Lipophilic Triphenylphosphonium Cations Inhibit Mitochondrial Electron Transport Chain and Induce Mitochondrial Proton Leak

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Abstract

Background

The lipophilic positively charged moiety of triphenylphosphonium (TPP\textsuperscript{+}) has been used to target a range of biologically active compounds including antioxidants, spin-traps and other probes into mitochondria. The moiety itself, while often considered biologically inert, appears to influence mitochondrial metabolism.

Methodology/Principal Findings

We used the Seahorse XF flux analyzer to measure the effect of a range of alkylTPP\textsuperscript{+} on cellular respiration and further analyzed their effect on mitochondrial membrane potential and the activity of respiratory complexes. We found that the ability of alkylTPP\textsuperscript{+} to inhibit the respiratory chain and decrease the mitochondrial membrane potential increases with the length of the alkyl chain suggesting that hydrophobicity is an important determinant of toxicity.

Conclusions/Significance

More hydrophobic TPP\textsuperscript{+} derivatives can be expected to have a negative impact on mitochondrial membrane potential and respiratory chain activity in addition to the effect of the biologically active moiety attached to them. Using shorter linker chains or adding hydrophilic functional groups may provide a means to decrease this negative effect.

Introduction

Lipophilic cations based on the triphenylphosphonium moiety (TPP\textsuperscript{+}) have been widely used to target various biologically active substances such as antioxidants [1–6], spin traps [7–10] or various other chemical probes into mitochondria [11, 12]. The accumulation of TPP\textsuperscript{+}...
derivatives in mitochondria was first described in 1970 [13, 14]. It relies on the electric potential difference maintained across the inner mitochondrial membrane by the action of the respiratory chain and the fact that this membrane can be permeated by large hydrophobic cations.

Assuming a perfectly Nernstian behaviour, a membrane-permeable cation will accumulate in a negatively charged compartment approximately ten-fold for each 60 mV of potential difference. In the case of TPP+ derivatives this ideal behaviour is complicated by the fact that the hydrophobicity of the derivative affects both the extent and the rate of accumulation, more lipophilic derivatives accumulate faster and to higher concentrations than the more hydrophilic ones [15, 16].

The TPP+ moiety itself is often assumed not to exhibit any significant biological activity, however, its high affinity for phospholipid membranes [17–19] makes it likely to disrupt membrane integrity [20–22] especially in mitochondria where such compounds accumulate, which could also alter the function of mitochondrial membrane proteins such as complexes of the respiratory chain [23].

It has been previously observed that some TPP+ compounds negatively affect mitochondrial and cellular respiration [24–26] and may increase proton leak across the inner mitochondrial membrane, e.g. by enhancing the uncoupling effect of palmitate [27], or anionic protonophores[28]. Most published studies used TPP+ derivatives with chemically active moieties [29] making it difficult to separate the effect of the TPP+ moiety itself. One notable exception is a recent paper by Reily et al. [30], who studied not only the impact on mitochondrial function of biologically active TPP+ compounds MitoQ, MitoTEMPOL and MitoE but also ‘inactive’ alkyl derivatives methyl-, butyl- and decylTPP+. Their results from Seahorse measurements of MES-13 cells show a general inhibitory effect of all TPP+ derivatives on basal respiration accompanied by signs of mitochondrial uncoupling for decylTPP+. This study, while useful in highlighting the significant effects of alkylTPP+ on cellular respiration, relied on only one source of data, namely the measurements of oxygen consumption and extracellular acidification. These parameters make it difficult to separate effects on membrane potential and respiratory chain activity.

In the present study we decided to use a range of ‘inactive’ TPP+ derivatives, namely alkyltriphenylphosphonium bromide salts, and employ additional assays to study the mechanisms of their toxic effects on mitochondrial respiration. We show a clear negative effect of TPP+ derivatives on the respiratory chain complexes, on mitochondrial membrane potential and ATP synthesis. We also provide further support for the previous suggestions that these negative effects increase with increasing hydrohobicity of TPP+ compounds.

Materials

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. Decylubiquinol was prepared by dissolving decylubiquinone in acidified ethanol (pH 4), adding a few grains of sodium borohydride (NaBH₄) and vortexing until the solution became colourless. Aliquots were stored at −20°C under argon. Ferrocyanochrome c was freshly prepared by adding few grains of sodium dithionite to 1 mM stock of ferricytochrome c.

Collection of rat tissues

Wistar rats 13–15 weeks old weighing 200–300 gm were obtained from AnLab Ltd., Prague, Czech Republic. Animals were sacrificed by diethylether overdose prior to tissue isolation. We collected both gastrocnemii muscles to prepare a homogenate enriched in the mitochondrial fraction. This was approved by the Committee for the Protection of Laboratory Animals of the Third Faculty of Medicine, Charles University in Prague.
Cell culture conditions
C2C12 cells were obtained from Sigma-Aldrich and grown in Dulbecco-modified Eagle’s medium (DMEM, Life Technologies) containing 1 g/l D-glucose and supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate. All cultures were incubated at 37°C in an atmosphere of 95% humidity and 5% CO₂. Cells were passaged every 48 hours.

Preparation of muscle homogenate enriched in mitochondrial fraction
We prepared a muscle homogenate by modifying a previously described protocol [31]. A freshly removed rat gastrocnemius was washed three times by ice-cold buffer (250 mM sucrose, 5 mM Tris, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N′,N′,N′-tetraacetic acid (EGTA), 0.1% fatty acid free bovine serum albumin (BSA), pH 7.4) then flash frozen in liquid nitrogen, and stored at -80°C. On the preparation day, the visible fat and connected tissue were removed by a scalpel blade, then the muscle was finely dissected into small fragments in a glass dish on ice. The muscle pieces were diluted 1:10 in ice-cold muscle homogenization medium (250 mM sucrose, 20 mM Tris, 40 mM KCl, 2 mM EGTA, pH 7.4) then the suspension was transferred to a glass tube and chopped with an UltraTurrax blender followed by homogenisation in a Dounce homogeniser with a motor-driven Teflon plunger at 500 r.p.m (≈ 10 passes). The homogenate was then centrifuged for 15 min at 600 x g at 4°C. The supernatant was transferred into new tubes on ice then flash frozen in liquid nitrogen and stored at −80°C. Protein concentration in the homogenate was determined using the bicinchoninic acid (BCA) assay.

Analysis of metabolism
Cellular respiration was measured using the XF-24 analyzer (Seahorse Bioscience). We performed mitochondrial bioenergetic assays based on published protocols [32]. The XF assay medium (bicarbonate-free modified DMEM, Seahorse Bioscience) was supplemented with 4 mM L-glutamine, 1 mM pyruvate, and 1 g/l D-glucose. The pH was adjusted with 1 M NaOH to 7.4 at 37°C. Cells were seeded at a density of 20,000 cells per well and left overnight to attach and proliferate to obtain a monolayer of cells before measurement. After measuring the basal respiration TPP⁺ derivatives or vehicle were injected and a mitochondrial respiration test was performed by sequential additions of 1 μM oligomycin, 0.5 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 1 μM rotenone and antimycin A. Proton leak-induced respiration was calculated as the difference between respiration achieved after oligomycin addition and non-mitochondrial oxygen consumption following rotenone-antimycin A treatment. Maximal respiration induced by FCCP uncoupler was corrected by subtracting the non-mitochondrial respiration values. For each well the highest measurement value was selected for each type of measurement and compared to the highest reading for the control.

Analysis of respiratory chain enzymatic activity
Mitochondrial respiratory chain enzymatic activity was assessed in a homogenate prepared from rat skeletal muscle. Prior to enzymatic assays this homogenate was exposed to three cycles of rapid freeze-thawing. We modified previously described protocols to measure the activity of complex I and II [33], complex III [34], and complex IV [35] to fit in a microplate reader.

We wanted to use TPP⁺ concentrations equivalent to those in energised mitochondria exposed to micromolar external concentrations therefore we assumed approximately a 1000-fold accumulation according to the Nernst equation and normal mitochondrial membrane potential. However, high concentrations of decyl- and dodecyl-TPP⁺ (≥ 300 μM) appear to interfere
with complex I and II assays due to the formation of a complex with 2,6-dichloroindophenol (DCIP) with a shifted absorbance maximum, and with the complex IV assay as the addition of ferricyanide to the reaction mixture induces the formation of a precipitate complicating the reading of absorbance. Instead of adding 1 mM alkylTPP⁺ derivatives to the complete assay mixture we therefore decided to preincubate the muscle homogenate with the compounds at this concentration and then add the rest of the assay mixture and thus decrease the final concentration of the TPP⁺ to avoid this interference. It is therefore possible that the effects observed in this study underestimate the real effects in intact cells.

**Complex I.** Complex I assay was performed in an assay mixture composed of 25 mM potassium phosphate, 3.5 g/l BSA, 2 mM ethylenediaminetetraacetic acid (EDTA), 60 μM DCIP, 70 μM decylubiquinone, 1 μM antimycin A and 0.2 mM reduced nicotinamide adenine dinucleotide NADH, pH 7.8. Changes in absorbance were followed at 600 nm. Rotenone sensitive activity was calculated by subtracting the activity of wells with 10 μM rotenone.

**Complex II.** Complex II activity was measured in an assay mixture containing 80 mM potassium phosphate, 1 g/l BSA, 2 mM EDTA, 10 mM succinate, 80 μM DCIP, 50 μM decylubiquinone, 1 μM antimycin A and 3 μM rotenone, pH 7.8. Changes in absorbance were followed at 600 nm. Malonate sensitive activity was calculated by subtracting the activity of wells with 20 mM malonate.

**Complex III.** Complex III activity was measured in an assay mixture containing 50 μM ferricytochrome c, 25 mM potassium phosphate, 4 mM sodium azide, 0.1 mM EDTA, 0.025% Tween² 20 and 50 μM decylubiquinol, pH 7.4. Changes in absorbance were followed at 550 nm. Antimycin A sensitive activity was calculated by subtracting the activity of wells with 10 μM antimycin A.

**Complex IV.** Complex IV activity was measured in an assay buffer containing 30 mM potassium phosphate and 25 μM of freshly prepared ferrocytochrome c, pH 7.4. Changes in absorbance were followed at 550 nm. The absorbance of samples oxidised with 10 μl of 0.5 M potassium hexacyanoferrate(III) was subtracted from all measurements, then the natural logarithm absorbance was plotted against time and compared to untreated control.

Detection of changes in mitochondrial membrane potential ($\Delta\psi_m$)

Qualitative changes in $\Delta\psi_m$ were determined as the changes in tetramethylrhodamine methyl ester (TMRM) fluorescence [36, 37] in non-quench mode [38] in C2C12 myoblasts. Cells were allowed to grow and reach ≈ 80% confluence, then we washed them twice with warm PBS and detached using trypsin for harvesting. The cell suspension was centrifuged at 500 xg at room temperature for 5 minutes. The pellet was resuspended in warm DMEM containing 50 nM TMRM (Life Technologies) for 20 minutes at 37°C with continuous gentle shaking. The cell suspension was then centrifuged and the pellet was resuspended in warm DMEM and exposed for 10 minutes to tested compounds or vehicle. A FACSCalibur flow cytometer (BD Biosciences) was used to read fluorescence with an excitation wavelength of 488 nm.

Statistical analyses

Data are presented as mean and 95% confidence interval (CI). One-way ANOVA with Tukey’s multiple comparisons test was performed using GraphPad Prism version 6.0d for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. Differences found statistically significant are marked with an asterisk. The number of independent experiments is denoted as n.
Results

‘Inactive’ TPP⁺ compounds impair mitochondrial function in intact cells

Firstly we evaluated the effects of TPP⁺ compounds on mitochondrial respiration of intact cells. Basal mitochondrial respiration is controlled by two processes: ATP production and proton leak. We can block ATP synthase by oligomycin, which allows us to measure proton leak-driven respiration. The addition of a highly effective protonophore (FCCP) removes the regulatory effect of the membrane potential and allows us to measure the maximal respiratory rate at a given substrate availability, which will detect any inhibition of the respiratory chain complexes. The extracellular acidification rate (ECAR) is usually interpreted as the production of lactate in glycolysis [32].

We observed three main effects: the longer-chain alkylTPP⁺ derivatives increased proton leak, decreased maximal respiration (Fig 1) and induced an increase in ECAR (Fig 2). The addition of 1 μM decyl- or dodecylTPP⁺ lead to ≈ 10 fold increase in proton leak-driven (oligomycin-inhibited) respiration, compared to controls treated with the vehicle alone. HeptylTPP⁺
exhibited a more modest effect, while the shortest alkyl derivative, propylTPP⁺ had no effect at this concentration (Fig 3A).

The effect on maximal respiration was somewhat less pronounced at the 1 μM concentration but a similar trend of increasing efficacy with increasing alkyl chain length was apparent (Fig 3B) with only decyl- and dodecylTPP⁺ exhibiting a significant inhibitory effect. Of course, under conditions of complete uncoupling due to FCCP it is likely that the effective concentrations of TPP⁺ derivatives inside mitochondria are much lower than under basal conditions suggesting that the impact of TPP⁺ on the respiratory chain activity could be much larger than shown by these data.

Both increased proton leak and decreased activity of the respiratory chain can be expected to decrease the mitochondrial membrane potential and therefore to have a deleterious effect on mitochondrial ATP synthesis, which may result in a stimulation of glycolysis to make up for ATP deficit. In Fig 3C we show a stimulatory effect of longer chain alkylTPP⁺ derivatives on ECAR, which once again follows the same relationship between alkyl chain length and the effect size. The addition of oligomycin completely blocks oxidative phosphorylation and leads to a further increase of ECAR under basal conditions and we observed no further effects of alkylTPP⁺ derivatives on this rate (Fig 3D).
We were also interested whether this effect of alkylTPP⁺ derivatives on mitochondrial proton leak and maximal respiration is dose-dependent. Fig 4 shows a clear relationship between both effects and the concentration of dodecylTPP⁺ to which cells were exposed. The proton leak stimulation appears to be much stronger than the inhibitory effect on maximal respiration.

In order to elucidate further these two effects of alkylTPP⁺ derivatives we decided to investigate which respiratory complexes are inhibited by these chemicals, and to measure directly their effect on the mitochondrial membrane potential.

**Inhibition of respiratory chain complexes**

We measured the effect of alkylTPP⁺ compounds on the enzymatic activity of individual mitochondrial respiratory chain complexes in freeze-thawed rat skeletal muscle homogenate.
enriched in the mitochondrial fraction. This model allows a direct access to the respiratory chain in the absence of a mitochondrial membrane potential.

**Fig 5** shows a significant inhibitory effect of long alkyl chain TPP⁺ derivatives on all four complexes. Complexes I and III appear to be the most sensitive to TPP⁺ inhibition, while complex II seems to be relatively resistant. The effect of TPP⁺ derivatives on complex IV activity is rather curious. There is a significant inhibitory effect of the vehicle (1% dimethylsulfoxide (DMSO) during preincubation, 0.05% during assay), which is further potentiated by decyl- and dodecylTPP⁺. The shorter chain derivatives, propyl- and heptylTPP⁺, on the other hand, appear to alleviate the toxic effect of DMSO.

For the two longest derivatives we also investigated the dose-dependency of the inhibition of individual complexes (**Fig 6**). DodecylTPP⁺ virtually completely inhibited complexes I, III and IV at a 0.5 mM concentration, while complex II is only inhibited by about 50% at this concentration. DecylTPP⁺ exhibits a similar pattern with a substantially lower potency.

**TPP⁺ derivatives decrease mitochondrial membrane potential**

We sought to strengthen our data on proton leak-driven respiration by using the negatively charged, membrane-permeable fluorescent dye TMRM to estimate the effect of TPP⁺ derivatives on the mitochondrial membrane potential. TMRM accumulates in mitochondria proportionately to the membrane potential and therefore cells with a higher membrane potential will fluoresce with a higher intensity. We detected fluorescence in individual cells using flow cytometry.

**Fig 7** shows a typical fluorescence intensity histogram and **Table 1** summarises the mean fluorescence intensities measured in our experiments. There is a clear trend towards lower fluorescence intensities as the alkyl chain length increases. While 1 μM propyTPP⁺ virtually doesn’t affect the membrane potential heptyl-, decyl- and dodecylTPP⁺ decrease it significantly. Intriguingly, the two longest chain derivatives appear to collapse the mitochondrial membrane potential even more effectively than an equal concentration of the uncoupler FCCP. This could
potentially be explained by the combined action of an uncoupling effect and respiratory chain inhibition.

Discussion

The TPP⁺ moiety of mitochondrially targeted compounds is often considered to be without a significant biological activity. Here we show a clear evidence that TPP⁺ derivatives with simple alkyl chains in place of ‘active’ chemical moieties may significantly affect mitochondrial bioenergetics.

In particular, we observed a significant potentiation of proton leak with an ensuing decrease in the mitochondrial membrane potential and an inhibition of the respiratory chain complexes.
Our expectation that the magnitude of these effects may correlate with the alkyl chain length and therefore hydrophobicity of the compounds was also supported by the data.

TPP⁺ compounds are well known to have a high affinity to biological membranes [39]. This affinity will further increase with an increasing hydrophobicity of the derivative [15, 16]. A plausible explanation of our observations therefore may be that both the increase in proton leak and the inhibition of respiratory chain complexes is mediated by an incorporation of alkylTPP⁺ molecules into the inner mitochondrial membrane and the resulting disruption of
its normal function. Since the respiratory complexes are known to be sensitive to their lipid environment and require phospholipid molecules for their activity \([40–42]\), a high proportion of alkylTPP\(^+\) molecules in the membrane could impair both the insulant properties of the membrane allowing protons to leak back into the matrix and the membrane structure required for the functioning of the protein complexes.

Data from intact cells presented in this work or previously published \([30]\) are useful to identify broad effects on mitochondrial bioenergetics but additional assays are required to pinpoint more precise mechanisms of effect of TPP\(^+\) derivatives. Spectrophotometric assays of individual respiratory chain complexes in tissue homogenate enriched in mitochondrial fraction

![Graph](image)

**Table 1. Mean fluorescence intensity of TMRM in C2C12 cells treated with 1 \(\mu\)M TPP\(^+\) derivatives.**

| Treatment  | Fluorescence intensity (% of untreated control) |
|------------|-----------------------------------------------|
| DMSO       | 94.50 [87.56,101.4]                           |
| propylTPP\(^+\) | 93.75 [88.18,99.32]                         |
| heptylTPP\(^+\) | 35.50 [25.07,45.93]\(^*\)           |
| decylTPP\(^+\) | 19.25 [2.39,36.11]\(^*\)              |
| dodecylTPP\(^+\) | 18.00 [0.71,35.29]\(^*\)              |
| FCCP       | 43.50 [25.47,61.53]\(^*\)               |

Data are geometrical means of fluorescence intensity expressed as the percentage of the untreated control [95\% CI], \(n = 4\).

\(^*\) indicates \(p < 0.05\) when compared to the DMSO treated group.

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allowed us to confirm direct inhibition of all complexes by the longer-chain derivates with complex II being the least sensitive and establish dose-dependency of these effects. We also provide independent determination of a negative effect on the mitochondrial membrane potential.

The exact mechanism of respiratory chain inhibition can only be speculated about based on our data. The fact that the inhibitory effect is not specific to any one derivative or any one complex suggests a non-specific binding of the TPP⁺ derivates to the inner mitochondrial membrane, which affects membrane integrity causing both the breakdown of its insulating properties and impairment of the phospholipid milieu faced by the respiratory complexes.

In summary, TPP⁺ derivates impair mitochondrial function with an increasing potency as their hydrophobicity increases. This may help explain some effects of existing mitochondrially targeted compounds and should be taken into account when designing new ones for use as diagnostic probes or therapeutic agents.

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Author Contributions

Conceived and designed the experiments: JT ME MA. Performed the experiments: JT ME. Analyzed the data: JT ME. Wrote the paper: JT ME MA.

References

1. Smith RA, Porteous CM, Coulter CV, Murphy MP. Selective targeting of an antioxidant to mitochondria. Eur J Biochem. 1999 August; 263(3):709–716. doi: 10.1046/j.1432-1327.1999.00543.x PMID: 10469134
2. Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, et al. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. J Biol Chem. 2001 February; 276(7):4588–4596. doi: 10.1074/jbc.M009093200 PMID: 11092892
3. Filipovska A, Kelso GF, Brown SE, Beer SM, Smith RA, Murphy MP. Synthesis and characterization of a triphenylphosphonium-conjugated peroxidase mimetic. Insights into the interaction of etsbelen with mitochondria. J Biol Chem. 2005 June; 280(25):24113–24126. doi: 10.1074/jbc.M501148200 PMID: 15831495
4. Brown SE, Ross MF, Sanjuan-Pia A, Manas AB, Smith RA, Murphy MP. Targeting lipoic acid to mitochondria: synthesis and characterization of a triphenylphosphonium-conjugated alpha-lipoyl derivative. Free Radic Biol Med. 2007 June; 42(12):1766–1780. doi: 10.1016/j.freeradbiomed.2007.02.033 PMID: 17512456
5. Trnka J, Blaikie FH, Smith RA, Murphy MP. A mitochondria-targeted nitroxide is reduced to its hydroxylamine by ubiquinol in mitochondria. Free Radic Biol Med. 2008 January; 1(44):1406–1419. doi: 10.1016/j.freeradbiomed.2007.12.036
6. Kelso GF, Maroz A, Cochemé HM, Logan A, Prime TA, Peskin AV, et al. A mitochondria-targeted macrocyclic Mn(II) superoxide dismutase mimetic. Chem Biol. 2012 October; 19(10): 1237–1246. doi: 10.1016/j.chembiol.2012.08.005 PMID: 23102218
7. Murphy MP, Echtya KS, Blaikie FH, Asin-Cayuela J, Cochemé HM, Green K, et al. Superoxide activates uncoupling proteins by generating carbon-centered radicals and initiating lipid peroxidation: studies using a mitochondria-targeted spin trap derived from alpha-phenyl-N-tet-butyl nitrone. J Biol Chem. 2003 December; 278(49):48534–48545. doi: 10.1074/jbc.M308529200 PMID: 12972420
8. Hardy M, Rockenbauer A, Vásquez-Vivar J, Felix C, Lopez M, Srinivasan S, et al. Detection, characterization, and decay kinetics of ROS and thyl adducts of mito-DEPMPO spin trap. Chem Res Toxicol. 2007 June; 20(7):1059–1060. doi: 10.1021/tx700101d PMID: 17559235
9. Xu Y, Kalyaranaram B. Synthesis and ESR studies of a novel cyclic nitrone spin trap attached to a phosphonium group-a suitable trap for mitochondria-generated ROS? Free Radic Res. 2007 January; 41(1):1–7. doi: 10.1080/10707060600911147 PMID: 17164173

10. Quin C, Trnka J, Hay A, Murphy MP, Hartley RC. Synthesis of a mitochondria-targeted spin trap using a novel Parham-type cyclization. Tetrahedron. 2009 September; 65(39):8154–8160. doi: 10.1016/j.tet.2009.07.081 PMID: 1988470

11. Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, et al. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc Natl Acad Sci USA. 2006 October; 103 (41):15038–15043. doi: 10.1073/pnas.0601945103 PMID: 17015830

12. Cocheme HM, Quin C, McQuaker SJ, Cabreiro F, Logan A, Prime TA, et al. Measurement of H2O2 within living Drosophila during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. Cell Metab. 2011 March; 13(3):340–350. doi: 10.1016/j.cmet.2011.02.003 PMID: 21356523

13. Grinius LL, Jasaitas AA, Kadziauskas YP, Liberman EA, Skulachev VP, Topali VP, et al. Conversion of biomembrane-produced energy into electric form. I. Submitochondrial particles. Biochim Biophys Acta. 1970 August; 216(1):1–12. doi: 10.1016/0005-2728(70)90153-2 PMID: 4395700

14. Bakeeva LE, Grinius LL, Jasaitas AA, Kuliene VV, Levitsky DO, Liberman EA, et al. Conversion of biomembrane-produced energy into electric form. II. Intact mitochondria. Biochim Biophys Acta. 1970 August; 216(1):13–21.

15. Ross MF, Kelso GF, Blakie FH, James AM, Cocheme HM, Filipovska A, et al. Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology. Biochemistry (Mosc). 2005 Feb; 70(2):222–230. doi: 10.1074/s10541-005-0104-5

16. Ross MF, Prime TA, Abakumova I, James AM, Porteous CM, Smith RA, et al. Rapid and extensive uptake and activation of hydrophobic triphenylphosphonium cations within cells. Biochem J. 2008 May; 411(3):633–645. doi: 10.1042/Bj20080063 PMID: 18294140

17. Trnka J. Mitochondria-targeted antioxidants and spin traps [PhD Thesis]. University of Cambridge; 2008.

18. Trnka J, Blakie FH, Logan A, Smith RA, Murphy MP. Antioxidant properties of MitoTEMPOL and its hydroxylamine. Free Radic Res. 2009 January; 43(1):4–12. doi: 10.1080/10715760802582183 PMID: 19058062

19. James AM, Cochemé HM, Smith RA, Murphy MP. Interactions of mitochondria-targeted and untargeted ubi- quinones with the mitochondrial respiratory chain and reactive oxygen species. Implications for the use of exogenous ubiquinones as therapies and experimental tools. J Biol Chem. 2005 March; 280 (22):21295–21312. doi: 10.1074/jbc.M501527200 PMID: 15788391

20. Leo S, Szabadkai G, Rizzuto R. The mitochondrial antioxidants MitoE(2) and MitoQ(10) increase mito- chondrial matrix. Cell Metab. 2011 March; 13(3):340–350. doi: 10.1016/j.cmet.2011.02.003 PMID: 21356523

21. O’Malley Y, Fink BD, Ross NC, Prisinzano TE, Sivitz WI. Reactive oxygen and targeted antioxidant administration in endothelial cell mitochondria. J Biol Chem. 2006 December; 281(52):39766–39775. doi: 10.1074/jbc.M608268200 PMID: 17060316

22. Wingrove DE, Gunter TE. Kinetics of mitochondrial calcium transport. II. A kinetic description of the sodium-dependent calcium efflux mechanism of liver mitochondria and inhibition by ruthenium red and by tetraphenylphosphonium. J Biol Chem. 1986 November; 261(32):15166–15171. PMID: 2429966

23. Brand MD. Measurement of mitochondrial protonmotive force. In: Brown GC, Cooper CE, editors. Bioenergetics - A practical approach. IRL PRESS; 1995. p. 39–62.

24. Patkova J, Andel M, Trnka J. Palmitate-induced cell death and mitochondrial respiratory dysfunction in myoblasts are not prevented by mitochondria-targeted antioxidants. Cell Physiol Biochem. 2014 May; 33(5):1438–1451. doi: 10.1159/000358709 PMID: 24854098

25. Severin FF, Severina II, Antonenko YN, Rokitskaya TI, Cherepanov DA, Mokhova EN, et al. Penetrating cation/fatty acid anion pair as a mitochondria-targeted protophoroph. Proc Natl Acad Sci USA. 2010 Jan; 107(2):663–668. doi: 10.1073/pnas.0910216107 PMID: 2008732
28. Antonenko YN, Khailova LS, Knorre DA, Markova OV, Rokitskaya TI, Ilyasova TM, et al. Penetrating cations enhance uncoupling activity of anionic protonophores in mitochondria. PLoS ONE. 2013; 8(4): e61902.

29. Plecita-Hlavata L, Jezek J, Jezek P. Pro-oxidant mitochondrial matrix-targeted ubiquinone MitoQ10 acts as anti-oxidant at retarded electron transport or proton pumping within Complex I. Int J Biochem Cell Biol. 2009 Aug-Sept; 41 (8–9): 1697–1707. doi: 10.1016/j.biocel.2009.02.015 PMID: 19433311

30. Reily C, Mitchell T, Chacko BK, Benavides G, Murphy MP, Darley-Usmar V. Mitochondrially targeted compounds and their impact on cellular bioenergetics. Redox Biol. 2013; 1(1):86–93. doi: 10.1016/j.redox.2012.11.009 PMID: 23667828

31. Spinazzi M, Casarin A, Pertegato V, Salviali L, Angelini C. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. Nat protocols. 2012 May; 7(6):1235–1246. doi: 10.1038/nprot.2012.058 PMID: 22653162

32. Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, et al. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. Am J Physiol Cell Physiol. 2007 Jan; 292(1):125–136. doi: 10.1152/ajpcell.00247.2006

33. Janssen A, Trijbels F, Sengers R, Smeitink J, Heuvel L, Wintjes L, et al. Spectrophotometric Assay for Complex I of the Respiratory Chain in Tissue Samples and Cultured Fibroblasts. Clinical Chemistry. 2007; 53(4):729–734. doi: 10.1373/clinchem.2006.078873 PMID: 17332151

34. Luo C, Long J, Liu J. An improved spectrophotometric method for a more specific and accurate assay of mitochondrial complex III activity. Clinica Chimica Acta. 2008; 395:38–41. doi: 10.1016/j.cca.2008.04.025

35. Cooperstein SJ, Lazarow A. A microspectrophotometric method for the determination of cytochrome oxidase. The Journal of Biological Chemistry. 1951; 189(2):665–670. PMID: 14832284

36. Scaduto RC, Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J. 1999 January; 76(1 Pt 1):469–477. doi: 10.1016/S0006-3495(99)77214-0 PMID: 9876159

37. Floryk D, Houstek J. Tetramethyl rhodamine methyl ester (TMRM) is suitable for cytofluorometric measurements of mitochondrial membrane potential in cells treated with digitonin. Biosci Rep. 1999 February; 19(1):27–34. doi: 10.1023/A:1020193906974 PMID: 10379904

38. Nicholls DG. Flourescence measurement of mitochondrial membrane potential changes in cultured cells. Methods Mol Biol. 2012; 810:119–133. doi: 10.1007/978-1-61779-382-0_8 PMID: 22057564

39. Murphy PM. Selective targeting of bioactive compounds to mitochondria. Trends Biotechnol. 1997 August; 15(8):326–330. doi: 10.1016/S0167-7799(97)01068-8 PMID: 9263481

40. Cerletti P, Strom R, Giordano MG. Reactivation of succinic dehydrogenase by phospholipids. Biochem Biophys Res Commun. 1965 January; 18:259–263. doi: 10.1016/0006-291X(65)90750-3 PMID: 14282027

41. Fry M, Green DE. Cardiolipin requirement by cytochrome oxidase and the catalytic role of phospholipid. Biochem Biophys Res Commun. 1980 April; 93(4):1238–1246. doi: 10.1016/0006-291X(80)90622-1 PMID: 6249285

42. Fry M, Green DE. Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. J Biol Chem. 1981 February; 256(4):1874–1880. PMID: 6257690