OROBOROS INSTRUMENTS
high-resolution respirometry

O2k-Protocols

NO effect on mitochondrial oxygen kinetics at low oxygen

Oxygraph-2k Workshop Report (IOC22)
University of Alabama at Birmingham
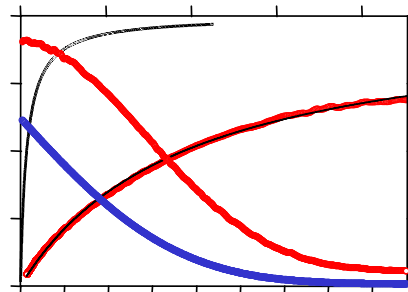
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Summary: A single pilot experiment was carried out during an Oxygraph-2k workshop on high-resolution respirometry (IOC22). Respiration of isolated rat liver mitochondria was inhibited by addition of NO, which increased the sensitivity to oxygen >25-fold when compared to the half-saturation oxygen pressure, p_{50}, in the absence of NO. Oxygen kinetics followed a monophasic hyperbolic function up to 2.2 kPa with NO (p_{50}=0.93 kPa), compared to the standard oxygen range to 1.1 kPa without NO (p_{50}=0.035 kPa).

1 Introduction

Nitric oxide, NO, is a reversible inhibitor of cytochrome *c* oxidase (COX), the terminal oxygen acceptor in the mitochondrial respiratory chain (Cleeter et al. 1994; Brown, Cooper 1994). Inhibition is highly effective with a K_i' of about 60 nM NO at an oxygen concentration of 30 µM (Brown, Cooper 1994; Koivisto et al. 1997). As a

result, it is assumed that *in vivo* part of cytochrome c oxidase is inhibited even at basal (nanomolar) NO concentrations (Brown, Cooper 1994; Clementi et al. 1999; Brunori et al. 1999). Two groups have put forward models for the competition between O_2 and NO in order to explain the uncommonly high reactivity of NO: Torres et al. (1995) suggested a unique reactivity of NO for the reduced Cu_B, whereas Giuffrè et al. (1996; Brunori et al. 1999) proposed a preferential binding of NO to reduced cytochrome a_3.
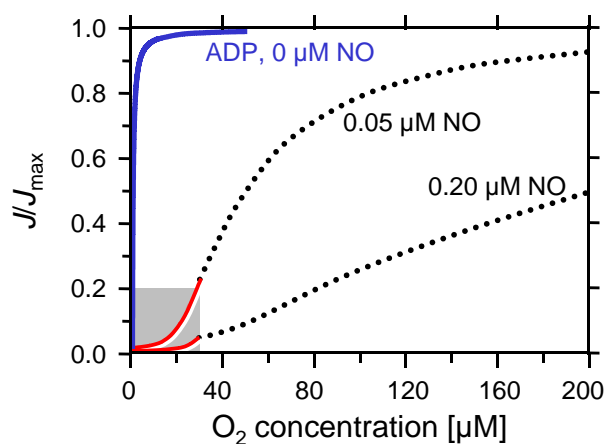

Figure 1. Oxygen dependence of mitochondrial respiration and competitive inhibition by NO. The full line shows oxygen kinetics at state 3 with pyruvate and malate in the absence of NO, measured in the physiological oxygen range (from Gnaiger et al. 1998a). Dotted lines show inhibition of respiration by the indicated NO concentrations, where measurements were performed with low-resoltion respirometry and are restricted to the high oxygen range (from Koivisto et al. 1977). Extrapolations into the physiological oxygen range (shaded region) suggest sigmoidal oxygen kinetics, which requires testing by direct measurements at low oxygen (modified after Gnaiger, Kuznetsov 2002).

Most kinetic studies on the effect of NO on respiratory flux in isolated mitochondria or cells were restricted to high oxygen levels (>20-30 µM O_2; Brown, Cooper 1994; Brown 1995, 1999, 2001; Cooper, Davis 2000; Koivisto et al. 1997). Others address the importance of low oxygen in augmenting the inhibitory effect of NO without quantitative evaluation of the kinetic response curve (Boveris et al. 1999; Cleeter et al. 1994; Griffiths, Garthwaite 2001; Lizasoain et al. 1996; Nishikawa et al. 1997; Shiva et al. 2001; Takehara et al. 1995). Physiological oxygen pressures in the microenvironment of the cell, however, may be as low as 2 % of air saturation under normoxia, i.e. 3 µM compared to about 200 µM in air-saturated solution (e.g. Molé et al. 1999; see Gnaiger et al. 1995, 1998b, 2000; Gnaiger, Kuznetsov 2002). Low intracellular oxygen levels suggest a significant regulatory role of nitric oxide, even at low (nanomolar) concentrations of NO expected under physiological conditions (Brown 1995).The actual degree of inhibition and the form of

inhibitions kinetics (monophasic or biphasic hyperbolic, sigmoidal) at these relevant low oxygen conditions, however, remains a matter of speculation (Fig. 1). The mechanism of inhibition requires re-investigation under low-oxygen conditions as relevant in active tissues (such as heart, liver or brain) and under pathological conditions of ischemia and hypoxia. Among other reasons, the limitation of previous studies to the high oxygen range has prohibited so far a sufficiently conclusive kinetic description of NO inhibition by COX (Brunori et al. 1999; Clementi et al. 1999; Cooper 2002; Giuffre et al. 1996).

In the following, an experiment is presented on the effect of nitric oxide on mitochondrial oxygen kinetics in the low oxygen range. This single experiment was carried out under rather uncontrolled boundary conditions during a lecture at a half-day Oxygraph-2k workshop at the Department of Biology, University of Alabama at Birmingham (UAB), USA, in collaboration with the Center for Free Radical Biology, UAB. Rather than presenting a definitive result, this short report illustrates the approach and application of HRR.
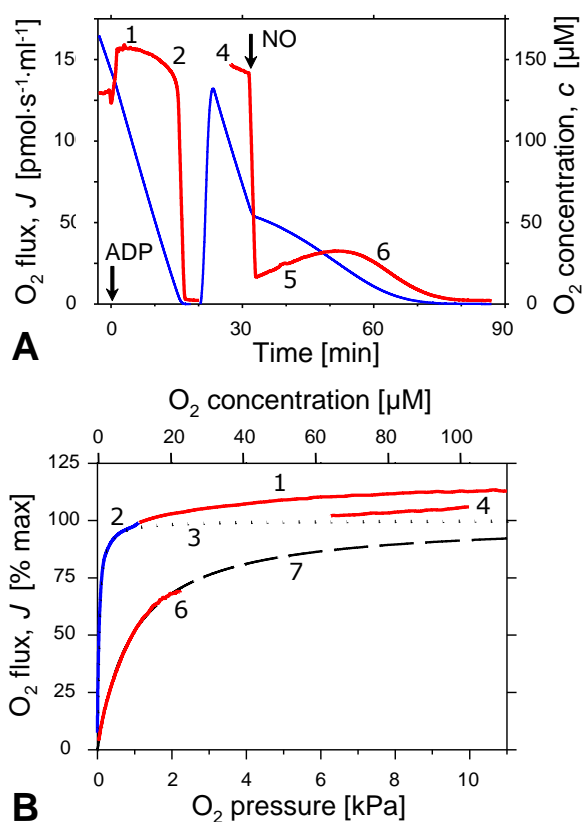

Figure 2. Measurement of oxygen kinetics of respiration in isolated rat liver mitochondria before and after addition of NO. **A**. Continuous traces of oxygen concentration (c, blue line) and oxygen flux (J, red line). **B**. Kinetic plots of oxygen flux as a function of oxygen concentration or oxygen pressure. Different sections of the experiment are indicated by numbers. (1) ADP-activated respiration in the high-oxygen range, -NO. (2) Aerobic-anaerobic transition, -NO, for calculation of oxygen kinetics <1.1 kPa. (3) Extrapolation of hyperbolic oxygen kinetics into the high-oxygen region. (4) Reoxygenation. (5) Inhibition of oxygen flux by NO and partial recovery due to degradation of NO. (6) Aerobic-anaerobic transition, +NO. (7) Extrapolation of hyperbolic oxygen kinetics.
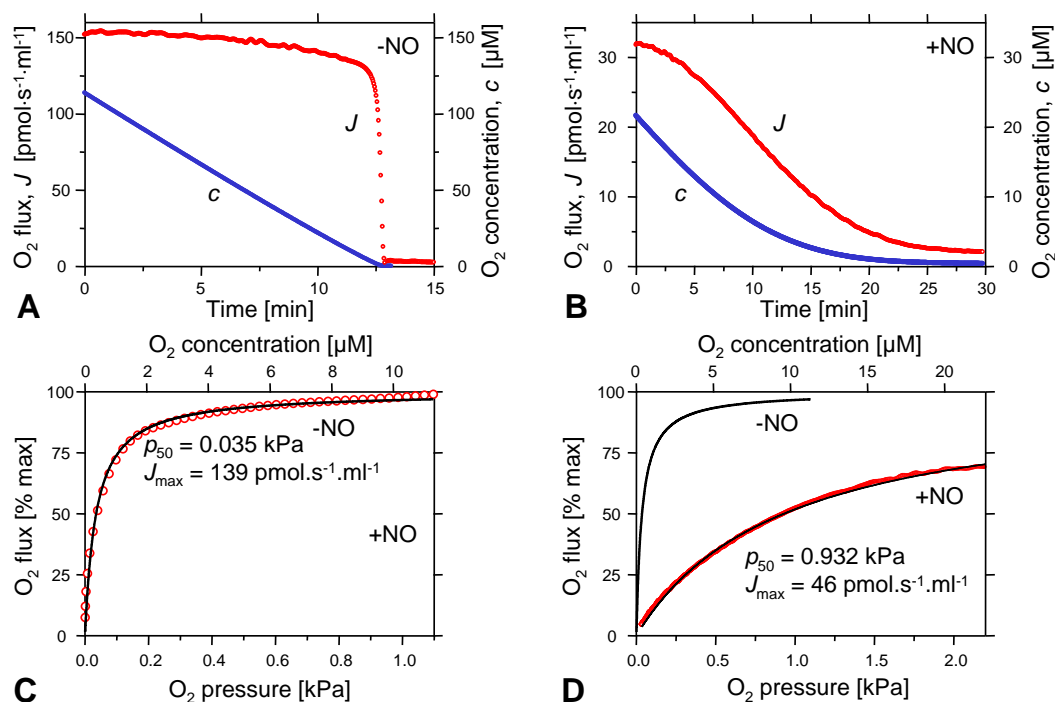
Figure 3. Oxygen kinetics of respiration in isolated rat liver mitochondria. **A** and **B**: Zoom into the aerobic-anaerobic transitions before and after addition of NO, showing oxygen concentration, *c*, and volume-specific oxygen flux, *J*. **C** and **D**: Kinetic plots of oxygen flux as a function of oxygen pressure, showing two ranges of oxygen pressure for hyperbolic fitting, up to 1.1 kPa (-NO) and 2.2 kPa (+NO).

2   The O2k - Demo Experiment

The OROBOROS Oxygraph-2k was operated at 37 °C with a 2 ml chamber volume. Isolated rat liver mitochondria had to be transferred from the lab into the lecture hall and were, therefore, mainly uncoupled (low stimulation by ADP). DatLab 3.1 was used for data acquisition and simultaneous on-line recording of oxygen concentration and oxygen flux (respiration), while presenting PowerPoint slides from the same laptop. Data analysis was performed offline, using a specific routine of DatLab for oxygen kinetics (Gnaiger et al. 1995; Gnaiger 2001).

Figure 2A presents the overview of the experiment, as displayed by automatic on-line data analysis. Mitochondrial respiration was quite stable, revealed by the comparison of flux before and after reoxygenation (Fig. 2; sections 1 and 4). Addition of 1 μl NO solution exerted an immediate inhibitory effect, followed by recovery of respiration to some extent (Fig.

2A, section 5). Figure 2B shows oxygen kinetics in the range up to 100 µM O_2 (approximately 10 kPa or 75 mmHg). Oxygen fluxes are expressed as % of the maximal rates in the absence and presence of NO. Figure 3 represents a zoom into the experimental sections relevant for oxygen kinetics.

In direct agreement with the p_{50} for uncoupled rat liver mitochondria (at 30 °C; Gnaiger et al. 1998a), the p_{50} before addition of NO was 0.035 kPa (Fig. 2, section 2; Fig. 3B; kinetic analysis in the standard low oxygen range up to 10 µM O_2). At higher oxygen levels, there is a non-hyperbolic further increase of oxygen flux with oxygen pressure (Fig. 1, section 1), which cannot be fully accounted for by the time-dependent decline of respiratory rate. This biphasic response to oxygen is known in isolated mitochondria (Gnaiger et al. 1995), and particularly in cultured cells (Gnaiger 2003; Hütter et al. 2002; Steinlechner et al. 1996).

After addition of NO, the p_{50} increased to 0.93 kPa (Fig. 2, section 6; Fig. 3D). The kinetics demonstrates a simple hyperbolic function after inhibition with NO.

3   Discussion

The single experiment shown in this report of an Oxygraph-2k workshop illustrates the potential for a detailed study of the oxygen kinetics of mitochondrial respiration at various concentrations of NO. The p_{50} of 0.035 kPa obtained for loosely coupled mitochondrial respiration in the absence of NO corresponds well with previous results on uncoupled rat liver mitochondria at 30 °C (Gnaiger et al. 1998a). NO metabolism by the mitochondria is indicated by the partial recovery of respiration after addition of NO (Fig. 2; section 5). From previous studies (Koivisto et al. 1997), one might expect strongly sigmoidal oxygen kinetics at high NO concentrations (Fig. 1). Despite of strong inhibition by NO and a 25-fold increase of the p_{50}, however, the kinetic response remained monophasic hyperbolic at low oxygen, without any significant sigmoidal component (Fig. 3D). Previous publications fail to provide accurate kinetic results at oxygen concentrations <10 to 20 µM, due to instrumental limitations (sensitivity of the oxygen sensor, signal drift, time resolution, oxygen back-diffusion in materials such as perspex or teflon). An extension of the studies presented here will contribute to our

understanding of the kinetic mechanism of inhibition of cytochrome *c* oxidase by NO. The kinetics in the low-oxygen range is of direct implications for mitochondrial bioenergetics in vivo (Gnaiger et al. 1998b, 2000), particularly under pathological conditions (Brown 1997; Dai et al. 2001; Stumpe et al. 2001), and for models on intracellular diffusion of NO under physiological and pathological conditions (Thomas et al. 2001). Adding a NO sensor to the Oxygraph-2k will increase the potential of these approaches. Importantly, the Oxygraph-2k and DatLab software are designed to accomodate additional channels in the extension to the OROBOROS O2k-Multisensor System.

Acknowledgement

4   References

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