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Kinetic model of the inhibition of respiration by endogenous nitric oxide in intact cells

Enara Aguirre [a,1], Félix Rodríguez-Juárez [a,1], Andrea Bellelli [b], Erich Gnaiger [c], Susana Cadenas [a,d,*]

[a] Department of Regenerative Cardiology, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Melchor Fenández Almagro 3, 28029 Madrid, Spain
[b] Dipartimento di Scienze Biochimiche and Istituto di Biologia e Patologia Molecolari del CNR, Università di Roma 'Sapienza', 00185 Roma, Italy
[c] Department of General and Transplant Surgery, D. Swarovski Research Laboratory, Medical University of Innsbruck, 6020 Innsbruck, Austria
[d] Servicio de Inmunología, Hospital Universitario de La Princesa, Diego de León 62, 28006 Madrid, Spain

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ABSTRACT

Nitric oxide (NO) inhibits mitochondrial respiration by decreasing the apparent affinity of cytochrome *c* oxidase (CcO) for oxygen. Using iNOS-transfected HEK 293 cells to achieve regulated intracellular NO production, we determined NO and O_2 concentrations and mitochondrial O_2 consumption by high-resolution respirometry over a range of O_2 concentrations down to nanomolar. Inhibition of respiration by NO was reversible, and complete NO removal recovered cell respiration above its routine reference values. Respiration was observed even at high NO concentrations, and the dependence of IC_{50} on [O_2] exhibits a characteristic but puzzling parabolic shape; both these features imply that CcO is protected from complete inactivation by NO and are likely to be physiologically relevant. We present a kinetic model of CcO inhibition by NO that efficiently predicts experimentally determined respiration at physiological O_2 and NO concentrations and under hypoxia, and accurately predicts the respiratory responses under hyperoxia. The model invokes competitive and uncompetitive inhibition by binding of NO to the reduced and oxidized forms of CcO, respectively, and suggests that dissociation of NO from reduced CcO may involve its O_2-dependent oxidation. It also explains the non-linear dependence of IC_{50} on O_2 concentration, and the hyperbolic increase of c_{50} as a function of NO concentration.

1. Introduction

Nitric oxide (NO) is a fundamental cell messenger produced in cells by NO synthases (NOS). Many physiological actions of NO are mediated through activation of soluble guanylate cyclase and subsequent production of cGMP [1,2]. NO is also an effective inhibitor of mitochondrial respiration [3–5]: it reversibly inhibits cytochrome *c* oxidase (CcO), the terminal electron acceptor of the mitochondrial respiratory system, in a process which occurs in competition with oxygen (O_2). NO, like other inhibitors that raise the K'_m of CcO, induces O_2 limitation under apparently normoxic conditions. The inhibition of mitochondrial respiration by NO has been implicated in a wide range of physiological processes, including the regulation of the affinity of mitochondrial respiration for O_2 [6–10], the control of mitochondrial generation of superoxide [11] and hydrogen peroxide [12], the activation of hypoxia-inducible factor [13], the activation of AMPK in astrocytes [14], the modulation of O_2 delivery to tissues [15], the modulation of calcium-mediated cell signaling in the brain [16] and the maintenance of constant cerebral O_2 consumption at varying blood flow [17]. The interaction between CcO and NO has been implicated in various pathophysiological situations [18,19].

The molecular mechanism by which NO inhibits CcO has not been fully defined (for a review see [20]). The enzyme belongs to the superfamily of heme-copper oxidases and contains a highly conserved bimetallic active site composed of a high-spin heme a_3 and a copper ion Cu_B. The binuclear center is the binding site of the physiological substrate O_2 and of other ligands such as CN^-, CO, and NO [21]. O_2-binding demands the complete reduction of the active site: two electrons donated by cytochrome *c* enter CcO at Cu_A, from which they are transferred, via cytochrome *a*, to the binuclear center. Conflicting hypotheses have been proposed to explain the mechanism of CcO inhibition by NO [22–24]; however, recent studies demonstrate that NO interactions with CcO cannot be adequately described by a simple competitive model and that only one NO molecule binds per binuclear center [25]. The enzyme can bind NO either at the reduced heme a_3 iron (competitive with O_2) or at the oxidized Cu_B (non-competitive with O_2)

Abbreviations: NO, nitric oxide; NOS, NO synthase; CcO, cytochrome *c* oxidase; O_2, oxygen; CN^-, cyanide; CO, carbon monoxide; iNOS, inducible NOS; DMEM, Dulbecco's modified Eagle's medium; Tet-iNOS 293, tetracycline-inducible iNOS-expressing HEK 293 cells; S-EITU, S-ethylisothiourea; FCCP, carbonylcyanide *p*-(trifluoromethoxy) phenylhydrazone; HBSS, Hanks balanced salt solution; HbO_2, oxyhemoglobin

* Corresponding author. Servicio de Inmunología, Hospital Universitario de La Princesa, Diego de León 62, 28006 Madrid, Spain. Tel.: +34 915202386; fax: +34 915202374.
E-mail address: susana.cadenas@salud.madrid.org (S. Cadenas).

[1] These authors contributed equally to this work.

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[25–28]. NO binds to reduced heme a_3 very quickly and with high affinity, to yield a Fe^{2+}-NO nitrosyl-adduct. Through binding to oxidized Cu_B, donation of an electron to the metal and subsequent hydration, NO is oxidized to nitrite. The predominance of one inhibitory mechanism over the other is controlled by the electron flux through the enzyme [29]. NO and its derivative peroxynitrite have also been reported to irreversibly decrease the affinity of CcO for O_2 [30], although this effect is unlikely to occur at physiological NO levels.

The complexity of the cellular environment provides the ideal setting for studying how the interplay between O_2 and NO concentrations determines the extent of respiratory inhibition and the O_2-dependence of iNOS-mediated NO production. However, because of technical limitations, previous studies have not provided accurate kinetic data at low [O_2]. Additionally, in many studies sensitivity of O_2 consumption to NO donors has been examined at [O_2] much higher than those likely to exist *in vivo*. High-resolution respirometry allows accurate determination of respiration at low O_2 concentrations and during transition to anoxia [31]. Here we present a detailed study of the O_2 kinetics of cellular respiration in intact cells producing controlled amounts of NO [32]. Insertion of an NO sensor into the Oxygraph-2k respirometer chamber allowed simultaneous recording of NO production and mitochondrial respiration over an extended [O_2] range. Based on our results, we present a pseudo-equilibrium kinetic model of inhibition of respiration by NO that accounts for both competitive and uncompetitive inhibition of CcO and has predictive value.

2. Materials and methods

2.1. Cell culture and reagents

Tetracycline-inducible HEK 293 cells stably expressing human iNOS (Tet-iNOS 293) were generated as described [32]. Cells were cultured in DMEM (Invitrogen, Barcelona, Spain) containing 4.5 g/l D-glucose, 10% (v/v) fetal-calf serum, 200 μg/ml hygromycin B and 15 μg/ml blasticidin, at 37 °C in a humidified atmosphere with 5% CO_2. S-ethylisothiourea hydrobromide (S-EITU; NOS inhibitor), L-arginine, oligomycin and FCCP were from Sigma-Aldrich (St. Louis, MO). Hygromycin B and blasticidin were from Invitrogen. Tetracycline was from Calbiochem (Darmstadt, Germany).

2.2. Induction of endogenous NO production

Expression of iNOS was induced as previously described [33]. Briefly, cells were incubated overnight in a complete growth medium (without selection antibiotics) containing tetracycline (5–50 ng/ml) and 500 μM S-EITU, a potent inhibitor of iNOS activity. Cells were washed with L-arginine-free DMEM supplemented with 1% dialysed fetal-calf serum to eliminate any traces of L-arginine. Cells were incubated in L-arginine-free medium for 1 h to completely eliminate S-EITU and tetracycline without activating NO production. Cells were trypsinized and resuspended at 1×10^7 cells/ml in HBSS containing 25 mM Hepes. Endogenous NO production was triggered by addition of L-arginine (1 mM).

2.3. Immunoblot analysis of iNOS expression

For preparation of total protein extracts, cells were trypsinized and resuspended at 1×10^7 cells/ml in HBSS containing 25 mM Hepes. Cells were spun (300 *g*, 5 min, 4 °C) and the pellet was resuspended in 60 μl ice-cold lysis buffer (20 mM Hepes, pH 7.5, 400 mM NaCl, 20% (v/v) glycerol, 0.1 mM EDTA, 10 mM NaF, 10 μM Na_2MoO_4, 1 mM $NaVO_3$, 10 mM PNPP (*p*-nitrophenyl phosphate), and 10 mM β-glycerophosphate) supplemented with 1 mM dithiothreitol, 1 mM pefablock, pH 7.4, and a protease-inhibitor cocktail (Roche Diagnostics, Barcelona, Spain). After 15 min on ice, cleared whole cell extracts were obtained by recovering the supernatant after centrifugation (16,000 *g*, 15 min, 4 °C). Protein samples (200 μg) were resolved by SDS/7.5%-(w/v)-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) non-fat dry milk in TBS-T (20 mM Tris/HCl, pH 7.2, 150 mM NaCl, 0.1% Tween 20) and incubated overnight with polyclonal anti-iNOS antibody (1:2000, Transduction Laboratories, Erembodegem, Belgium) in a blocking solution at 4 °C. Protein bands were detected by incubation for 1 h with horseradish peroxidase-coupled goat anti-rabbit IgG (1:5000; Vector Laboratories, Burlingame, CA) in blocking solution at room temperature, followed by enhanced chemiluminescence (GE Healthcare, Amersham, UK).

2.4. Simultaneous measurement of O_2 consumption and NO production

O_2 consumption at physiological tissue [O_2] was determined by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Cells were trypsinized after overnight treatment with tetracycline and resuspended in HBSS containing 25 mM Hepes at 1×10^7 cells/ml. The instrumental background flux was calculated as a linear function of [O_2] and the experimental data were corrected for the whole range of [O_2] using DatLab software (Oroboros Instruments) [31]. To separate mitochondrial and residual O_2 consumption, all results were corrected for residual O_2 consumption of 0.5 pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells, measured at 35 μM O_2 (Table 1). Compared with CcO, residual oxygen uptake has a lower affinity for oxygen [34]. This was accounted for by assigning a 5-fold higher c_{50} to residual respiration as a basis for application of the residual O_2 consumption–correction over the entire experimental [O_2] range. At air saturation, 37 °C and local barometric pressure (92.6 kPa), the [O_2] in culture medium (HBSS) was 175.7 μM (O_2 solubility factor 0.92). Measurements were taken in cell suspensions (2 ml) gently agitated at 37 °C in parallel Oxygraph-2k chambers; NO production was initiated in one chamber by addition of 1 mM L-arginine; a control run (in cells treated with the same amount of tetracycline) was simultaneously performed in the other chamber. Because light reverses NO-induced inhibition of respiration [35–37] O_2 consumption was measured in the dark, except for analysis of inhibition reversibility, when cell suspensions were illuminated with cold light (Intralux 5000-1; Volpi, Switzerland). Reversibility was also studied by adding HbO_2 to quench NO or S-EITU (1 mM) to inhibit iNOS activity.

For the simultaneous measurement of endogenous NO production, an NO sensor (ISO-NOP; World Precision Instruments, Stevenage, UK) was inserted into the Oxygraph-2k chamber. For this, a second capillary was drilled into the PVDF stopper to tightly fit the 2 mm diameter sleeve of the NO sensor. The opening of the second capillary did not increase O_2 backdiffusion into the chamber at low [O_2]. The NO sensor was calibrated by addition of known $NaNO_2$ concentrations under reducing conditions (KI/H_2SO_4) at 37 °C. To initiate NO production, 1 mM L-arginine was added to the chamber at 60 μM O_2. This [O_2] is sufficient to sustain iNOS activity [38,39]. NO concentrations were monitored until O_2 was exhausted and the NO signal returned to basal. The NO signal

Table 1
Routine respiration, electron transport capacity, and resting and residual respiration in Tet-iNOS 293 cells. Respiration of control cells in the absence of NO (cells were not treated with tetracycline and no arginine was added). Routine: basal respiration using endogenous substrates. Resting: addition of oligomycin (80 ng/ml) to measure respiration in the absence of ATP synthesis. Uncoupled: addition of optimum FCCP concentration (0.35 μM) for maximum flow to measure the capacity of the electron transport system. Corrections were made for residual O_2 consumption (1 mM KCN at 30 to 40 μM O_2). Measurements were made at 37 °C at 1×10^7 cells/ml in the chambers of a high-resolution respirometer. Values are means ± SD (number of independent experiments in parentheses).

Respiratory state endogenous substrates	Respiration [pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells]	Flux control ratio per uncoupled rate
Routine	14.5 ± 1.9 (8)	0.31 ± 0.03
Resting (oligomycin)	4.2 ± 0.4 (3)	0.089 ± 0.004
Uncoupled (FCCP)	47.4 ± 7.1 (8)	
Residual (KCN)	0.51 ± 0.08 (5)	0.011 ± 0.003