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Palmitate treated human hepatocellular carcinoma HuH7 cells require higher digitonin concentration for plasma membrane permeabilization

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Introduction

Mitochondrial physiology analysis is central to cell metabolism research and understanding of many human diseases. High resolution respirometry represents an efficient approach to study mitochondrial physiology in isolated mitochondria, both permeabilized and intact cells, as well as in tissue biopsy samples. We use digitonin for controlled plasma membrane permeabilization to perform extended functional OXPHOS analysis by O2k Oxygraph in palmitate treated cells, an *in vitro* model of insulin resistance and lipotoxicity. Digitonin has high affinity for cholesterol, thus low digitonin concentration will completely and selectively permeabilize plasma membrane (richer in cholesterol), compared to mitochondrial and endoplasmic reticulum membranes (low in cholesterol), so the latter are affected only at higher digitonin concentrations. It is known that molecular composition of plasma membrane differs between cell types and that modification in plasma membrane lipid composition occurs in tumor cells [1]. Thus, every cell line requires previous optimization of adequate digitonin concentration, which is sufficient to permeabilize plasma membrane and which allows entrance of mitochondrial substrates and inhibitors, while keeping mitochondria intact and functional in their intracellular environment.

Methods

HuH7 (human hepatocellular carcinoma) cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/l), L-Glutamine and Sodium Pyruvate, supplemented with 10% fetal bovine serum (FBS) and 1% of antibiotic/antimycotic mixture (all ordered from Capricorn Scientific). Cells were seeded in 100 mm Petri dishes (0.6×10^6 cells) and next day exposed to chronic palmitate treatment: 24h at 0.4 mM. Palmitic acid (Sigma-Aldrich) was dissolved at 80 mM in absolute ethanol, then diluted (at 37°C) to 3.2 mM in DMEM with 10% FBS and 20% BSA.After treatment cells were detached with trypsin/EDTA, resuspended in mitochondrial respiration medium MiR05 (EGTA 0.5 mM, $MgCl_2\times6H_2O$ 3 mM, lactobionic acid 60 mM, taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, D-sucrose 110 mM, BSA 1g/l, pH 7.1) and number of live cells

was estimated with trypan blue exclusion test. Substrate-uncoupler-inhibitor titration (SUIT) protocol was adapted from [2]: after optimal digitonin concentration, pyruvate (8.8 mM), malate (4.4 mM), ADP (2.5 mM), rotenone (0.5 µM), succinate (10 mM), antimycinA (2.5 µM) were added. Digitonin was optimized acoording to the protocol [3,4]. All reagents for high-resolution respirometry were purchased from Sigma-Aldrich. The viability assays were done according to [5], acid phosphatase converts p-nitrophenil phosphate to p-nitrophenol which was detected by measuring absorbance at 405 nm, absorbance value is directly proportional to number of viable cells. Data for calculation of respirometric viability index described in [4] was taken from digitonin optimization protocol and inserted into formula 1-(S-Rot)/(Dig-Rot) giving the percent of viable cells in O2k chamber.

Results and discussion

We found that optimal number of HuH7 cells for respirometry is 0.5×10^6 per O2k chamber, having ROUTINE respiration of approximately 81±17 pmol O_2/(s*10^6 cells). Optimal digitonin concentration was 3 µg per chamber (2 ml) with 0.5×10^6 cells [1.5 µg/mL]. Using palmitate-treated cells in SUIT protocol, we noticed that digitonin concentration optimal for control cells was insufficient to achieve adequate permeabilization of palmitate treated cells plasma membrane (Fig. 1A) and that they require an additional digitonin optimization. Figure 1B shows plots from digitonin optimization protocol done in parallel in control and palmitate-treated cells. According to this experiment, optimal digitonin concentration for permeabilization of control cells was 1.5 µg/mL whereas for palmitate treated cells it was 3 µg/mL. Finally, in Figure 2 plots from the SUIT protocol with optimal digitonin concentration in treated and untreated cells are shown. The time necessary for digitonin to induce permeabilization can be observed as a decrease in respiration due to dilution of endogenous substrates upon plasma membrane permeabilization, as shown in Figure 2.

Chronic palmitate treatment (0.4 mM, 24 h) caused decrease in cell viablity to 77±17%, estimated by acid phosphatase assay. Even though in palmitate-treated cells prepared for respirometry analysis in MiR05, trypan blue dye-exclusion test showed 90±3% viable cells (most of dead cells were washed out with PBS prior to trypsinization). The calculated respirometric viability index was 0.56±0.15 (56% of viable cells). Because of this toxic effect of palmitate, one would expect that treated cells are more prone to digitonin permeabilization. On the contrary, palmitate-treated cells needed double the digitonin concentration compared to controls. One of the possible explanations might be that chronic palmitate treatment somehow affects composition and/or dynamics of plasma membrane, including cholesterol content or exposure, thus influencing digitonin affinity. However, whether this phenomenon is present in different cell types warrants further investigation.

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Figures

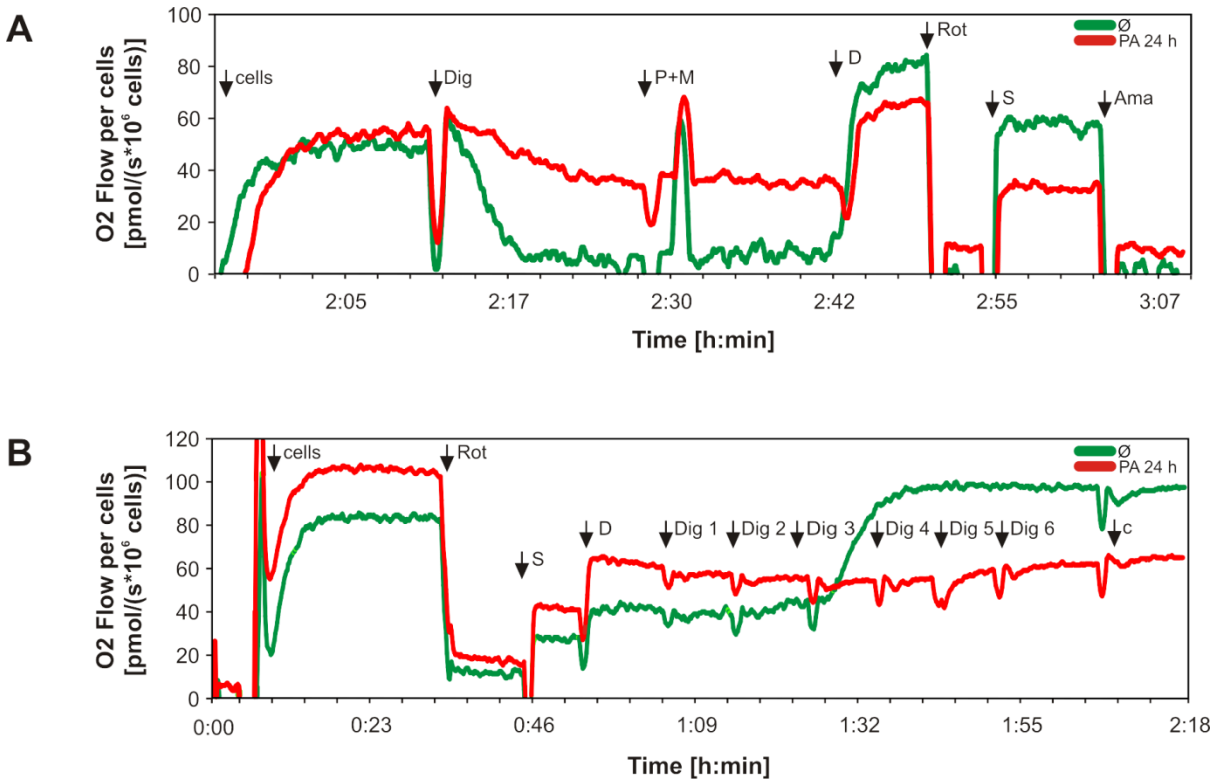

Figure 1. Digitonin concentration optimal for control cells was insufficient to achieve adequate permeabilization of palmitate treated HuH7 cells. Plots from SUIT protocol (A) and digitonin optimization protocol (B) done in palmitate treated (PA 24 h)-red plot and control cells –green plot. (A) An insufficient permeabilization of treated cells can be observed as higher respiration after digitonin (Dig) titration, caused by presence of endogenous substrates inside the cells. Titrations were made in following order: Dig (digitonin), Pyr+Mal (Pyruvate + Malate), D (ADP), Rot (Rotenone), S (Succinate), Ant A (Antimycin A). (B) Increase in respiration after adding Rot, S, D and Dig is more slowly in treated cells and requires six titration (Dig 1-6) for respiration to increase, while in controls respiration increases after third titration (Dig 1-3). Every digitonin titration contains 1 µg of digitonin. Cytochrome C (c) was added as a final step to check the intactness of mitochondria.

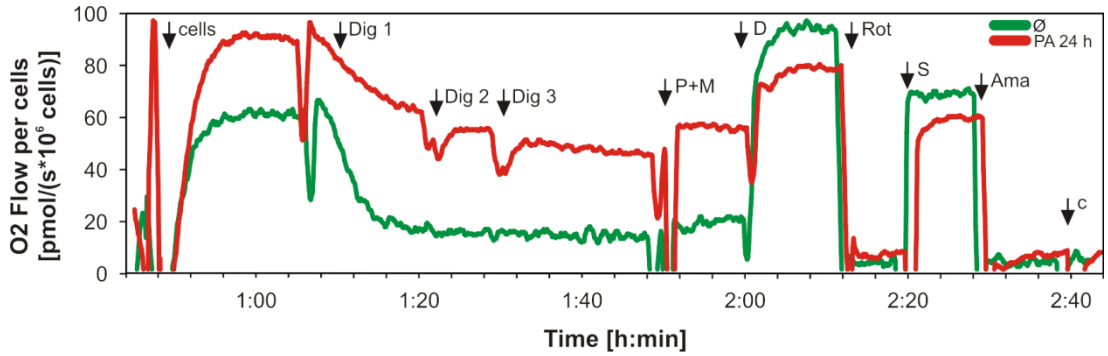

Figure 2. Palmitate treated HUH7 cells need two times higher digitonine concentration than controls, in order to be sufficiently permeabilized for SUIT protocol. Dig 1 (3 µg of digitonine in control cells and 4 µg in palmitate treated (PA 24h) cells), Dig 2 and Dig 3 (1 µg of Dig each titration) added only to PA 24h cells. Optimal Dig concentration was 1.5 µg/mL for HuH7 controls and 3 µg/mL for palmitate treated cells per 0.5×10^6 per O2k chamber. Titrations were made in the following order: Dig (digitonin), Pyr+Mal (Pyruvate + Malate), D (ADP), Rot (Rotenone), S (Succinate), Ant A (Antimycin A), and c (Cytochrome C) was added at the end to check intactness of mitochondria.