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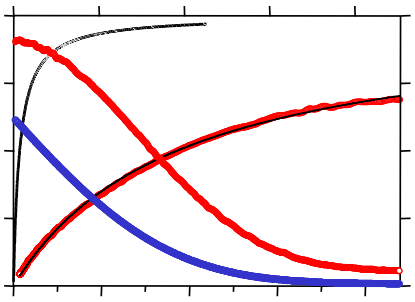
NO effect on mitochondrial oxygen kinetics at low oxygen

Oroboros O2k Workshop Report (IOC22)
University of Alabama at Birmingham



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Summary: A single pilot experiment was carried out during an O2k 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.

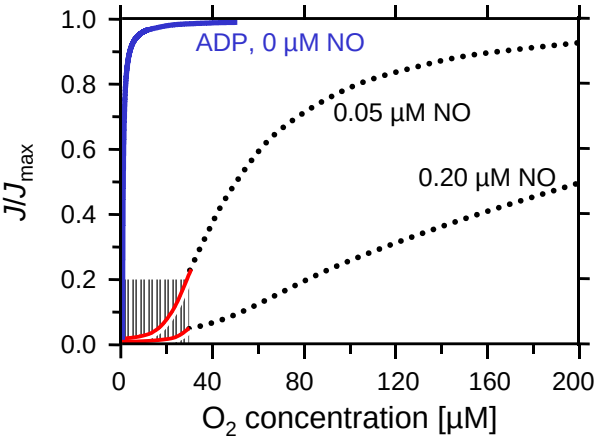

Figure 1. Oxygen dependence of mitochondrial respiration and competitive inhibition by NO. The full line shows oxygen kinetics at state 3 with pyruvate and malate in the absence of NO, measured in the physiological oxygen range (from Gnaiger et al. 1998a). Dotted lines show inhibition of respiration 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.

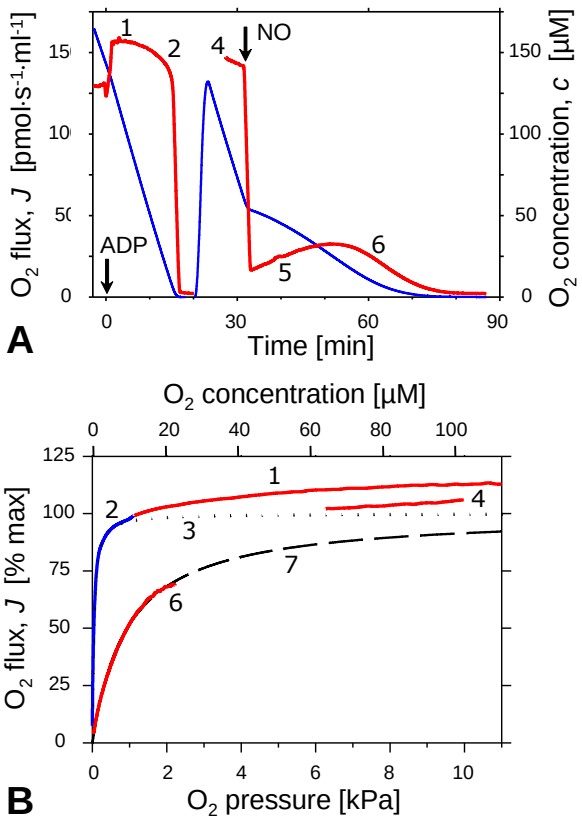

Figure 2. Measurement of oxygen kinetics of respiration in isolated rat liver mitochondria before and after addition of NO. **A**. Continuous traces of oxygen concentration (*c*, blue line) and oxygen flux (*J*, red line). **B**. Kinetic plots of oxygen flux as a function of oxygen concentration or oxygen 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.

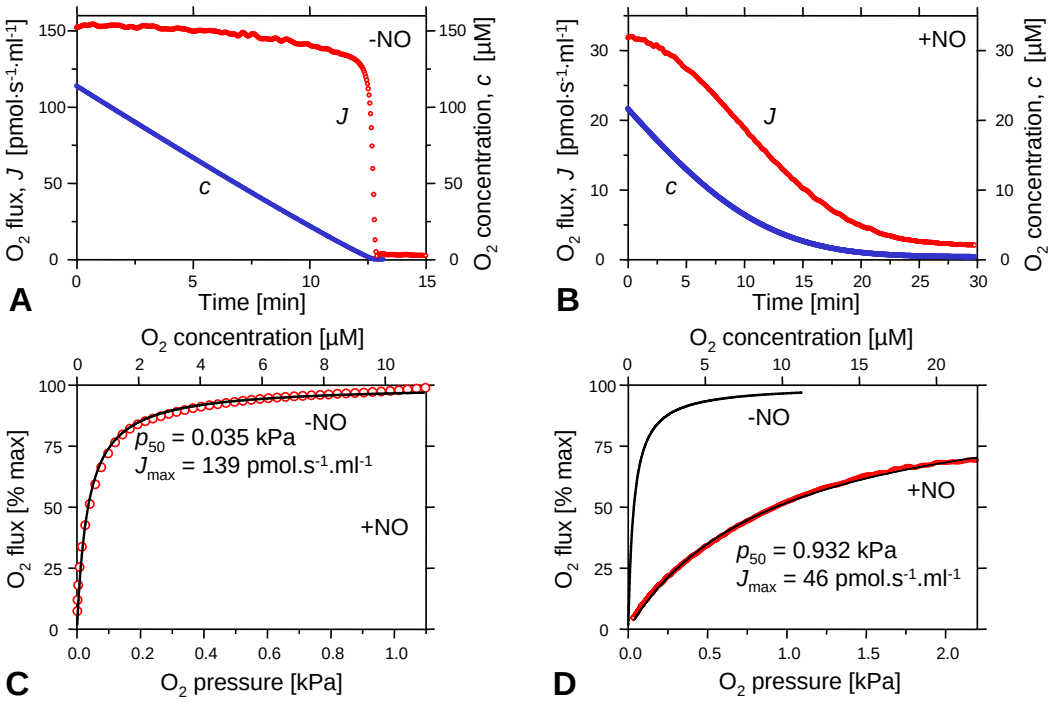

Figure 3. Oxygen kinetics of respiration in isolated rat liver mitochondria. **A** and **B**: Zoom into the aerobic-anaerobic transitions before and after addition of NO, showing oxygen concentration, *c*, and volume-specific oxygen flux, *J*. **C** and **D**: Kinetic plots of oxygen flux as a function 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 O2k 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 O2k 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 O2k 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 O2k-Multisensor System.

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4 References

Boveris A, Costa LE, Cadenas E, Poderoso JJ (1999) Regulation of mitochondrial respiration by ADP, O_2 and NO. Methods Enzymol 301: 188-198.

Brown GC, Cooper CE (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome c oxidase. FEBS Lett 356: 295-298.

Brown GC (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome c oxidase. FEBS Lett 369:136-139.

Brown GC (1997) Nitric oxide inhibition of cytochrome oxidase and mitochondrial respiration: Implications for inflammatory, neurodegenerative and ischaemic pathologies. Mol Cell Biochem 174: 189-192.

Brown GC (1999) Nitric oxide and mitochondrial respiration. Biochim Biophys Acta 1411: 351-369.

Brown GC (2001) Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochim Biophys Acta 1504, 46-57.

Brunori M, Giuffrè A, Sarti P, Stubauer G, Wilson MT (1999) Nitric oxide and cellular respiration. Cell Mol Life Sci 56: 549-557.

Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AHV (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. FEBS Lett 345: 50-54.

Clementi E, Brown GC, Foxwell N, Moncada S (1999) On the mechanism by which vascular endothelial cells regulate their oxygen consumption. Proc Natl Acad Sci U S A 96: 1559-1562.

Cooper CE (2002) Nitric oxide and cytochrome oxidase: substrate, inhibitor or effector? Trends Biochem Sci 27: 33-39.

Cooper CE, Davies NA (2000) Effects of nitric oxide and peroxynitrite on the cytochrome oxidase K_m for oxygen: implications for mitochondrial pathology. Biochim Biophys Acta 1459: 390-396.

Dai L, Brookes PS, Darley-Usmar VM, Anderson PG (2001) Bioenergetics in cardiac hypertrophy: mitochondrial respiration as a pathological target of NO•. Am J Physiol 281: H2261-H2269.

Giuffrè A, Sarti P, D'Itri E, Buse G, Soulimane T, Brunori M (1996) On the mechanism of inhibition of cytochrome c oxidase by nitric oxide. J Biol Chem 271: 33404-33408.

Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. Respir Physiol 128: 277-291.
Gnaiger E (2003) Oxygen conformance of cellular respiration. A perspective of mitochondrial physiology. Adv Exp Med Biol 543: 39-55.
Gnaiger E, Kuznetsov AV (2002) Mitochondrial respiration at low levels of oxygen and cytochrome *c*. Biochem Soc Trans 30: 242-248.
Gnaiger E, Lassnig B, Kuznetsov AV, Margreiter R (1998a) Mitochondrial respiration in the low oxygen environment of the cell: Effect of ADP on oxygen kinetics. Biochim Biophys Acta 1365: 249-254.
Gnaiger E, Lassnig B, Kuznetsov AV, Rieger G, Margreiter R (1998b) Mitochondrial oxygen affinity, respiratory flux control, and excess capacity of cytochrome *c* oxidase. J Exp Biol 201: 1129-1139.
Gnaiger E, Méndez G, Hand SC (2000) High phosphorylation efficiency and depression of uncoupled respiration in mitochondria under hypoxia. Proc Natl Acad Sci U S A 97: 11080-11085.
Gnaiger E, Steinlechner-Maran R, Méndez G, Eberl T, Margreiter R (1995) Control of mitochondrial and cellular respiration by oxygen. J Bioenerg Biomembr 27: 583-596.
Griffiths C, Garthwaite J (2001) The shaping of nitric oxide signals by a cellular sink. J Physiol 536: 855-862.
Hütter E, Renner K, Jansen-Dürr P, Gnaiger E (2002) Biphasic oxygen kinetics of cellular respiration and linear oxygen dependence of antimycin A inhibited oxygen consumption. Molec Biol Rep 29: 83-87.
Koivisto A, Matthias A, Bronnikov G, Nedergaard J (1997) Kinetics of the inhibition of mitochondrial respiration by NO. FEBS Lett 417: 75-80.
Lizasoain I, Moro MA, Knowles RG, Darley-Usmar V, Moncada S (1996) Nitric oxide and peroxynitrite exert distinct effects on mitochondrial respiration which are differentially blocked by glutathione or glucose. Biochem J 314: 877-880.
Molé PA, Chung Y, Tran TK, Sailasuta N, Hurd R, Jue T (1999) Myoglobin desaturation with exercise intensity in human gastrocnemius muscle. Am J Physiol 277: R173-R180.
Nishikawa M, Sato EF, Kuroki T, Inoue M (1997) Role of glutathione and nitric oxide in the energy metabolsm of rat liver mitochondria. FEBS Lett 415: 341-345.
Shiva S, Brookes PS, Patel RP, Anderson PG, Darley-Usmar VM (2001) Nitric oxide partitioning into mitochondrial membranes and the control of respiration at cytochrome *c* oxidase. Proc Natl Acad Sci U S A 98: 7212-7217.
Steinlechner-Maran R, Eberl T, Kunc M, Margreiter R, Gnaiger E (1996) Oxygen dependence of respiration in coupled and uncoupled endothelial cells. Am J Physiol 271: C2053-C2061.
Stumpe T, Decking UKM, Schrader J (2001) Nitric oxide reduces energy supply by direct action on the respiratory chain in isolated cardiomyocytes. Am J Physiol 280: H2350-H2356.
Takehara Y, Kanno T, Yoshioka T, Inoue M, Utsumi K (1995) Oxygen-dependent regulation of mitochondrial energy metabolism by nitric oxide. Arch Biochem Biophys 323: 27-32.
Thomas DD, Liu X, Kantrow SP, Lancaster JR (2001) The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O_2. Proc Natl Acad Sci U S A 98: 355-360.
Torres J, Darley-Usmar V, Wilson MT (1995) Inhibition of cytochrome *c* oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. Biochem J 312:169-173.
Torres J, Cooper CE, Wilson MT (1998) A common mechanism for the interaction of nitric oxide with the oxidized binuclear centre and oxygen intermediates of cytochrome c oxidase. J Biol Chem 273: 8756-8766.