**Mitochondrial stress test using LUHMES cells**

Introduction

Aim

This protocol describes the analysis of how to assess the utilized and spare mitochondrial activity of LUHMES cells (human neuronal).

Purpose

Many toxic substances impair mitochondrial function. Also many compounds in research for pharmaceutical use inherit mitochondrial off-target effects. This assays aims to identify direct or indirect impairment of mitochondrial function.

Limitations

This assay asses only the mitochondrial and non-mitochondrial oxygen consumption, but gives not directly an explanation why these parameters might be impaired. E.g. mitochondrial pyruvate uptake inhibitors might result in the same reduction in oxygen consumption as complex I inhibitors. Additionally, redox cyclers might result in the same increased oxygen consumption as mitochondrial uncouplers. Therefore follow-up experiments might be needed.

Method outline

Cells are cultured in Seahorse assay plates (100,000 cells per well in 24 well plates) and allowed to equilibrate to their environment. At the day of the assay, their normal cell culture medium gets replaced by Seahorse assay medium, supplemented with pyruvate (1 mM), glucose (18 mM), glutamine (2 mM), N2 supplement (1x) and tetracycline (2.25 µM) 1 h prior to the assay.

Then the Agilent Seahorse Mitostress test is performed according to the manufacturer’s recommendation. Port A is used for the injection of the compound of interest or the solvent control, while ports B-D are used for oligomycin, FCCP and rotenone/antimycin a, respectively.

Finally, mitochondrial oxygen consumption is analyzed simultaneously in treated samples relative to solvent control samples, based on the oxygen consumption using the Seahorse device, as the manufacturer recommends.

Method description

Chemicals and buffers

Agilent Seahorse Mitostress test:

Oligomycin: final concentration on cells is 1 µM

FCCP: final concentration on cells is 1.5 µM

Rotenone/antimycin A: final concentration on cells is 0.5/0.5 µM

Agilent Seahorse basal DMEM

Glucose, pyruvate, glutamine, N2 supplement and tetracycline

Preparation upfront

* Hydrate seahorse cartridge
* Have cells at in coated (normal PLO/fibronectin LUHMES coating, optionally with additional laminin) Seahorse plates ready, cultured at least 18 h in these plates for equilibration
* Substrates and tool compounds

Experimental procedure

1. Change the normal cell culture medium to the assay medium, place the plate in a 37°C non-CO2 incubator for at least 1 h before the start of the experiment
2. Prepare the cartridge with the compounds solutions (10x solution in ports A/B/C/D, 56 µl/62 µl/69 µl/77 µl, respectively), incubate in a 37°C non-CO2 incubator for at least 1 h before the start of the experiment
3. Set up the Seahorse analyzer and the measurement program, use mix/wait/measure times of 3/2/3 minutes, respectively. Enable “calibrate” and “equilibrate”
4. Calibrate the cartridge and when the instrument is ready, the utility plate is removed from the analyzer and, while the cartridge remained in the instrument.
5. Finally, the cell culture plate was inserted into the XF analyzer and the assay was started.

Data analysis

* Each experiment has to contain control treated (solvent) samples
* Oxygen levels have to be checked before deeper data analysis (mmO2, not OCR!)
* If oxygen doesn’t get depleted, the data can be analyzed.
* First, basal respiration of all wells gets normalized to the last measurement value before compound injection via port A
* Then the oxygen consumption rate of the first measurement cycle after each tool inhibitor injection undergoes comparison. Inhibition results in OCR values smaller in treated samples than in control samples, uncoupling vice versa.
* Compound effects are expressed as percent inhibition relative to control
* The manufacture’s Excel sheets were used for data analysis.