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The mitochondria-targeted imidazole substituted oleic acid 'TPP-IOA' affects mitochondrial bioenergetics and its protective efficacy in cells is influenced by cellular dependence on aerobic metabolism

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ABSTRACT

A variety of mitochondria-targeted small molecules have been invented to manipulate mitochondrial redox activities and improve function in certain disease states. 3-Hydroxypropyl-triphenylphosphonium-conjugated imidazole-substituted oleic acid (TPP-IOA) was developed as a specific inhibitor of cytochrome *c* peroxidase activity that inhibits apoptosis by preventing cardiolipin oxidation and cytochrome *c* release to the cytosol. Here we evaluate the effects of TPP-IOA on oxidative phosphorylation in isolated mitochondria and on mitochondrial function in live cells. We demonstrate that, at concentrations similar to those required to achieve inhibition of cytochrome *c* peroxidase activity, TPP-IOA perturbs oxidative phosphorylation in isolated mitochondria. In live SH-SY5Y cells, TPP-IOA partially collapsed mitochondrial membrane potential, caused extensive fragmentation of the mitochondrial network, and decreased apparent mitochondrial abundance within 3 h of exposure. Many cultured cell lines rely primarily on aerobic glycolysis, potentially making them less sensitive to small molecules disrupting oxidative phosphorylation. We therefore determined the anti-apoptotic efficacy of TPP-IOA in SH-SY5Y cells growing in glucose or in galactose, the latter of which increases reliance on oxidative phosphorylation for ATP supply. The anti-apoptotic activity of TPP-IOA that was observed in glucose media was not seen in galactose media. It therefore appears that, at concentrations required to inhibit cytochrome *c* peroxidase activity, TPP-IOA perturbs oxidative phosphorylation. In light of these data it is predicted that potential future therapeutic applications of TPP-IOA will be restricted to highly glycolytic cell types with limited reliance on oxidative phosphorylation.

1. Introduction

Cell death via apoptosis can contribute to pathologies associated with various diseases. For example, the loss of post-mitotic terminally differentiated cells that have little regenerative capacity (e.g. neurons, cardiomyocytes) can occur via excessive apoptotic cell death in a variety of disease states such as neurodegenerative disorders of the brain and ischemia/reperfusion injury of highly aerobic tissues [21, 41]. Preventing apoptotic cell death in these contexts, and thus preserving tissue mass and function, is a medical goal.

Mitochondria play an important role in the intrinsic apoptotic pathway that is initiated in many pathological disease states [81]. A pivotal event in mitochondria-mediated apoptosis is the release of cytochrome *c* into the cytosol. Cytochrome *c* release from mitochondria proceeds by a two-step process, whereby it first loses its affinity for the inner mitochondrial membrane, and then translocates into the cytosol after permeabilization of the outer membrane [49]. Cytosolic cytochrome *c* is a core component of the 'apoptosome' — the multimeric complex with Apaf-1 that activates Caspases to complete cell death [37, 80].

Cytochrome *c*'s interactions with cardiolipin play a key role in its release from mitochondria. Cardiolipin is predominantly localized in the inner mitochondrial membrane [23,33] where it can interact with cytochrome *c* via its negatively charged head group. Cardiolipin binding to cytochrome *c* is thought to involve electrostatic interactions between positively charged lysine residues of cytochrome *c* and the negatively charged phosphate groups on cardiolipin, as well as hydrophobic interactions between a polyunsaturated fatty acid residue on cardiolipin and a hydrophobic pocket of cytochrome *c* [5,53]. The former interaction involves a loosely bound protein conformation and is believed to facilitate electron transport activity [78]. Alternatively, the hydrophobic interaction is associated with a tightly bound conformation with partial unfolding of the protein that is important for mitochondrial involvement in apoptosis. This interaction with cardiolipin involves the loss of the Met80-heme iron coordination, and opens the heme iron catalytic site, making it accessible to small oxidizing equivalents, like H_2O_2 [28, 78]. Using such oxidizing equivalents for catalysis, the heme iron

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exhibits measurable peroxidase activity that is specific to bound polyunsaturated species of cardiolipin [28]. The accumulation of peroxidized cardiolipin species within mitochondria is recognized as an essential event for both outer mitochondrial membrane permeabilization during apoptosis, and the loss of cytochrome *c* from the inter membrane space of mitochondria [28,32,47,49,52,78].

Considering that the peroxidase activity exhibited by cytochrome *c*/cardiolipin complexes is crucial to cytochrome *c* release, the design and characterization of agents that inhibit this specific activity are important goals. Imidazole-substituted long chain fatty acids have been invented for this purpose [3,29]. They were designed on the basis of their hydrophobic acyl chain being able to protrude into the hydrophobic pocket within the protein that provides accessibility to the heme iron site. Consequently, the imidazole attached to the oleate chain could potentially interact with the heme-iron of the catalytic site, thus fulfilling heme-iron hexa-coordination and lessening the catalytic site's availability [3,29]. Indeed, imidazole-substituted oleic (IOA) and stearic acids (ISA) with the imidazole moiety located 7 carbons away from the terminal methyl group are able to interact with the heme-iron of cytochrome *c* [3]. Furthermore, these imidazole-fatty acid derivatives are effective inhibitors of the peroxidase activity of pure cytochrome *c*/tetra-oleoyl-cardiolipin complexes [3].

To achieve mitochondrial targeting of these inhibitors, they were chemically conjugated to a lipophilic triphenylphosphonium cation-containing molecule, an approach that has been widely used to target bioactive molecules to mitochondria [1,4,7,11,13,18,19,26,27,30,31,39,46,55,59,61,67,70]. Indeed, 3-hydroxypropyl-triphenylphosphonium-conjugated imidazole-substituted oleic acid (TPP-IOA) administration to cultured mouse cells resulted in rapid accumulation of TPP-IOA within mitochondria [3]. Notably, in addition to the fully intact molecule being present, the two hydrolysis products, 3-hydroxypropyl-TPP and the imidazole-oleic acid moiety were each also detectable, indicating that TPP-IOA undergoes hydrolytic processing [3]. A single bolus intraperitoneal injection of TPP-IOA to live mice resulted in rapid detectable accumulation within radiosensitive tissues of interest (bone marrow, small intestine; [3]), indicating successful *in vivo* uptake. Injected mice were resistant to radiation-induced death.

TPP-IOA has potential as a therapeutic agent in pathologies involving cell death through the mitochondrial pathway. However, since TPP-IOA is targeted to mitochondria and interacts directly with cytochrome *c*, it has the potential to interfere with oxidative phosphorylation, which would limit its protective efficacy in cell types that are heavily reliant on this pathway for ATP supply. We therefore used pure cytochrome *c*, isolated mitochondria, and live cells to compare the dose response to TPP-IOA in terms of inhibiting peroxidase activity and interfering with other aspects of mitochondrial bioenergetics. These investigations indicate significant perturbation of mitochondrial function in the same concentration range that is associated with inhibition of cytochrome *c* peroxidase activity. Furthermore, TPP-IOA effectively inhibited apoptotic cell death in cultured cells able to utilize glucose fermentation but not in cells with increased reliance on oxidative phosphorylation. These findings limit the utility of TPP-IOA as an anti-apoptotic agent for highly oxidative tissues.

2. Materials and methods

2.1. Materials

3-Hydroxypropyl-triphenylphosphonium (3-hydroxypropyl-TPP), cytochrome *c* (Cat. # C3131), cardiolipin solution (in ethanol, Cat. #C1649), ascorbic acid, *tert*-butyl hydroperoxide aqueous solution, Dulbecco's Modified Eagle Medium (DMEM) powdered media with glucose (4500 mg/L), L-glutamine, and sodium pyruvate (Cat. #D7777), supplement-free DMEM powdered media (Cat #5030), fetal bovine serum, nonessential amino acids, penicillin/streptomycin solution, 0.25% trypsin/EDTA solution, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, USA). Dimethylsulfoxide (DMSO), DL-dithiothreitol (DTT), Bradford reagent, D-galactose, L-glutamine, HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and Trypan Blue were obtained from BioShop (Burlington, ON, Canada). Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) was obtained from Cayman Chemical (Ann Arbor, MI, USA). The Caspase-3 fluorogenic substrate Ac-DEVD-AMC was obtained from BD Biosciences (Franklin Lakes, NJ, USA). MitoTracker Red CMXRos dye and Lipofectamine 2000 transfection reagent were purchased from Life Technologies Incorporated (Burlington, ON, Canada). Goat (C-11) polyclonal Actin antibody (sr-1615) raised against a peptide fragment of the C-terminus of human Actin was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal VDAC antibody (600-401-882) raised against a synthetic peptide corresponding to amino acids 185–197 of human VDAC1 and infrared dye-conjugated secondary (IgG) antibodies to rabbit (611-132-122) and goat (605-732-125) were all purchased from Rockland Immunochemicals (Gilbertsville, PA, USA).

2.2. Reagents and buffers

TPP-IOA was prepared as described in Atkinson et al. [3]. Synthesis of TPP-OA is described below (Section 2.3). TPP-IOA, TPP-OA, and 3-hydroxypropyl-TPP (Fig. 1) were dissolved in sterile-filtered DMSO. For isolation of mitochondria, SHE buffer consisted of 250 mM sucrose, 10 mM HEPES, and 1 mM EGTA, final pH adjusted to 7.5, and SHE + BSA buffer consisted of SHE buffer with 0.5% (*w*/*v*) bovine serum albumin (delipidated, fraction V), final pH adjusted to 7.5. Mitochondrial respiration buffer consisted of 125 mM sucrose, 65 mM KCl, 10 mM HEPES, and 2 mM KH_2PO_4, pH adjusted to 7.2. Trypan Blue solution (0.4% *w*/*v*) was prepared in 0.81% NaCl and 0.06% K_2HPO_4.

2.3. Synthesis of 3-hydroxypropyl-triphenylphosphonium-conjugated oleic acid ((Z)-(3-(oleoyloxy)propyl)triphenylphosphonium bromide)

To a clear solution of oleic acid (400 mg, 1.416 mmol) in 10 mL acetone and 3 mL distilled water at room temperature was added potassium hydroxide (79.4 mg, 1.41 mmol). The solution was stirred vigorously for 10 min. (3-bromopropyl)Triphenylphosphonium bromide (454.6 mg, 1.133 mmol) was added and stirred for 36 h at room temperature. The acetone was evaporated and dichloromethane was added to the reaction mixture and washed with distilled water. The phases were separated and the water phase was washed two times with dichloromethane. The combined organic phases were dried over anhydrous sodium sulphate. Column chromatography silica eluting with a solvent gradient from 1:1 hexane:dichloromethane to 100% dichloromethane afforded 385 mg of a clear, amorphous solid. Yield: 40.9% R_f: 0.24 TLC: 9:0.5 dichloromethane:methanol. NMR: ^{1}H NMR: (400 MHz, $CDCl_3$) δ: 7.916–7.697 (m, 15H), 5.371–5.337 (m, 2H), 4.420–4.389 (t, $J = 6$ Hz, 2H), 4.166–4.093 (dt, $J = 6$ Hz, 2H), 2.255–2.236 ($J = 7.6$ Hz, 2H), 2.081–1.990 (m, 6H), 1.51–1.50 (m, $J = 7.2$ Hz, 2H) 1.40–1.20 (m, 20H) 0.910–0.876 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (400 MHz, $CDCl_3$) 173.49, 135.04, 135.01, 133.83, 133.73, 130.56, 130.43, 130.04, 129.72, 118.70, 117.85, 63.20, 63.03, 34.18, 31.91, 29.77, 29.72, 29.53, 29.33, 29.21, 29.13, 27.23, 27.18, 24.86, 22.69, 22.36, 22.33, 19.84, 19.32, 14.13. MS: (ESI) *m*/*z* $C_{39}H_{54}O_2PBr$ (M^+) calculated 663.296 (M^+) found 585 = (M^+ − Br), 303 ($PPh_3CH_2CH_2CH^{+\cdot}$), 262 (PPh_3^+).

2.4. Cytochrome c/cardiolipin complex peroxidase activity

Peroxidase activity of pure cytochrome *c* in the presence of cardiolipin was assessed with Amplex Red reagent essentially as described in Atkinson et al. [3] and Birk et al. [9] using a Varian Cary Eclipse fluorescence spectrophotometer equipped with microplate reader (Agilent Technologies, Santa Clara, CA, USA). This spectrofluorometric assay measures the formation of resorufin — the fluorescent oxidation