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Original research article

The profiles of mitochondrial respiration and glycolysis using extracellular flux analysis in porcine enterocyte IPEC-J2

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ABSTRACT

The porcine intestinal mucosa require large amounts of energy for nutrient processing and cellular functions and is vulnerable to injury by weaning stress involving bioenergetics failure. The mitochondrial bioenergetic measurement in porcine enterocytes have not been defined. The present study was to establish a method to measure mitochondrial respiratory function and profile mitochondrial function of IPEC-J2 using cell mito stress test and glycolysis stress test assay by XF24 extracellular flux analyzer. The optimal seeding density and concentrations of the injection compounds were determined to be 40,000 cells/well as well as 0.5 μM oligomycin, 1 μM carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) and 1 μM rotenone & antimycin A, respectively. The profiles of mitochondrial respiration and glycolysis confirmed that porcine enterocyte preferentially derived much more energy from glutamine than glucose. These results will provide a basis for further study of mitochondrial function and bioenergetics of the porcine small intestine.

1. Introduction

The porcine intestinal mucosa requires large amounts of energy for nutrient processing and other basic cellular functions and accounts for 25% of total body oxygen consumption (Vaugelade et al., 1994). At the same time, the intestinal mucosa is vulnerable to injury in morphology and function after weaning, which may involve bioenergetics failure induced by mitochondrial dysfunction and subsequently short energy supply (Lacza et al., 2001; Tan et al., 2010, 2015; Wang et al., 2013, 2015; Xiao et al., 2013). Xiong et al. (2015) demonstrated that early-weaning lead to the down-regulations of proteins involved in the tricarboxylic acid cycle, beta-oxidation and the glycolysis pathway in the upper and middle villus of piglets but up-regulations of glycolysis-related proteins. Intestinal epithelia cell restitution including crypt cell proliferation, differentiation and migration after injury appear to depend strongly on energy availability during the first 2 days post-weaning (Lallès et al., 2004; Van Beers-Schreurs et al., 1998).

Mitochondria are considered as the major suppliers of ATP to maintain biological function. Substrates such as oxygen, glucose, glutamine, and so on were uptaken and subsequently conversed into energy through a series of enzymatically controlled oxidation and reduction reactions, resulting in the production of ATP (Nicholls et al., 2010). Some methods have applied to measure mitochondrial function and dysfunction and the extracellular flux analysis is the best assay for intact cells (Brand and Nicholls, 2011; Salabei et al., 2014), but the mitochondrial bioenergetic measurement in porcine enterocytes have not been defined. Therefore, the aim of the present study was to establish a method to measure mitochondrial respiratory function and profile mitochondrial

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function of IPEC-J2 under normal conditions using extracellular flux analysis, which will provide a basis for further study of mitochondrial function and bioenergetics of the porcine small intestine.

2. Materials and methods

2.1. Materials

XF cell mito stress test kit including oligomycin, carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP), rotenone & antimycin A and XF glycolysis stress test kit including glucose, oligomycin, 2-deoxyglucose (2-DG) were obtained from Seahorse Bioscience Inc. (Billerica, MA, USA). XF24 cell culture plates, sensor cartridgeas and XF base medium were also purchased from Seahorse Bioscience Inc. Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) and fetal bovine serum (FBS) were procured from GIBCO (Langley, OK, USA) and plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). Unless indicated differently, all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell preparation

IPEC-J2 cells were obtained from GuangZhou Jennio Biotech Co., Ltd (Guangzhou, China). Cells were seeded in XF24 cell culture plates in 100 μL growth medium (DMEM-HG medium containing 10% FBS) and placed in 37°C incubator with 5% CO_2. After cells adhere within 3 h, add 150 μL growth medium and return to 37°C incubator with 5% CO_2. For optimizating cell number, 10,000, 20,000, 40,000 or 80,000 cells/well were seeded and then an optimal seeding density was used for other experiments.

2.3. Cell mito stress test assay

After a 24-h incubation, growth medium from each well were removed, leaving 50 μL of media. And cells were washed twice with 1,000 μL of pre-warmed assay medium (XF base medium supplemented with 25 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate; pH 7.4) and 1,000 μL was removed as above then 475 μL of assay medium (525 μL final) was added. For the study of energy substrates on mitochondrial respiration, XF base medium (Energy-), XF base medium supplemented with individual glutmaine, glucose, sodium pyruvate or all these energy substrates (Energy+) were replaced assay medium.

Cells were incubated in 37°C incubator without CO_2 for 1 h to allow to pre-equilibrate with the assay medium. Load pre-warmed oligomycin, FCCP, rotenone & antimycin A into the injector ports A, B and C of sensor cartridge, respectively. The final concentrations of injections were as follows: optimizating cell number experiment, 0.25 μM oligomycin, 1 μM FCCP, 1 μM rotenone & antimycin A; optimizating oligomycin experiment, 0, 0.25, 0.5 or 1 μM oligomycin, 1 μM FCCP, 1 μM rotenone & antimycin A; optimizating FCCP experiment, 0.5 μM oligomycin, 0, 1, 2 or 4 μM FCCP, 1 μM rotenone & antimycin A; experiment of energy substrates on mitochondrial respiration, 0.5 μM oligomycin, 1 μM FCCP, 1 μM rotenone & antimycin A.

The cartridge was calibrated by the XF24 analyzer (Seahorse Bioscience, Billerica, MA, USA), and the assay continued using cell mito stress test assay protocol as described by Nicholls et al. (2010). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were detected under basal conditions followed by the sequential addition of oligomycin, FCCP, as well as rotenone & antimycin A. This allowed for an estimation of the contribution of individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production (Tan et al., 2015).

2.4. Glycolysis stress test assay

After a 24-h incubation, the media was changed to assay medium (XF base medium DMEM supplemented with 2 mM glutamine), and cells were incubated in a non-CO_2 incubator at 37°C for 1 h before the assay. Injections of glucose (10 mM final), oligomycin (0.5 μM final) and 2-DG (100 mM final) were diluted in the assay medium and loaded into ports A, B and C, respectively. The machine was calibrated and the assay was performed using glycolytic stress test assay protocol as suggested by the manufacturer (Seahorse Bioscience, Billerica, MA, USA). ECAR was measured under basal conditions followed by the sequential addition of 10 mM glucose, 0.5 μM oligomycin, and 100 mM 2-DG (Glu > Oli > 2-DG). Assay medium was injected instead of glucose, oligomycin and 2-DG serving as the control. This allowed for an estimation of the contribution of individual parameters for non-glycolytic acidification, glycolysis, glycolytic capacity, glycolytic reserve of IPEC-J2.

2.5. Data analysis

The XF mito stress test report generator and the XF glycolysis stress test report generator automatically calculate the XF cell mito stress test and XF glycolysis stress test parameters from Wave data that have been exported to Excel. Respiration and acidification rates are presented as the mean ± SEM of 4 independent experiments in all experiments performed with 4 to 10 replicate wells in the Seahorse XF24 analyzer. For experiment of energy substrates on mitochondrial respiration, significance level was determined by performing ANOVA on the complete data set with Tukey's post-hoc testing. The results were considered significant at $P < 0.05$.

3. Results

3.1. Optimization of XF assay for IPEC-J2

The seeding density and concentrations of the injection compounds were optimized. The OCR was increased with increasing cell number from 10,000 to 80,000 per well under basal condition, but begans to level off at 40,000 cells for FCCP stimulated OCR rates. For both basal and FCCP stimulated rates, the ECAR value also does not increase from 40,000 to 80,000 cells (Fig. 1). A seeding density of 40,000 cells/well was chosen for the subsequent experiments.

After oligomycin injection, the OCR was decreased with the increasing the concentration of oligomycin from 0 to 0.5 μM, but 1 μM did not decrease the OCR compared with 0.25 or 0.5 μM (Fig. 2A). For FCCP stimulated OCR rates, the higher peak point at 1 μM was observed and increasing the concentration of FCCP beyond 1 μM did not increase the OCR (Fig. 2B). Therefore, the optimal concentrations for oligomycin and FCCP were determined to be 0.5 and 1 μM, respectively, for the subsequent experiments.

3.2. Effects of energy substrates on mitochondrial respiration of IPEC-J2

The general scheme of mitochondrial stress test is shown in Fig. 3A. Sequential injections of oligomycin, FCCP, rotenone and antimycin A measure basal respiration, ATP production, proton leak,