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Measurement of mitochondrial respiration in trophoblast culture

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Abstract

Pregnancy is a state of oxidative stress, which becomes exaggerated under pathological conditions, such as preeclampsia, IUGR, diabetes and obesity, where placental mitochondrial dysfunction is observed. The majority of investigations utilize isolated mitochondria when measuring mitochondrial activity in placenta. However, this does not provide a complete physiological readout of mitochondrial function. This technical note describes a method to measure respiratory function in intact primary syncytiotrophoblast from human term placenta.

Keywords

placenta; mitochondria; trophoblast; oxygen consumption; hypoxia

Introduction

The placenta transports gases and nutrients between mother and fetus, and releases hormones that influence maternal metabolism and fetal growth and development [1]. The transport and endocrine functions are mainly carried out by the syncytiotrophoblast, formed by fusion of mononuclear cytotrophoblasts [2–3]. The increasing metabolic activity of placental mitochondria throughout gestation results in excessive production of reactive oxygen species (ROS) and oxidative stress [4–5], which becomes exaggerated in pregnancies complicated by preeclampsia [6–7], IUGR [8–9], gestational diabetes [10] and maternal obesity [11–12]. Several lines of evidence indicate that placental mitochondrial dysfunction is central to these pathological conditions [13–14]. In addition to being a major site for ROS production, mitochondria also become a target for ROS-induced damage and can be severely compromised by prolonged oxidative stress. Thus, mitochondrial abnormalities and ROS formation could be part of a vicious cycle and represent a mechanism of placental dysfunction. Given the crucial roles of mitochondria in normal and complicated pregnancies, the importance of being able to comprehensively assess mitochondrial function cannot be overemphasized.

Most investigations use measurement of single mitochondrial enzymes as indices of mitochondrial function. However, this does not provide a complete physiological readout of mitochondria. Important concerns are the disruption of the mitochondrial three-dimensional

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network or reticular structure [15] and lack of interaction with other cellular compartments (e.g., sarcoplasmic reticulum, cytoskeleton, lipid droplets) following the isolation of mitochondria [16].

Recent evidence suggests that mitochondrial morphology is closely associated with various functional aspects [17]. As such, we postulate that standard mitochondrial isolation procedures could have quite dramatic effects on mitochondrial function.

The goal of the present work was to establish a method to measure mitochondrial respiratory function in intact syncytiotrophoblast cells from human term placenta. We profiled mitochondrial function under normal and hypoxic conditions using a XF24 Extracellular Flux Analyzer from Seahorse Bioscience.

Materials and Methods

Materials

Oligomycin, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone FCCP, and Antimycin A solutions were obtained from Seahorse (Billerica, MA) as the Mito Stress Test Kit; Desferrioxamine (DFO) was purchased from Sigma.

Cytotrophoblast preparation and culture

All tissues were collected after obtaining informed consent under a protocol approved by the UTHSCSA Institutional Review Board. Cytotrophoblasts were purified from the placenta of uncomplicated term pregnancies obtained immediately after c-section (in the absence of labor) as we have previously described [18] and plated in Seahorse XF 24 well plates.

Measurement of cellular energetics

Cellular energetics were measured using a Seahorse Bioscience XF24 extracellular flux analyzer as described previously [19]. Data are expressed as the rate of oxygen consumption (OCR) in pmol/min. Total cellular protein was measured following each experiment by the Bradford method to normalize mitochondrial oxygen consumption.

Statistical Analysis

Data are reported as means $\pm$ SEM. Comparisons between two groups were performed with Student's *t*-test. One-way analysis of variance (ANOVA) with Bonferroni correction was used where appropriate. The null hypothesis was rejected when $p < 0.05$.

Results and Discussion

All cell cultures were predominantly trophoblast being 90–92% cytokeratin-18 positive and less than 9% positive for vimentin (data not shown). In the first series of experiments, the optimal number of cells needed to obtain a measurable and reproducible OCR was established. OCR increased with increasing cell number from 1.25×10^5 to 1×10^6 per well (Figure 1A), after which high variability in oxygen consumption and increased cell death were observed. A seeding density of 8×10^5 gave the best resolution for basal and maximal values of OCR and was chosen for the remainder of the experiments.

In many studies with the XF24, a straightforward bioenergetic assay, a stress test, has been used [20–21]. This assay uses inhibitors of respiratory complexes and uncoupling agents to examine and quantify different components of mitochondrial function. The general scheme of stress test is shown in Figure 1B. To estimate the proportion of the basal OCR coupled to ATP synthesis, ATP synthase (Complex V) was inhibited by oligomycin (0.5 μ M). It

decreased the OCR rate to the extent to which the cells are using mitochondria to generate ATP. The remaining OCR is ascribed to proton leak across the mitochondrial membrane. To determine the maximal OCR that the cells can sustain, the proton ionophore FCCP (0.75 μM) was injected. Lastly, antimycin A (1.5 μM) was injected to inhibit electron flux through Complex III which causes a dramatic suppression of the OCR. The remaining OCR is attributable to O_2 consumption due to the formation of mitochondrial ROS and non-mitochondrial sources. The reserve respiratory capacity was calculated as the maximal rate minus the basal rate and presents a parameter which is available to cells for increased work to cope with stress.

To assess the effect of syncytialization on cellular energetics, the stress test was performed as described on both single cytotrophoblasts 24 hours after plating and syncytiotrophoblast at 72hr (Fig. 1C), and individual parameters were calculated (Fig. 1D). Syncytiotrophoblasts showed significantly higher levels of basal and non-mitochondrial respiration, and ATP coupling when compared to cytotrophoblasts but with no change in maximal respiration and reserve capacity.

Finally, we examined the effects of chemically-induced hypoxia on mitochondrial bioenergetics by exposing the cells for 24 hours to different concentrations of the iron chelating agent desferrioxamine (DFO), which induces the accumulation of hypoxia-inducible factor-1 α protein [22–24] (Fig. 2). DFO treatment significantly reduced the maximal flux through electron transport chain in a concentration-dependent manner from 25 μM to 200 μM . The trophoblasts exposed to DFO maintained, however, the basal (up to 200 μM of DFO) and ATP-linked oxygen consumption.

In summary, we showed here that the XF24 extracellular flux analyzer presents a reliable tool to address a number of questions related to placental mitochondrial competence.

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Figure 1.

A, Optimization of cell number for measuring OCR. Basal OCR was measured and plotted as a function of cell seeding number (triplicate measurements at each cell density taken from same culture of primary syncytiotrophoblasts and representative of 3 individual experiments).

B, Mitochondrial Stress Test. OCR was measured under basal conditions followed by the sequential addition of oligomycin (1 μg/ml), FCCP (1 μM), and antimycin A (10 μM), as indicated. Each data point represents an OCR measurement as mean ± SE (n=6 cultures of primary syncytiotrophoblast each from a different placenta).

C, D, Effect of syncytialization of cytotrophoblasts on mitochondrial respiration. C, OCR was measured by sequential addition of inhibitors as shown in panel B on single cytotrophoblast cells at 24 hrs and syncytialized trophoblasts at 72 hrs after plating. D, Individual parameters for basal respiration, ATP coupling, non-mitochondrial OCR, reserve capacity, and maximal respiration were derived from the assay in panel C, mean ± SE, *p < 0.05 vs. 24 hours measurements, n=6 placentas.

Figure 2. Effect of addition of Desferrioxamine (DFO) on mitochondrial function

Cytotrophoblasts were seeded at 8×10^5 cells/well in Seahorse XF 24 well plates. DFO treatment was initiated at 42 hrs after plating, continued for 24 hrs and cell bioenergetics analyzed by XF 24. Following basal OCR readings, OCR was measured with oligomycin (0.5 μM), FCCP (0.75 μM) and Antimycin A (1.5 μM), n=6 cultures of primary syncytiotrophoblast each comes from a different placenta), *p<0.005 vs. untreated control.