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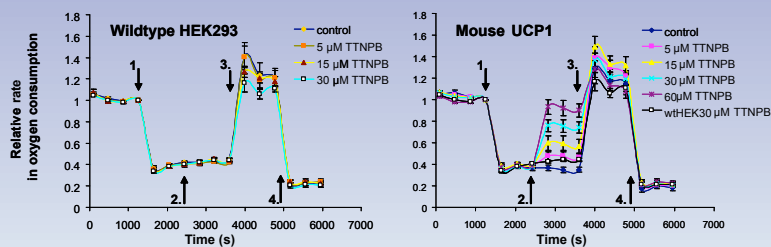
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Introduction

Mammalian uncoupling protein 1 (UCP1), and putatively the novel paralogous proteins UCP2 and UCP3, dissipate proton motive force by uncoupling substrate oxidation from ATP synthesis. UCP1 enables brown fat mitochondria to increase proton conductance and respire at maximal rates in the absence of ATP-synthesis thus resulting in heat production¹. The physiological role of other UCPs is still debated² and as these cannot be activated with fatty acids, they may have a function different from UCP1. Their physiological role may be unravelled by identifying modulating molecules that regulate their function. To solely preserve an environment that may be essential to display physiological protein function, we aimed to measure bioenergetic parameters (respiration and mitochondrial membrane potential) in intact cells. In this study, we tested the suitability of novel *in situ* techniques in HEK293 cells that ectopically express mouse UCP1 to evaluate UCP1 function in the intact cell. These techniques will contribute significantly to identifying functional regulators of other UCPs assisting in the elucidation of their physiological roles.

A. Effect of the retinoic acid analog TTNPB on leak respiration of mouse UCP1 expressing HEK293 cells measured by plate-based respirometry using the Seahorse XF24 Extracellular Flux Analyzer

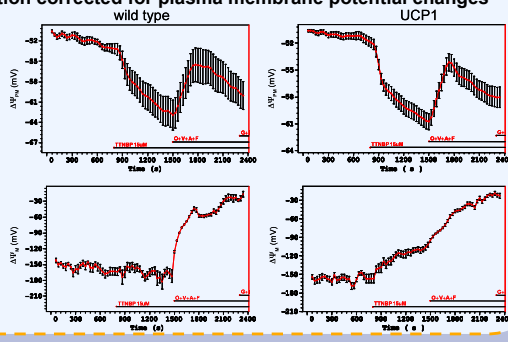


Graphs show oxygen consumption rates normalized to basal respiration. Wildtype and mouse UCP1 HEK293 cells were grown on Seahorse V7 microplates. At the start of each measurement, basal respiration was determined, subsequently oligomycin was added to inhibit the ATP synthase (1), then different concentrations of TTNPB (2), then FCCP (3) to completely uncouple mitochondria and finally a rotenone/myxothiazol (4) cocktail to determine non-mitochondrial respiration. **Notably, TTNPB increased leak respiration in UCP1-containing cells but not in wildtype.**

B. Effect of TTNPB on mitochondrial membrane potential of HEK293 cells expressing mouse UCP1 assessed by mitochondrial TMRM accumulation corrected for plasma membrane potential changes

Plasma membrane ($\Delta\psi_{PM}$) and mitochondrial membrane potentials ($\Delta\psi_M$) were followed by wide field fluorescence microscopy of PMPI and TMRM*. Fluorescence values were converted to millivolts by a compartment model based deconvolution method.

15 μ M TTNPB decreased mitochondrial membrane potential selectively in UCP1 containing cells (see lower panel).
Valinomycin/oligomycin/FCCP/antimycin A cocktail served to completely dissipate $\Delta\psi_M$ and gramicidine/iodoacetate, to discharge $\Delta\psi_{PM}$ allowing calculation of absolute potential values.



Conclusions and Outlook

TTNPB is a known activator of UCP1, so far only investigated in isolated tissue and yeast mitochondria³. Here, we show that state 4 respiration of HEK293 cells ectopically expressing mouse UCP1 can be selectively increased with very low amounts of TTNPB under *in vivo* conditions (A).

By simultaneous measurements of plasma/mitochondrial membrane potential, we confirmed that the increase in state 4 respiration is due to uncoupling activity (B): TTNPB lowered mitochondrial membrane potential in UCP1 cells thus dissipating proton motive force.

This study demonstrates that UCP1 function can be accessed *in situ* in a cell culture system and provides a system for future studies searching for novel modulators of UCP1 activity and exploring potential regulators of other UCPs with so far unknown function.

Materials and Methods

HEK293 stably expressing mouse UCP1 — HEK cells were transfected with pcDNA3.1 vector (INVITROGEN) containing the coding sequence of mouse UCP1 (NM 009463). A stable transfected clone was kindly provided by the Philipps Universität of Marburg.

Measurement of cell respiration — 1×10^6 cells were seeded on a Seahorse V7 microplate and grown for 48h prior to the experiment. Cells were washed with Seahorse assay buffer (3.5 mM KCl, 120 mM NaCl, CaCl₂·H₂O, 0.4 mM KH₂PO₄, 1.2 mM Na₂SO₄, 20 mM TES, 5 mM NaHCO₃, 15 mM D-Glucose, 2 mM MgSO₄, 15 mM Na-Pyruvate, 0.4% defatted BSA, pH 7.4), incubated for an hour and assayed in the XF24 flux analyzer (Seahorse biosciences). Basal respiration was monitored, injections were made of: 4 μ M oligomycin to shift mitochondrial respiration in state 4, then TTNPB at different concentrations or buffer, then 4 μ M FCCP to achieve maximum respiration rates and rotenone/myxothiazol to measure non-mitochondrial respiration).

Measurement of mitochondrial membrane potential — Cells were incubated in Seahorse assay buffer two hours prior to experiments. $\Delta\psi_M$ was determined and separated from $\Delta\psi_{PM}$ by a modification of the technique described by David Nicholls⁴ (A.A.G. unpublished results). TMRM* (10 nM) plus tetraphenylboron (1 μ M) and the plasma membrane potential indicator PMPI (Molecular Devices #R0242 FLIPR Membrane Potential Assay Kit at 1:200 dilution of the Loading Buffer*) were included into the assay buffer. PMPI is a lipophilic anion, therefore it is expelled from the cytosol by the $\Delta\psi_{PM}$, exhibiting Nernstian distribution with a charge of -1. The extracellular fluorescence of PMPI is attenuated by a quencher reagent, therefore the PMPI fluorescence measures the amount of dye present in the cytosol. TMRM* is a lipophilic cation distributed across the plasma and mitochondrial membranes according to the Nernst equation with a charge of +1. To calculate $\Delta\psi_{PM}$ and $\Delta\psi_M$ the Nernstian behavior and the slow redistribution of the fluorophores through the plasma membrane is considered:

$$\frac{\partial [PMPI]_c}{\partial t} = k_p [PMPI]_{ec} - k_p e^{-\frac{\Delta\psi_{PM}}{zF}} [PMPI]_c, \quad \frac{\partial [TMRM]_c}{\partial t} = k_r e^{-\frac{\Delta\psi_M}{zF}} [TMRM]_{ec} - k_r a_c [TMRM]_c, \quad \Delta\psi_{PM} = -b \ln \frac{a_c [TMRM]_c}{a_c [TMRM]_c}, \quad b = \frac{RT}{zF}$$

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