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Selective Uncoupling of Individual Mitochondria within a Cell Using a Mitochondria-Targeted Photoactivated Protonophore

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Supporting Information

ABSTRACT: Depolarization of an individual mitochondrion or small clusters of mitochondria within cells has been achieved using a photoactivatable probe. The probe is targeted to the matrix of the mitochondrion by an alkyltriphenylphosphonium lipophilic cation and releases the protonophore 2,4-dinitrophenol locally in predetermined regions in response to directed irradiation with UV light via a local photolysis system. This also provides a proof of principle for the general temporally and spatially controlled release of bioactive molecules, pharmacophores, or toxins to mitochondria with tissue, cell, or mitochondrion specificity.

Mitochondria are central to eukaryotic cells. As well as consuming ~95% of the O_2 inspired to generate most of the cell's ATP, they control cell death, contain much of central metabolism, and modulate calcium and redox signaling. Consequently there is considerable interest in designing mitochondria-targeted molecules to report on and manipulate mitochondrial function.[1] The use of lipophilic cations such as the alkyltriphenylphosphonium (TPP) moiety has enabled the delivery of a wide range of molecules to mitochondria without the need to alter genes or protein structure.[1,2] However, this procedure is not selective for particular mitochondria within an organism. The challenge remains of how to use this system to deliver molecules to mitochondria within individual tissues or cells or to individual mitochondria in a cell at a specific time. The latter point is significant as the organization and localization of mitochondria within cells is important for their function[3−7] and subpopulations of mitochondria contribute differently to cell processes depending on their location within the cell.[6−11] Furthermore, temporal control is vital to understanding cellular mechanisms, so that transient changes can be observed and causal relationships established. To address this need we have developed a procedure to target molecules to mitochondria by coupling them to the lipophilic TPP cation via a photocleaveable linker,[12] enabling the release of the molecule within chosen mitochondria at a specific moment by selective irradiation of those mitochondria.

As a first example of this class of molecule here we describe MitoPhotoDNP, which can release a protonophore within selected mitochondria thereby stopping ATP production in and calcium ion uptake by a chosen single mitochondrion or localized subpopulation of mitochondria within a cell (Figure 1).

MitoPhotoDNP has three constituent parts, a caged protonophore, a photocleavable linker and a mitochondria-targeting unit. We chose to target a protonophore to mitochondria as this is a way of selectively decreasing the mitochondrial membrane potential, which is at the heart of mitochondrial function. To produce ATP by oxidative phosphorylation the mitochondrial electron transport chain uses the oxidation of substrates by oxygen to pump protons out of the matrix to generate a proton electrochemical potential gradient across the mitochondrial inner membrane (MIM), which is composed mainly of a membrane potential (150−170 mV, negative inside). Protons flow down this gradient through the ATP synthase to form ATP. Ca^{2+} uptake into the mitochondria through the calcium uniporter is also driven by the membrane potential and provides a mechanism of modulating the mitochondrial and cytosolic Ca^{2+} concentration locally, which is necessary for many cellular processes.[13] Oxidation can be uncoupled from ATP synthesis and Ca^{2+} transport by dissipating the membrane potential, typically by mildly acidic lipophilic compounds, which act as protonophores. These protonophores, or uncouplers, are deprotonated in the mitochondrial matrix to form lipophilic anions, which can cross the MIM, pick up a proton, and return, so abolishing the membrane potential and switching off mitochondrial ATP production. The classic mitochondrial uncoupler is dinitrophenol (DNP)[14−16] (pK_a = 4.1[17]) and in MitoPhotoDNP DNP is caged by linking to an *o*-nitrobenzyl group, which is a well-tried photoactivatable linker.[14,18,19] The TPP cation is included as a mitochondria-targeting group because it easily permeates biological membranes due to its hydrophobicity and large ionic radius, and it accumulates several hundred fold in the mitochondrial matrix within cells *in vivo* due to the large membrane potential across the MIM, in accordance with the Nernst equation.[1,2] It also has very low toxicity; indeed, a TPP-containing antioxidant, MitoQ, has been fed long-term to both animals and humans without toxicity at its therapeutic dose.[20]

The most scalable and reproducible synthesis of MitoPhotoDNP involved alkylation of a commercially available phenol **1**

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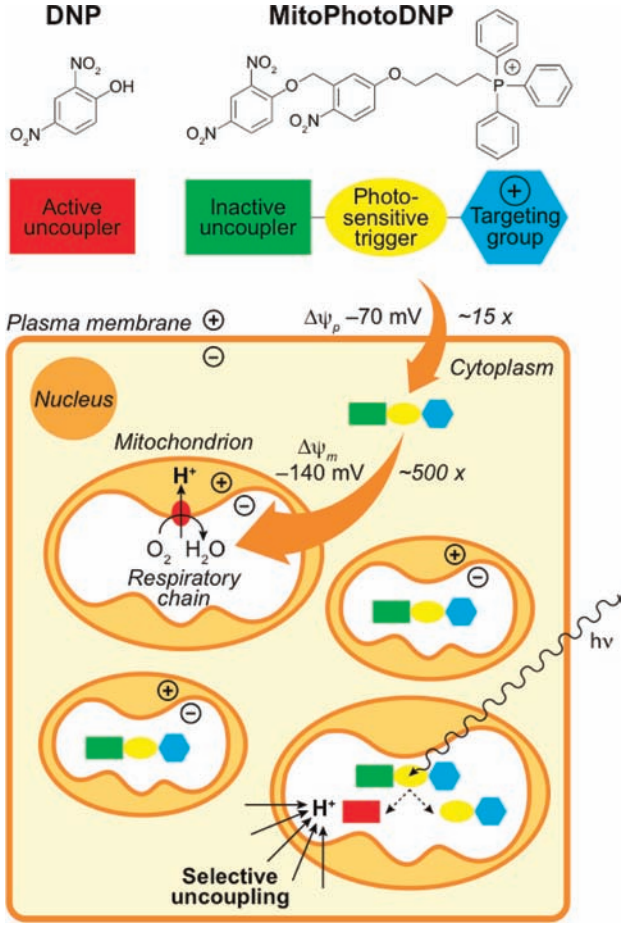
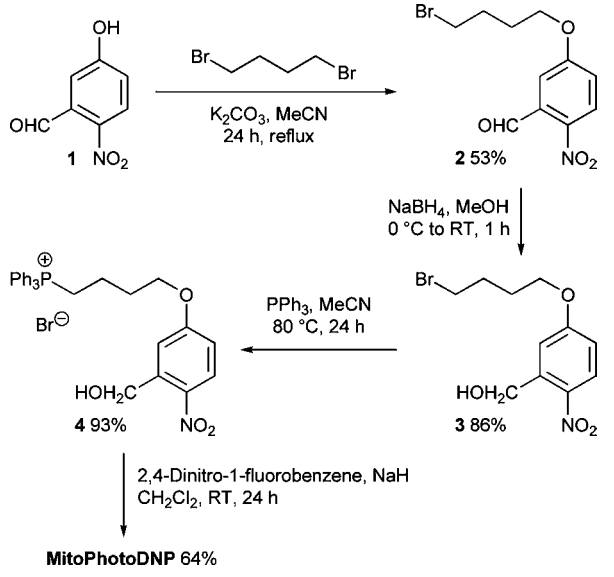


Figure 1. Schematic representing MitoPhotoDNP accumulation within the mitochondrial matrix of all mitochondria and selective activation within a single mitochondrion to depolarize the inner membrane and uncouple electron transport from ATP synthesis and calcium transport.

(Scheme 1), and then reduction of the resulting aldehyde **2** to give alcohol **3**. This was converted into the phosphonium salt **4**

Scheme 1. Synthesis of MitoPhotoDNP



and the caged uncoupler introduced to complete the synthesis of MitoPhotoDNP. UV irradiation of a solution of MitoPhotoDNP in $CDCl_3$ led to release of DNP, detected by ^{1}H NMR spectroscopy.

To assess whether MitoPhotoDNP was taken up by mitochondria in response to the membrane potential across the inner membrane we constructed an electrode that responded selectively to the TPP moiety of MitoPhotoDNP.[21] When mitochondria were added in the presence of the respiratory inhibitor rotenone to prevent development of a membrane potential there was a decrease in the concentration of MitoPhotoDNP due to the expected adsorption of the hydrophobic TPP compound to mitochondrial membranes.[22] Subsequent addition of the respiratory substrate succinate led to the rapid and extensive accumulation of MitoPhotoDNP by energized mitochondria, and this was reversed by addition of FCCP [carbonylcyanide 4-(trifluoromethoxy)-phenylhydrazone] to abolish the mitochondrial membrane potential.[14,23] This semiquantitative approach showed that MitoPhotoDNP is rapidly and extensively accumulated by energized mitochondria in response to the membrane potential, as expected for a TPP cation.[1,2]

Next, we demonstrated that MitoPhotoDNP can be photoactivated within cells to uncouple selectively an individual mitochondrion or a small group of mitochondria. Freshly isolated colonic smooth muscle cells were loaded with the mitochondrial membrane potential-sensitive dye tetramethylrhodamine ethyl ester (TMRE) and showed a punctate fluorescent staining of the mitochondria, consistent with energized mitochondria with high membrane potentials. A short (85 ms) irradiation of site 1 with UV at 355 nm in the absence of MitoPhotoDNP did not lead to any change in the pattern of TMRE fluorescence indicating no change in mitochondrial membrane potential in response to UV alone (Figure 2, i). There was no change when the cells were exposed to a low concentration of MitoPhotoDNP (200 nM), indicating that MitoPhotoDNP itself did not disrupt mitochondrial function. However, when site 2 was irradiated with UV light in the presence of MitoPhotoDNP, there was localized loss of TMRE fluorescence (red region in panel ii). The depolarization was maintained for the duration of the experiment, which was typically limited to 30 min, a period during which there was little TMRE bleaching and cell performance was unaltered. When all of the cell's mitochondria were depolarized with rotenone an irreversible loss of TMRE fluorescence was observed throughout the cell (panel iii), confirming that the depolarization caused by MitoPhotoDNP and UV light was selective. In control experiments, smooth muscle cells treated with 200 nM MitoPhotoDNP for the same length of time (30 min) showed no alterations in gross cellular morphology, basal cytosolic [Ca^{2+}] or responsiveness to calcium-generating agonists, and the compound was not toxic to C2C12 myoblasts following chronic exposure (overnight) to concentrations below 5 μM.

The mitochondrial depolarization remained localized and was not reversed during the period for which observation of an individual mitochondrion is feasible (up to 1 h). Further experiments confirmed that the sustained depolarization was not the result of local depletion of ATP or mitochondrial metabolites. Cells were patch clamped[3] in whole cell configuration and using the access afforded by the electrode, ATP (3 mM), phosphocreatine (5 mM), malate (2.5 mM), and pyruvate (2.5 mM) were introduced into the cell. Irradiation of

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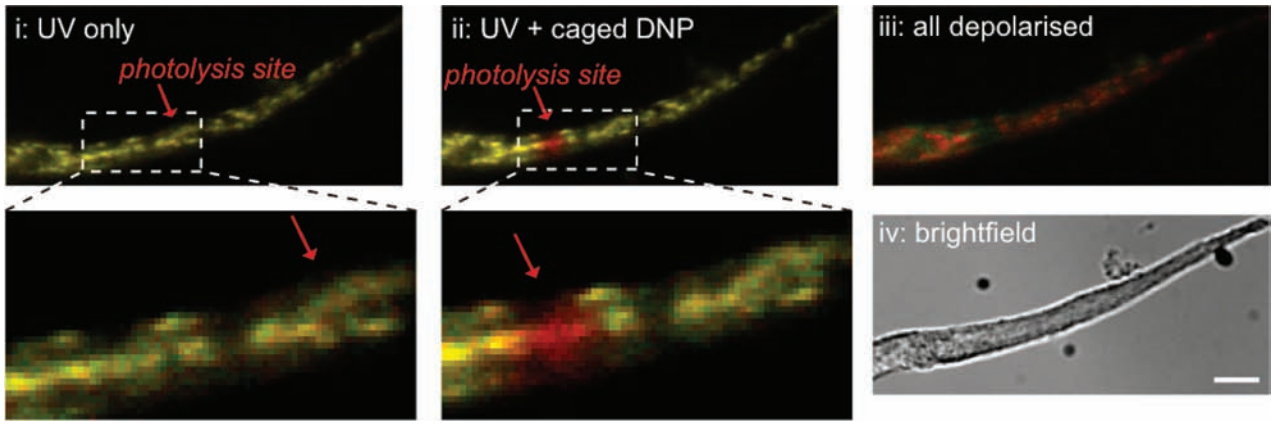

Figure 2. Localized mitochondrial depolarization following localized photolysis of MitoPhotoDNP. Freshly isolated colonic smooth muscle cells loaded with TMRE (10 nM) and wortmannin (10 μM, to prevent cell contraction) display a punctate fluorescent staining (i). The cell displayed underwent (1) a brief, localized exposure to UV laser light (355 nm for 85 ms in region shown in panel i in the absence of MitoPhotoDNP; (2) MitoPhotoDNP (200 nM) was washed into the cell's bathing medium, allowed to equilibrate for 15 min and then a second region of the cell was exposed to UV light (85 ms, in region shown in panel ii); (3) finally the mitochondrial inhibitors rotenone (2 μM) plus oligomycin (3 μM) were washed into the bathing medium (panel iii). TMRE fluorescence was detected before and after each of the three treatments and any differences highlighted by overlaying artificially colored images in which "before" is red and "after" is green, such that no change results in yellow regions, loss of TMRE staining (hence mitochondrial depolarization) results in red regions, and any gain of fluorescence results in green. A bright-field image of the cell plus 10 μm scale bar is shown in panel iv. This is a typical result of more than 20 similar experiments.

a region of the cell again resulted in depolarization that was localized and sustained, and a flickering event[4] in the unaffected region further confirmed that if repolarization had occurred, it would have been observed (see Supporting Information).

The several-hundred-fold accumulation of MitoPhoto-DNP within the mitochondrial matrix in cells is vital for the selective depolarization of mitochondria. This is supported by the fact that addition of 200 nM DNP has no effect on mitochondrial membrane potential, with 90 μM DNP being required to decrease the ratio of fluorescence of TMRE to Mitotracker (a mitochondrial dye whose uptake is not reversed upon mitochondrial membrane depolarization) to half maximal within 10 min. The importance of targeting is further supported by the finding that no UV-induced depolarization of mitochondria was observed when an untargeted photoactivatable DNP **5** (Figure 3) was employed at a concentration

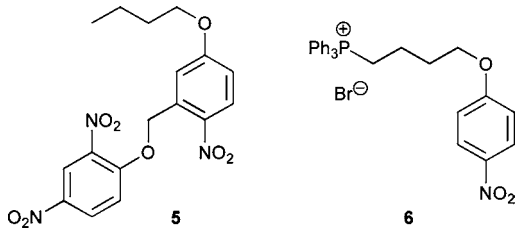

Figure 3. Control compounds.

of 200 nM or even when used at a concentration of 30 μM (an observation which is consistent with the limited effectiveness of an earlier untargeted photoactivatable uncoupler[24]).

While UV alone does not cause depolarization of mitochondria, it could be suggested that the TPP targeting group or the nitroarene trigger unit of MitoPhotoDNP, in conjunction with UV, sensitized mitochondria to photodamage and thus led to uncoupling. This possibility was eliminated by incubating cells with 3 μM of the TPP-targeted nitro compound **6** (Figure 3) that cannot release an uncoupler and showing that the mitochondria were not depolarized upon irradiation. As well as releasing DNP, irradiation of MitoPhotoDNP will also generate TPP-conjugated side products from the caging group. To determine whether these side products could affect mitochondrial polarization, cells were incubated with 3 μM TPP-conjugated *o*-nitrobenzylic alcohol **4** and irradiation of a small cluster of mitochondria as before gave no depolarization. *o*-Nitrobenzylic alcohol **4** is expected to release water upon irradiation, and HPLC confirmed that it gives a similar distribution of TPP-conjugated side-products to MitoPhoto-DNP under the irradiation conditions.

In summary, in this proof of concept study, we have shown that we can locally activate a mitochondria-targeted compound within mitochondria inside a cell by combining a mitochondria-targeting group with a photolyzable linker to deliver a cargo. We showed that this effectively delivered a protonophore to mitochondria within cells and thereby led to the selective uncoupling of either individual or a small number of mitochondria within a cell when used in conjunction with fluorescence imaging. This demonstrated exquisite spatial and temporal control of mitochondrial function. The probe itself should also allow the investigation of the role of subpopulations of mitochondria in controlling local ATP supply and calcium ion regulation in specific parts of cells, particularly in highly differentiated cells such as neurons and smooth muscle cells. In neurons, for example, mitochondria cluster at locations with high energy demand and control subplasma membrane $[Ca^{2+}]$ to manage exocytosis in nerve terminals[25] and adrenal chromaffin cells,[26] but current techniques that involve inhibiting mitochondrial activity throughout the entire cell cannot elucidate the effect of the localized activity of the organelle. Thus, we expect MitoPhotoDNP to be an important investigational tool for relating intracellular organization of mitochondria to complex functions. More generally, our approach could be extended to deliver selectively a wide range of functional molecules including specific inhibitors or activators of mitochondrial processes to study intracellular processes and also provide a means of introducing tissue or cell specificity to mitochondria-targeted pharmacophores not previously achievable.

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■ ASSOCIATED CONTENT

Supporting Information

Complete refs 2a and 20b; experimental details for the preparation of **4**–**6** and MitoPhotoDNP; UV–vis spectra; NMR spectra showing release of DNP from MitoPhotoDNP in response to UV irradiation; electrode responses for uptake of MitoPhoto-DNP into isolated mitochondria; details of experiments with intact cells (including pictures and a movie); HPLC of irradiated MitoPhotoDNP and **4**; toxicity tests; and ^{1}H and ^{13}C NMR spectra of all compounds synthesized. This material is available free of charge via the Internet at http://pubs.acs.org).

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