tBID/BAX PERMEABILIZATION OF THE MITOCHONDRIAL OUTER MEMBRANE AS SENSITIZED BY CARDIOLIPIN HYDROPEROXIDE TRANSLOCATION: MECHANISTIC IMPLICATIONS FOR THE INTRINSIC PATHWAY OF OXIDATIVE APOPTOSIS

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Supplemental Experimental Procedures

Radiolabeling of Cellular CL and Determination of IM-to-OM Translocation of CL Species Under Oxidative Stress - Rat H9c2 cardiomyocytes at ~30% confluency in 15-cm dishes under DME medium containing 10% fetal bovine serum were grown to ~90% confluency in the presence of 15 μCi sodium [1-^{14}C]acetate (NEN Life Sciences). The cells were then washed twice with PBS and incubated with 100 μM antimycin A in serum free medium for 90 min at 37 ^{o}C, after which they were scraped into ice-cold PBS for isolation of mitochondria by differential centrifugation (1). For isolating IM (mitoplasts) and OM (2-4), mitochondria at a protein concentration of 2-5 mg/ml were swollen by stirring for 20 min in ice cold 10 mM potassium phosphate (pH 7.4) containing BSA (0.2 mg/ml) and protease inhibitors. The mitochondria were then disrupted by 20 passages through a 25-gauge needle. The resulting suspension was diluted to ~1 mg protein/ml and centrifuged for 10 min at 3000 x*g*, the recovered supernatant being considered as the crude OM fraction and the mitoplast-containing pellet as the crude IM fraction. Western blotting using an antibody against IM cytochrome c oxidase and OM monoamine oxidase was carried out to check for any possible cross contamination of fractions. Lipids were extracted from the IM and OM fractions using chloroform/methanol (2:1 by vol) and aliquots corresponding to an equal protein content of pre-extraction samples were recovered from the organic phases and analyzed by two-dimensional high-performance thin layer chromatography (2D-HPTLC), using Silica gel-60 plates (EM Sciences, Gibbstown, NJ) and as mobile phase, chloroform/methanol/ammonium hydroxide (65:35:5 by vol) for the first direction and chloroform/acetone/methanol/water/glacial acetic acid (55:45:20:10:10 by vol) for the second direction. Resulting chromatograms were subjected to phosphorimaging and quantitative analysis was accomplished using ScanQuant™ software. Identification of resolved phospholipid analytes, including species in the cardiolipin (CL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) families was based on the migration of standard phospholipids and confirmed by reference to published 2D-HPTLC analyses (5,6).

In a parallel setting, cells grown in 6-well culture plates to ~80% confluency were treated with 100 μM antimycin A in serum-free medium. After various incubation times at 37 ^{o}C, cells from triplicate wells were recovered by scraping and analyzed for caspase-3/7 activity as described (7), using the fluorogenic substrate Ac-DEVD-AMC (Calbiochem, Gibbstown, NJ).

References

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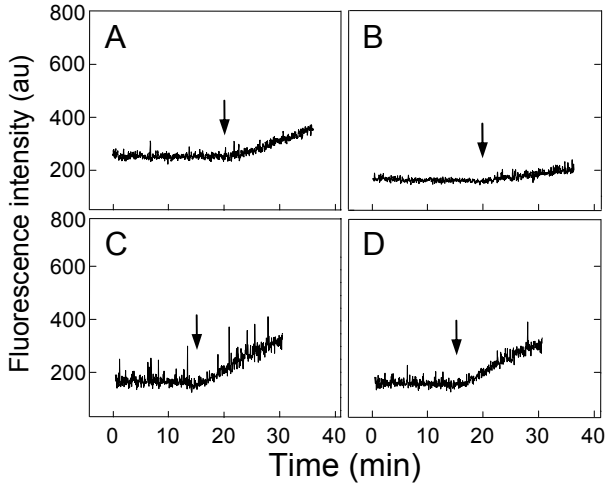

FIGURE S1. **Effect of varying CL or CLOOH content on rate of ANTS/DPX release from LUVs.** Each reaction mixture contained ANTS/DPX-bearing POPC/CL or POPC/CLOOH LUVs (50 μM total lipid) and 10 mM $CaCl_2$ in 25 mM HEPES buffer (pH 7.4) at 37 ^{o}C. tBid (40 nM) was introduced at the indicated times (arrows). (A) 5 mol % CL; (B) 2 mol % CL; (C) 5 mol % CLOOH; (D) 2 mol % CLOOH. Other details were as described in Fig. 1.

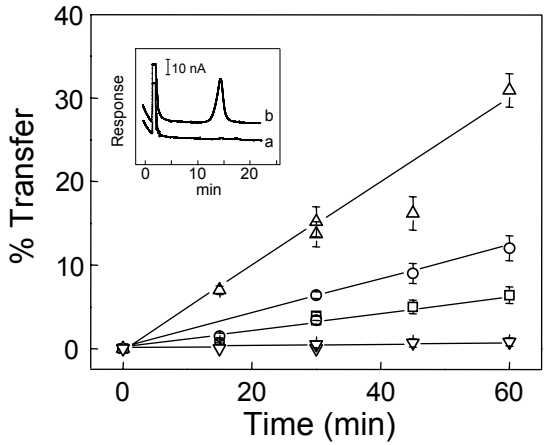

FIGURE S2. **Rate of intermembrane CLOOH transfer as a function of nsLTP concentration.** Reaction mixtures in 25 mM HEPES (pH 7.4) at 37 °C contained POPC/CL/CLOOH/LacPE/Ch (7:1:1:1:0.2 by mol) SUV donors (5 mM total lipid), POPC LUV acceptors (2.5 mM lipid), and nsLTP at the following concentrations (μg/ml): 0 (▽); 10 (□); 20 (○); 50 (△). At the indicated times, samples were quenched with ice-cold PBS, treated with RCA_{120} and centrifuged to separate SUVs from LUVs, and lipid extracts of the LUV fractions were analyzed for CLOOH by HPLC-EC(Hg). Plotted values are means ± SE (n = 3). *Inset*: HPLC-EC(Hg) profiles (detector response *vs.* retention time) for 50 μg nsLTP/ml after 0 min (a) and 60 min (b) of transfer incubation.

Fig. S3

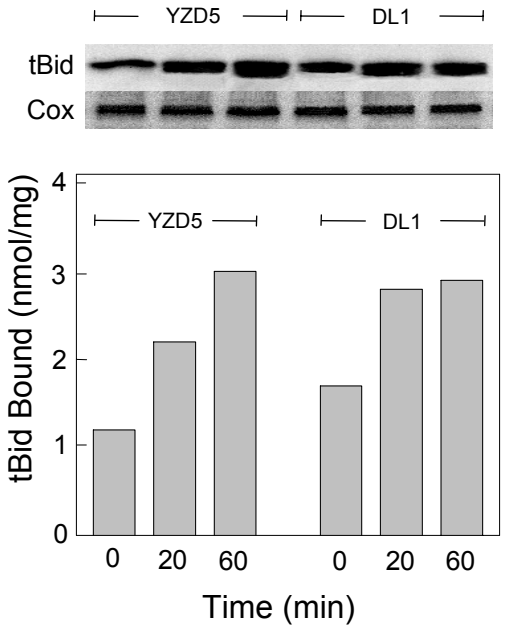

FIGURE S3. **tBid binding by isolated mitochondria.** Suspensions containing [^{14}C]POPC/CLOOH/DCP (7:3:0.1 by mol) SUVs (0.3 mM lipid), YZD5 or DL1 Mito (1.4 mg protein/ml), and nsLTP (50 μg/ml) in 200 mM mannitol/50 mM sucrose/1 mM KH_2PO_4/2 mM $MgCl_2$/1 mM EGTA/0.05 mM DFO/5 mM MOPS (pH 7.2) were incubated for 20 min at 37 ^{o}C, then centrifuged and washed. Recovered Mito (1.2 mg/ml) were incubated with tBid (4.5 ng/ml) for 15 min at 30 ^{o}C, then pelleted and analyzed by Western blotting using an anti-tBid antibody. Top: immunodetected tBid and cytochrome c oxidase (Cox, used as a loading standard). Bottom: Integrated (Cox-normalized) tBid band intensities.

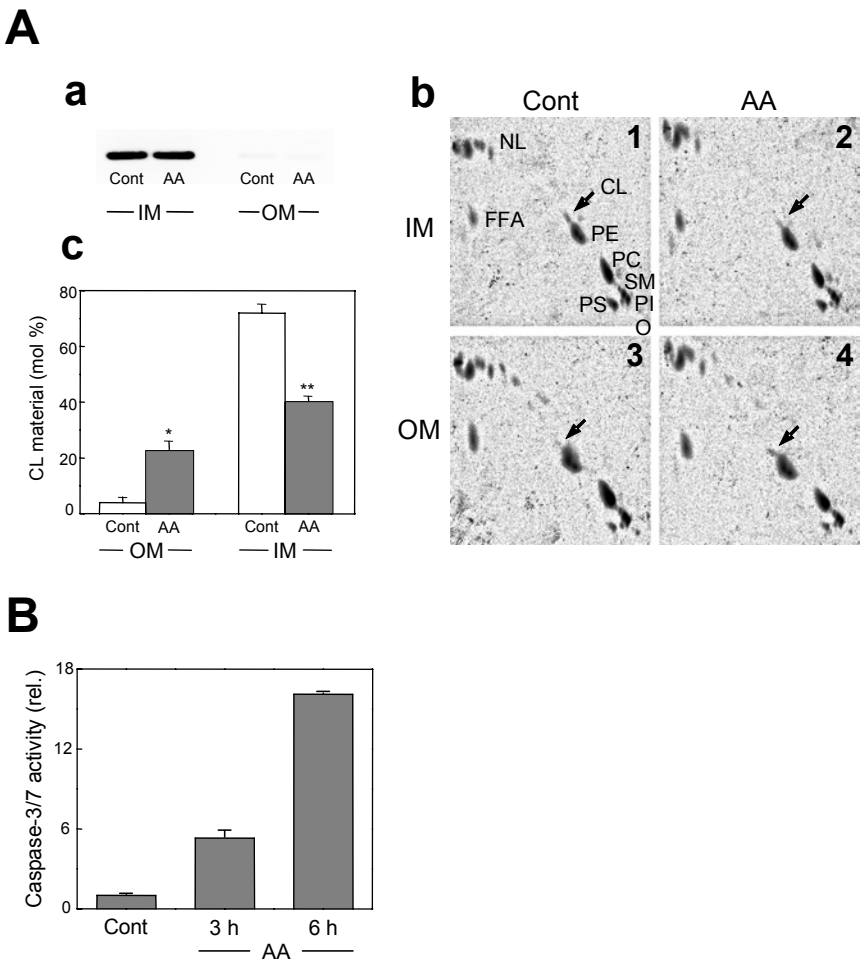

FIGURE S4. **IM-to-OM translocation of CL material and caspase activation in oxidatively stressed cells.** (A) H9c2 cardiomyocytes were either not treated (control) or treated with 100 µM Antimycin A (AA) for 1.5 h in serum-free DME medium, after which cells were scraped into ice-cold PBS. Mitochondria were isolated and IM and OM fractions prepared. (a) Western blot of IM and OM fractions probed with an antibody against subunit-IV of cytochrome c oxidase; load of total IM or OM protein: 1.5 µg per lane. (b) 2D-HPTLC chromatogram of lipid extracts from control [IM (1); OM (3)] and AA-treated [IM (2); OM (4)] cells. NL, neutral lipids; FFA, free fatty acids; CL, cardiolipin; PE, phosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; O, origin. (c) Levels of CL material in OM and IM fractions (mol % of total mitochondrial CL), as determined by quantitative phosphorimaging. Intensities of analyte spots were normalized to protein load reflecting distribution of IM and OM protein in intact mitochondria. Means ± SD of values from three separate experiments are shown. *P<0.005 compared with control OM; **P<0.01 compared with control IM. (B) Caspase-3/7 activation during treatment of H9c2 cells with 100 µM AA in serum-free DME medium. After 3 h and 6 h of incubation with AA or 6 h without AA (control), cells were harvested, lysed, and caspase-3/7 activity was determined using fluorogenic Ac-DEVD-AMC as the substrate.