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Analysis of respiratory capacity in brain tissue preparations: high-resolution respirometry for intact hippocampal slices

Cândida Dias[a], Cátia F. Lourenço[a], Rui M. Barbosa[a,b], João Laranjinha[a,b], Ana Ledo[a,*]

[a] Center for Neuroscience and Cell Biology, University of Coimbra, Portugal
[b] Faculty of Pharmacy, University of Coimbra, Portugal

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ABSTRACT

The evaluation of mitochondrial function provides the basis for the study of brain bioenergetics. However, analysis of brain mitochondrial respiration has been hindered by the low yield associated with mitochondria isolation procedures. Furthermore, isolating mitochondria or cells results in loss of the inherent complexity of the central nervous system. High-resolution respirometry (HRR), is a valuable tool to study mitochondrial function and has been used in diverse biological preparations ranging from isolated mitochondria to tissue homogenates and permeabilized tissue biopsies. Here we describe a novel methodology for evaluation of mitochondrial respiration using tissue preparations from the central nervous system, namely acute hippocampal slices from rodents, with HRR. By using acute intact hippocampal slices, tissue cytoarchitecture, intercellular communication and connectivity are preserved. Mitochondrial respiration was evaluated by using an adapted substrate-uncoupler-inhibitor titration (SUIT) protocol and the expected responses were observed. This methodology can be used to detect differences in mitochondrial function at the oxidative phosphorylation level and for studies with different brain oxidative substrates in physiological and neuropathological settings, by using a system that better represents the *in vivo* conditions than isolated mitochondria and/or cells.

Introduction

The brain is an organ with high index of metabolic activity and although it comprises only 2% of the human body mass, its oxygen and glucose requirements amount to 20% and 25% of the total body consumption, respectively [1]. This high energy demand results mainly from the maintenance and restoration of ion gradients, generation of action potentials and neurotransmitter uptake [2].

Mitochondrial oxidative phosphorylation, a major source of ATP, encompasses the transfer of electrons from NADH and $FADH_2$ (produced during glycolysis, fatty acid oxidation and the tricarboxylic acid chain) to oxygen (O_2) through an electron transport chain. Mitochondria also play several other key roles in the cell, including production and modulation of reactive oxygen species (ROS) and maintenance of calcium homeostasis, besides actively participating in apoptosis [3,4]. Furthermore, changes in mitochondrial function have been reported in several neurological and neurodegenerative disorders [5,6], hence the importance of studying mitochondrial function.

One widely used method for the evaluation of respiratory states in biological samples is the observation of changes in respiratory rates resulting from substrate-uncoupler-inhibitor-titration (SUIT) protocols, i.e., the successive addition of different substrates and drugs to either increase or inhibit the activity of the different complexes of the electron transport chain. Using a closed chamber, the measurement of the O_2 consumption rate can be accomplished with several methods, including the use of O_2 electrodes. The Clark electrode, developed by Leland Clark [7], employs amperometry to measure O_2 concentration within a closed chamber and has since been largely used in bioenergetics studies, mainly using isolated mitochondria. The Oxygraph-2k system (Oroboros Instruments, Innsbruck, Austria), comprises two separate amperometric O_2 sensors and allows the measurement of [O_2] with high resolution and accuracy, overcoming putative limitations associated with classical Clark-type electrodes [8–10]. The chamber design and construction materials combined with the O_2 sensor and electronics (highly stable temperature regulation) guarantee higher stability and lower noise of the amperometric signal resulting from the O_2 reduction reaction [8,11]. The requirement for smaller amounts of sample increases time resolution, as the rate of O_2 depletion from the medium is decreased [11].

The SUIT protocols used in respirometry studies require access of substrates and drugs to the complexes of the mitochondrial electron transport chain, leading most researchers to use isolated mitochondria

* Corresponding author. Center for Neuroscience and Cell Biology, Faculty of Medicine, University of Coimbra, Rua Larga, 3004-504, Coimbra, Portugal.
E-mail address: analedo@cnc.uc.pt (A. Ledo).

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in their studies [12–15]. However, the study of mitochondrial respiration in discrete structures of the rodent brain, such as the hippocampus, has been hindered by the impossibility of isolating adequate amounts of mitochondria to perform classical bioenergetics studies. The use of whole tissue preparations coupled to high-resolution respirometry affords a powerful tool to study mitochondrial respiration and metabolic fluxes in the brain tissue, because only a small amount of tissue (5–10 mg) is required to perform a SUIT protocol [16]. Furthermore, this approach allows the maintenance of cytoarchitecture, inter-cellular communication and connectivity. An additional advantage of using whole tissue preparations is that it avoids the loss of the physiological environment that occurs during isolation of mitochondria, under which both mitochondrial morphology and function are altered [17]. Mitochondria are extremely dynamic organelles and multiple mechanisms allow the maintenance of their homeostasis and function, including fission, fusion and movement through the cytoskeleton, among others [18]. Comparative studies using isolated mitochondria and permeabilized tissue have produced disperse results - aging, for example, has been shown to produce more pronounced effects on mitochondrial function when measurements were carried out in isolated mitochondria as compared to those found in permeabilized tissue [19]. Although permeabilized cells or tissue may be a better option as compared to isolated mitochondria, there is still loss of the physiological environment with permeabilization. The issues that arise from isolation or permeabilization can be overcome by using intact cells [20,21]. While maintaining mitochondria in a physiological environment, the use of intact cells presents other limitations, such as access of substrates and drugs to mitochondria, and the lack of intercellular interaction remains an issue of concern. The use of intact tissue, while challenging, is advantageous in the sense that cell-to-cell interactions are preserved.

The hippocampus is a brain structure of the medial temporal lobe involved in memory and learning [22] and is particularly affected in several neurodegenerative diseases. By using intact hippocampal slices as opposed to isolated mitochondria or neurons/astrocytes, tissue cytoarchitecture, intercellular communication and connectivity are maintained. Oxygen consumption in hippocampal slices has been measured before [23–27]. Here, we present a method that allows the study of brain bioenergetics intact tissue using high-resolution respirometry.

Materials and methods

Chemicals and reagents

Fatty acid free bovine serum albumin (FAF-BSA) and carboxyatractyloside were obtained from Calbiochem. Magnesium chloride hexahydrate was purchased from Fluka, sodium chloride from OmniPur, and carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) from Enzo Life Sciences. Carbox gas mixture (O_2/CO_2 95:5) was obtained from Linde. All other chemicals were analytical-grade and purchased from Sigma-Aldrich.

Media and solutions

The medium used in experiments was artificial cerebrospinal fluid (aCSF) with the following composition (in mM): 124 NaCl, 2 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 D-glucose, 10 sodium pyruvate. The medium was additionally supplemented with 20 mM HEPES for pH buffering and 5 mg/mL FAF-BSA to facilitate drug diffusion. For tissue dissection and recovery period, a modified aCSF with the following composition (in mM) was used: 124 NaCl, 2 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 1.5 $CaCl_2$, 0.2 L-ascorbic acid, 1 L-glutathione reduced, and 10 D-glucose. All aqueous solutions and media were prepared using ultra-pure deionized water ($R \geq 18.2\,M\Omega\,cm$), obtained from a Milli-Q system (Millipore Integral 10, MA, USA). Oligomycin (4 mg/mL), FCCP (10 mM), rotenone (1 mM) and antimycin A (5 mM) stock solutions were prepared with ethanol. Carboxyatractyloside (5 mM), succinate (1 M), sodium azide (4 M) and propidium iodide (1.5 mM) stock solutions were prepared in ultra-pure deionized water.

Animals

All the procedures used in this study were performed in accordance with the European Union Council Directive for the Care and Use of Laboratory Animals, 2010/63/EU and approved by local ethics committee (ORBEA). In the present work, we used 7 female Wistar rats (aged 8–10 weeks) and 5 male CD-1 mice (aged 8 weeks). All animals were purchased from Charles River Laboratory (Barcelona, Spain) and maintained at the local animal facility (Faculty of Medicine, University of Coimbra). Rooms for housing were kept under controlled environment: a temperature of 22–24 °C, a relative humidity of 45–65%, 15 air exchanges per hour and a 12:12 light/dark cycle. Animals were fed with a standard rodent chow and were provided chlorinated water, both *ad libitum*. Cage bedding of standard corn cob was changed three times a week and environmental enrichment was provided with tissue paper and a cardboard tube.

Hippocampal slices

Wistar rats (under anesthesia, isoflurane) and CD-1 mice were killed by cervical displacement. Following decapitation, the brain was rapidly removed and placed in ice-cold modified aCSF. The hippocampi were dissected and 250-μm (rat) or 400-μm (mouse) transverse slices were prepared using a vibratome (Vibroslice, World Precision Instruments, Inc., UK). The thickness of tissue for each species was optimized as to allow a compromise between tissue viability (ratio between surface damage and healthy core tissue) and access of substrates/drugs to the tissue core. The chamber of the vibratome was filled with ice-cold modified aCSF and continuously bubbled with humidified carbox (95% O_2/5% CO_2) for pH buffering (pH 7.4) and oxygenation during slice preparation. Slices were then transferred into a pre-incubation chamber (BSC-PC, Harvard Apparatus) containing modified aCSF at room temperature, also continuously bubbled with carbox and allowed to recover for at least 1 h under these conditions.

High-resolution respirometry

Tissue oxygen consumption rate was measured by high-resolution respirometry with the Oxygraph-2k system (OROBOROS Instruments, Innsbruck, Austria), at 32 °C. Air calibration was performed as described in the instrument manual. A manufactured slice-holder was used to sustain the tissue in the chamber during the recording to avoid slice damage by the stirring bar present in the recording chamber. This holder consisted of a mesh glued with cyanoacrylate onto a polypropylene plastic ring with a diameter of 1.6 cm (Fig. 1A) and a height of 3 mm. The holder positioning in the chamber is shown in Fig. 1B (fitted between the stirring bar and the stopper).

The DatLab software allows for a correction for instrumental background. This was performed prior to experiments with the same aCSF medium used during experiments and with each manufactured holder in place in the respective chamber. Initially, the O_2 concentration of the medium was increased to values near saturation with carbox. After stabilization, the O_2 concentration was decreased by briefly opening the chamber. This background correction experiment was performed within the O_2 levels used in the experiments (~750–900 nmol/mL), and stable fluxes were obtained for approximately 900, 750 nmol/mL and two concentrations in between these two values.

Measurements were carried out with continuous stirring (750 rpm) in 2 mL of aCSF supplemented with HEPES and FAF-BSA and at high O_2 concentration (between 750 and 900 nmol/mL) to ensure proper