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Comparative Biochemistry and Physiology, Part A 142 (2005) 111 – 117

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Differential requirements of calcium for oxoglutarate dehydrogenase and mitochondrial nitric-oxide synthase under hypoxia: Impact on the regulation of mitochondrial oxygen consumption☆

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Received 28 January 2005; received in revised form 26 April 2005; accepted 5 May 2005
Available online 21 June 2005

Abstract

Studies with isolated mitochondria are performed at artificially high pO_2 (220 to 250 μM oxygen), although this condition is hyperoxic for these organelles. It was the aim of this study to evaluate the effect of hypoxia (20–30 μM) on the calcium-dependent activation of 2-oxoglutarate dehydrogenase (or 2-ketoglutarate dehydrogenase; OGDH) and mitochondrial nitric-oxide synthase (mtNOS). Mitochondria had a P/O value 15% higher in hypoxia than that in normoxia, indicating that oxidative phosphorylation and electron transfer were more efficiently coupled, whereas the intramitochondrial free calcium concentrations were higher (2–3-fold) at lower pO_2. These increases were abrogated by ruthenium red indicating that the higher uptake via the calcium uniporter was involved in this process. Mitochondria at high calcium concentration microdomains may produce nitric oxide, given the $K_{0.5}$ of calcium for OGDH (0.16 μM) and mtNOS (~1 μM). Nitric oxide, by binding to cytochrome oxidase in competition with oxygen, decreases the rate of oxygen consumption. This condition is highly beneficial for the following reasons: i, these mitochondria are still able to produce ATP and support calcium clearance; ii, it prevents the accumulation of ROS by slowing the rate of oxygen consumption (hence ROS production); iii, the onset of anoxia is delayed, allowing oxygen to diffuse back to these sites, thereby ameliorating the oxygen gradient between regions of high and low calcium concentration. In this way, oxygen depletion at the latter sites is prevented. This, in turn, assures continued aerobic metabolism which may involve the activated dehydrogenases.

Keywords: Calcium; Cytochrome oxidase; Dehydrogenases; Hypoxia; Krebs cycle; Mitochondria; Nitric oxide; Oxygen; Mitochondrial nitric-oxide synthase; Normoxia; Nitric-oxide synthase

1. Introduction

Nitric oxide was first recognized as endothelial-derived relaxing factor in the mid-1980s (Ignarro et al., 1987; Martin et al., 1986). Since then, nitric oxide has been implicated in a variety of physiological responses. Aside from the role of nitric oxide in vascular regulation, this species is also involved in neural communication and in the immune host-defense system (Bredt and Snyder, 1994; Griffith and Stuehr, 1995).

The enzyme responsible for nitric-oxide formation is nitric-oxide synthase (NOS). NOS catalyzes the oxygen- and NADPH-dependent oxidation of L-Arg to nitric oxide and L-citrulline. Constitutively expressed NOS isoforms, eNOS and nNOS, are regulated by calcium/calmodulin (Bredt and Snyder, 1990; Abu-Soud and Stuehr, 1993). Calmodulin binds to the linker between the heme and reductase domains, resulting in a conformational switch that enables the flow of electrons from FMN to heme (Abu-Soud

☆ From: ***Nitric Oxide: Comparative aspects of respiratory and cardiovascular homeostasis***, an SEB symposium, organised by Prof. Bruno Tota (University of Calabria, Italy) and Prof. Tobias Wang (University of Aarhus, Denmark)—Capri, Italy, September 2004.

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doi:10.1016/j.cbpb.2005.05.004

and Stuehr, 1993). The iNOS, however, is controlled at the level of transcription and calmodulin is a permanent bound subunit (Cho et al., 1992).

It has been reported that the primary target for nitric oxide is guanylate cyclase. Activation of guanylate cyclase by nitric oxide (Ignarro et al., 1982) is directly related to well-known porphyrin-nitric oxide chemistry. Guanylate cyclase converts GTP to the signalling molecule cGMP (Hobbs, 1997) leading to the observed physiological effects of nitric oxide.

However, there are two examples in which nitric oxide exhibits its effect without interacting with guanylate cyclase. In the immune system nitric oxide acts as some other reactive product such as peroxynitrite (Huie and Padmaja, 1993; Beckman et al., 1990). The second example is the reversible inhibition of cellular respiration by nitric oxide, attained by binding to cytochrome oxidase in competition with oxygen (Brown and Cooper, 1994; Cleeter et al., 1994; Giulivi, 1998). This can be efficiently achieved by stimulating mitochondria to produce nitric oxide for they are endowed with a mitochondrial nitric-oxide synthase (mtNOS; Bates et al., 1995, 1996; Kobzik et al., 1995; Ghafourifar and Richter, 1997; Giulivi et al., 1998; Kanai et al., 2001; Giulivi, 2003). The mtNOS has been identified as the nNOS isoform alpha in rat liver mitochondria, precluding a novel alternative spliced product (Elfering et al., 2002). It is likely coded by the same gene as nNOS since neuronal nos (−/−) mice have no mtNOS (Kanai et al., 2001).

As a constitutive NOS isoform, mtNOS, requires calcium for its full activation (Ghafourifar and Richter, 1997; Tatoyan and Giulivi, 1998). We investigated the dual effect of calcium on mitochondrial oxygen consumption: on the one hand, calcium enhances the activity of calcium-sensitive mitochondrial dehydrogenases resulting in an increased oxygen consumption by mass action at the electron transport chain. On the other hand, calcium by activating mtNOS, decreases the oxygen consumption via the competitive binding of nitric oxide to cytochrome oxidase. These opposite effects of calcium were resolved by evaluating the concentration of calcium required to give half-maximum activation ($K_{0.5}$) for each process (Traaseth et al., 2004). The activation of dehydrogenases occurred at very low intra-mitochondrial free calcium concentrations ($K_{0.5}$ of 0.16 μM), whereas that of mtNOS required higher concentrations ($K_{0.5}$ of about 1 μM; Traaseth et al., 2004). All these higher concentrations fit within physiological concentrations, and are below those required to compromise any mitochondrial component or function (Andreyev and Fiskum, 1999; Traaseth et al., 2004).

A critical point that was not considered in our previous study was the effect of oxygen on these enzymatic activations. Typical studies with isolated mitochondria are performed at artificially high partial pressure of oxygen at air saturation (220 to 250 μM oxygen). This condition is effectively hyperoxic for these organelles. Therefore, oxygen-dependent reactions whose rates are negligible at the low oxygen pressure (2–3 mmHg) that mitochondria experience in tissues (Oshino et al., 1975), may become relevant under hyperoxic conditions, resulting in conclusions that may not be applicable to a physiological situation. For instance, the rate of superoxide anion generation by mitochondria in State 4 at 20 μM oxygen is one-sixth of that obtained at 250 μM (Boveris, 1977). Considering the promotion of ROS generation in hyperoxia, this shows the effects of oxidative stress may be over-emphasized when the 250 μM oxygen environment is extrapolated as a physiological model.

It is the aim of this study to evaluate the effect of hypoxia (20–30 μM) on the calcium-activation of mitochondrial dehydrogenases and mtNOS' activities, and compare these results with those obtained under normoxic conditions (220 μM).

2. Materials and methods

2.1. Chemicals and Biochemicals

All reagents used were of analytical grade. All chemicals and biochemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for sucrose and magnesium chloride (Mallinckrodt, Phillipsburg, NJ, USA), fatty-acids free bovine serum albumin (ICN Biochemicals, Irvine, CA, USA), EGTA (ACROS Organics, Belgium), and fura-2 (Molecular Probes, Eugene, OR, USA).

2.2. Isolation of rat liver mitochondria

Liver mitochondria were isolated from adult Wistar rats (*Rattus norvegicus*, 180–200 g) by differential centrifugation (Giulivi et al., 1998). The medium used to isolate mitochondria contained 220 mM mannitol, 70 mM sucrose, 2 mM HEPES (pH 7.4), 0.1% fatty acid-free albumin, and 0.5 mM EGTA. This medium at 0–4 °C prevents calcium uptake and/or redistribution that may occur during isolation (McCormack and Denton, 1984; McCormack, 1985; Traaseth et al., 2004). From this enriched-mitochondrial fraction, mitochondria were purified by Percoll gradient (Giulivi et al., 1998), subsequently washed with 150 mM KCl. Protein was determined by the Lowry assay using bovine serum albumin as a standard (Lowry et al., 1951).

2.3. Determination of respiratory rates

Rates of oxygen consumption were measured polarographically at 25 °C with a Clark oxygen electrode (Hansatech Instruments Limited, Kings Lynn, Norfolk, UK). Although 25 °C is not a physiological temperature for mitochondria, this setting was chosen because of the following reasons: first, the data could be compared to our previous measurements performed at 220 μM oxygen (Traaseth et al., 2004); second, even though according to