowed to attach overnight. The cells were γ -irradiated at a dose of 10 Gy using a Shepherd model 143-45A irradiator (J.L. Shepherd & Associates, CA). Ten min after irradiation, different concentrations of TPP-ISA or TPP-IOA (2.5 and 5 μM) were added and cells were incubated at 37°C in 5% CO_2 for 48 h. Then, cells were harvested for measuring phosphatidylserine externalization by Annexin V/PI kit and caspase 3/7 activity using Caspase-Glo[®] 3/7 Assay kit. Data are means \pm S.D., $n = 3$. * $p < 0.01$ vs irradiated only cells.

Exposure of mouse lung endothelial cells to rotenone. Mouse lung endothelial cells were maintained in 2% O₂, 5% CO₂, 93% nitrogen in Opti-MEM containing 10% FBS, 2 mM glutamine, 0.2% retinal derived growth factor, 10 U/mL heparin, 0.1 mM non-essential amino acid supplement and 55 μM β-mercaptoethanol. Cells at passage 4 to passage 6 were seeded on collagen/gelatin coated plates and incubated with 2 μM rotenone in growth factor depleted medium together with TPP-IOA (2.5, 5 μM) for 48 h, then cells were collected by trypsinization

Clonogenic assay. Cells were plated in 35-mm dishes with 2 ml culture medium at the appropriate density (between 100 and 1000 cells per dish). TPP-ISA (2.5 μM) or TPP-IOA (2.5 μM) was added to cell culture 30 min post γ-irradiation. Medium containing TPP-ISA or TPP-IOA was replaced with fresh complete culture medium after 4 hrs. Colonies were fixed and stained with 0.25% crystal violet and 10% formalin (35% v/v) in 80% methanol for 30 min after a 7-day incubation, and those with >50 cells were scored as survivors. The surviving fraction was calculated as the plating efficiency (= number of colonies counted/number of cells seeded x100%) of samples relative to that of control. The data were fitted to a single-hit multi-target model. D₀ - the dose needed to reduce cell surviving fraction to 37% (1/e) – was estimated from the curves fitted to evaluate the radiomitigative effects of TPP-ISA and TPP-IOA.

Isolation of mitochondria. Mitochondrial fractions were isolated by differential centrifugation. Briefly, cells were suspended in mitochondria isolation buffer (pH 7.4; MIB) containing mannitol (210 mM), sucrose (70 mM), HEPES (10 mM), EDTA (1 mM) and protease inhibitor cocktail. Cells were lysed by Dounce homogenization. Unbroken cells, nuclei and debris were removed by 5 min centrifugation at 600×g at 4 °C. Mitochondria, obtained by centrifugation at 10,000×g (10 min), were washed once with MIB.

Cell-free apoptosis system (S100 system). The cytosol extracts (S100) of mouse embryonic cells were obtained as described previously⁴⁵ (Liu et al., 1996) with minor modifications. Briefly, the cells were washed twice in cold phosphate-buffered saline, pH7.4, and the resulting pellet was resuspended in buffer containing 25 mM HEPES-KOH, pH 7.0, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% digitonin, and 1% protease inhibitor mixture for 2 min at 4 °C. Cells were centrifuged at 4 °C for 10 min at 10,000×g. The resulting supernatant was further centrifuged at 4 °C for 50 min at 100,000×g. The supernatant was collected as S-100 and kept at -80 °C until further use. For caspase-3 activation, S100 (5 μg of protein/μl) was incubated with 1 mM dATP and 1 μM cyt c for 90 min at 37 °C. The caspase-3 activity was evaluated using Enzchek caspase-3 assay kit as described in the manufacturer's manual (Invitrogen, Carlsbad, CA).

ATP measurements. ATP levels in cells were measured using ATP bioluminescent somatic cell assay kit (St. Louis, MO) according to the manufacturer's instruction.

Production of superoxide anion radicals. A superoxide-mediated oxidation-sensitive fluorogenic dye, dihydroethidium (DHE), was utilized for assessments of superoxide production. Briefly, cells were incubated with 5 μM of DHE for 30 min. Cells were collected by trypsinization and re-suspended in PBS. The fluorescence of 2-hydroxyethidium was measured using a FACScan flow cytometer (BD Biosciences, San Jose, CA) supplied with the CellQuest software. Mean fluorescence intensity from 10,000 cells was acquired using a 585/42-nm band-pass filter.

Assessment of (Z)-(3-(12-(1H-imidazol-1-yl)octadec-9-enoyloxy)propyl)triphenylphosphonium (TPP-IOA) in mouse embryonic cells and subcellular fractions. Mouse embryonic cells (2×10⁶) were seeded in 100 mm cell culture dishes and let attached overnight. Thereafter, the cells were incubated with TPP-IOA (10 μM) in PBS at 37 °C for 30 min, washed with PBS (2 x 5 mL), and collected by trypsinization. Mitochondria were isolated as described above. In mitochondria, TPP-IOA was analyzed by HPLC after precipitation of proteins with CH₃CN (final concentration, 70%; incubation time, 15 min at 4 °C; centrifugation, 5 min at 10,000×g). TPP-IOA from cytosolic fractions was extracted by following the protocol of Folch et al.⁴⁶ (Folch et al., 1957) and then re-dissolved in minimal volume of methanol.

Isocratic HPLC separation of TPP-IOA was achieved with 90% methanol containing 20 mM LiClO₄ at flow rate of 1 ml/min using Zorbax Eclipse XDB-C18 column (5 µm; 4.6x150mm; Agilent, Santa Clara, CA, USA). Detection of TPP-IOA was performed with a SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan) following the specific UV spectrum of the triphenylphosphonium function (λ_{max} =224, 264 and 274 nm; Fig. S3A, Inset). TPP-IOA was eluted as single peak with retention time of 4 min.

Assessment of imidazole substituted fatty acids by mass spectrometry. Mass spectral analysis of TPP-IOA was performed with a Finnigan LCQ Duo mass spectrometer (ThermoFisher Scientific, Co. West Palm Beach, FL). To exclude interference from LiClO₄, HPLC fractions were collected and analytes were extracted following the Folch procedure⁴⁶ (Folch et al., 1957). The dry residues obtained after evaporation of the organic phase were re-dissolved in CH₃OH and the corresponding solutions were directly infused in the mass spectrometer. For MS analysis of (*E*)-12-(1H-imidazol-1-ium-1-yl)octadec-9-enoic acid, the methanolic solution was supplemented with CH₃COOH (1%). All mass spectra were recorded in positive mode.

Selective reaction monitoring (SRM) and multiple reaction monitoring (MRM) analysis of TPP-IOA and its hydrolysis products. TPP-IOA and its hydrolysis products were extracted from plasma, small intestine and bone marrow tissue 10 min after TPP-IOA i.p. injection into animals (5 mg/kg body weight), using a standard Folch procedure for extraction of lipids⁴⁶. In one set of runs, intact TPP-IOA was assessed by SRM as described below. In an identical set of duplicate runs, hydrolyzed TPP-IOA (in the form of the TPP moiety, see below) was assessed by a different mass spectrometric method, MRM, as described below. TPP-IOA (m/z 651) was assessed by SRM in a LCQ-Duo ion trap mass spectrometer (Thermo, Inc., Waltham, MA). Chromatography was performed on an Eclipse XDB reverse phase C18 column (4.6mm x 15 cm, Agilent Technologies, Santa Clara, CA) using an isocratic solvent system consisting of acetonitrile:water:triethylamine:acetic acid (450:50:2.5:2.5, v/v/v/v) and a flow rate of 0.4 ml/min. The transition measured was 651>303 (TPP-IOA to TPP-propyl moiety – water) within a 0.5 Da window. Instrument conditions were as follows: spray voltage, 4.5 kV, positive mode; sheath gas 30; capillary temperature, 250°C; tube lens, 20; capillary voltage, 26. The instrument was tuned for the appropriate parent ion and all parameters were optimized to maximize the transition during the SRM including tuning under appropriate flow conditions. MRM conditions for assessment of hydrolyzed TPP-IOA utilized the same LC conditions. Transitions measured during MRM were as follows: 321 to 303 and 303 to 262 (TPP-propyl to TPP-propyl minus water to TPP, respectively). Instrument conditions were as follows: spray voltage, 4.5 kV, positive mode; sheath gas 20; capillary temperature, 300°C; tube lens, 20; capillary voltage, 26. The hydrolysis measured the appearance of the TPP-propyl/TPP group and not the appearance of the IOA. Based on our studies using IOA standard we concluded that measurements at the pg level could not be attained due to IOA's poor ionization efficiency. This was most likely due to the zwitterionic nature of the IOA, allowing a positive charge to be associated with the imidazole group and a negative charge to be associated with the carboxyl group. This prevented strong ionization in either the positive or negative mode at low abundances despite modifiers that were added to the mobile phase system. Additions of various mobile phase modifiers to the solvent system was not able to enhance ionization of the IOA in either positive or negative mode. To circumvent this problem, we also developed an LC-MS/MS method to assess the alternative product of the hydrolysis, namely the TPP-propyl group. Like the parent molecule (TPP-IOA), the strong positive charge allowed excellent ionization efficiencies and measurement at the pg level.

Assessment of oxidized molecular species of CL by mass spectrometry. For measurement of TLCL oxidation, cyt c (4 µM) was incubated with TLCL (100 µM) containing liposomes (TLCL/cyt c ratio - 20:1) and H₂O₂ (80 µM) for 10 min at 37°C. The reaction was stopped by catalase (150 units/ml) and CL was extracted using Folch procedure⁴⁶ (Folch et al., 1957). Analysis of (hydroperoxy- and hydroxy-) oxidized phospholipid species was performed as described⁴⁷ (Tyurin et al., 2008). TLCL and its oxidized molecular species were extracted by the Folch procedure⁴⁵ (Folch et al., 1957) and separated on a normal phase column (Luna 3 µm Silica 100A, 150 x 2 mm, Phenomenex, Torrance CA). Analytes were eluted at a flow rate of 0.2 mL/min using gradient of solvents A (chloroform : methanol : ammonium hydroxide (28%),

80:19.5:0.5 (v/v)) and B (chloroform : methanol : water : ammonium hydroxide, 60:34:5:0.5 (v/v); 48 (Malavolta et al., 2004). The column was flushed for the first 3 min isocratically with solvent B (10%), 3–15 min with a linear gradient from 10% solvent B to 37% solvent B, 15–23 min following a linear gradient to 100% solvent B, and then 23–45 min isocratically at 100% solvent B, 47–57 min isocratic at 10% solvent B for equilibrium column. To assess oxidized molecular species of CL, electro-spray-ionization-liquid chromatography mass-spectrometry (LC/ESI-MS) was performed using a Dionex Ultimate™ 3000 HPLC coupled on-line to an ESI ion source and a linear ion trap mass spectrometer (LXQ Thermo-Fisher) with the Xcalibur operating system (Thermo Fisher Scientific, San Jose, CA). The ESI probe was operated at a voltage differential of 3.5–5.0 kV in the negative ion mode. Capillary temperature was maintained at 150°C. Using range zoom (200–2000 m/z) in negative ion mode, the centroid spectra were acquired.

Small unilamellar liposomes were prepared from DOPC and TOCL (1:1 ratio) or DOPC and TLCL (4:1 ratio) by sonication in HEPES buffer (20 mM with 100 μ M DTPA (pH 7.4) 3 x 60sec, relaxation time 2 min, 4°C).

Peroxidase activity of cyt c. Peroxidase activity of cyt c was assessed by oxidation of Amplex Red, etoposide or TLCL.

Measurements of Amplex Red oxidation were performed using fluorescence of its oxidation product resorufin. Cyt c (1 μ M) was incubated with TOCL containing liposomes (CL/cyt c ratio 25:1). Then 50 μ M Amplex Red and 50 μ M H₂O₂ were added, and the incubation proceeded for an additional 20 min. Fluorescence was detected by employing a “Fusion α ” universal microplate analyzer and by using an excitation wavelength of 535 nm and an emission wavelength of 590 nm.

EPR spectra of etoposide phenoxyl radicals were recorded at 25 °C in gas permeable Teflon tubings (inner diameter, 0.8 mm; thickness, 0.013; Alpha Wire Corp., Elizabeth, NJ). Volume of samples was 60 μ L. The spectra were recorded under the following conditions: 335.3 mT, center field; 2 mT, sweep width; 0.04 mT, field modulation; 10 mW, microwave power; 0.1 s, time constant; 2 min, time scan. The time course of the etoposide radical EPR signal was obtained by repeated scanning of the field (0.15 mT, sweep width; 335.3 mT, center field) corresponding to a part of the EPR signal.

Peroxidase activity of mitochondria was determined by the formation of resorufin from Amplex Red. Isolated mouse liver mitochondria (0.6 mg/ml) were incubated in the presence of 100 μ M Amplex Red and 2 mM *tert*-butyl hydroperoxide (tBOOH). After incubation at room temperature for 20 min, fluorescence of resorufin was measured.

Low-temperature EPR measurements of protein-immobilized radicals. EPR spectra of protein-immobilized (tyrosyl) radicals were recorded after the addition of H₂O₂ (500 μ M) to cyt c (100 μ M) incubated with TOCL liposomes at room temperature. After incubation for 20 s, the reaction was stopped by freezing the samples in liquid nitrogen. EPR spectra were obtained on a JEOL-REIX spectrometer with 100 kHz modulation (JEOL, Kyoto, Japan). EPR spectra from frozen samples were recorded at 77 K under the following conditions: center field, 3230 G; sweep width, 100 G; field modulation, 5 G; microwave power, 1 mW; receiver gain, 10³; time constant, 0.1 s; time scan, 4 min.

Heme nitrosylation. Cyt c (100 μ M) was incubated with a nitroxyl donor, Angeli's salt (500 μ M), for 30 min at room temperature. The reaction was stopped by freezing the samples in liquid nitrogen. The EPR spectra of heme-nitrosylated cyt c were measured at 77 K under the following instrumental settings: center field, 3200 G; scan range, 500 G; field modulation, 5 G; microwave power, 10 mW; time constant, 0.1 s; scan time, 4 min; receiver gain, 10³.

Supplementary References:

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