Activation of Hsp90/NOS and increased NO generation does not impair mitochondrial respiratory chain by competitive binding at cytochrome C Oxidase in low oxygen concentrations

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Received: 5 March 2009 / Accepted: 2 April 2009 / Published online: 2 May 2009
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Abstract Nitric oxide (NO) is known to regulate mitochondrial respiration, especially during metabolic stress and disease, by nitrosation of the mitochondrial electron transport chain (ETC) complexes (*irreversible*) and by a competitive binding at O₂ binding site of cytochrome c oxidase (CcO) in complex IV (*reversible*). In this study, by using bovine aortic endothelial cells, we demonstrate that the inhibitory effect of endogenously generated NO by nitric oxide synthase (NOS) activation, by either NOS stimulators or association with heat shock protein 90 (Hsp90), is significant only at high prevailing pO₂ through nitrosation of mitochondrial ETC complexes, but it does not inhibit the respiration by competitive binding at CcO at very low pO₂. ETC complexes activity measurements confirmed that significant reduction in complex IV activity was noticed at higher pO₂, but it was unaffected at low pO₂ in these cells. This was further extended to heat-shocked cells, where NOS was activated by the induction/activation of (Hsp90) through heat shock at an elevated temperature of 42°C. From these results, we conclude that the entire attenuation of respiration by endogenous NO is due to irreversible inhibition by nitrosation of ETC complexes but

not through reversible inhibition by competing with O₂ binding at CcO at complex IV.

Keywords Nitric oxide synthases · Heat shock proteins · Oxygen metabolism · Electron transport chain

Introduction

Nitric oxide (NO) is an important signaling molecule that influences numerous physiological processes, especially in vascular endothelial relaxation/contraction and in many human diseases (Ignarro 2000). At physiological concentrations, NO is fairly unreactive; where the majority of its physiological events are mediated by the binding of NO to Fe²⁺ in the heme of soluble guanylyl cyclase (Ignarro 2000). This binding initiates the activation of soluble guanylyl cyclase and cyclic guanosine monophosphate production (Ignarro 2000). Numerous studies have shown that NO regulates cellular respiration, by interfering in mitochondrial electron transport chain (ETC) (Brown and Cooper 1994; Cooper and Davies 2000; Kinugawa et al. 2005; Palacios-Callender et al. 2004). These studies also establish that the ETC regulatory mechanisms of NO-induced inhibition can be conveniently determined by measuring the change in extracellular pO₂ due to respiration. NO can inhibit respiration by a reversible or irreversible mechanism, depending upon the relative concentrations of NO and O₂ present (Brown and Cooper 1994; Cleeter et al. 1994). A prolonged exposure to NO can lead to irreversible inhibition of cellular respiration by S-nitrosation of proteins in ETC complexes by peroxynitrite (Brown 2001). On the other hand, NO was also shown to inhibit respiration by directly binding at cytochrome c

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oxidase (CcO) of complex IV (Jacobson et al. 2005). Since CcO function can be manipulated by NO, it also has the ability to control the majority of energy production in cells (Babcock 1999; Brown 2001). Both exogenously added and endogenously generated [by activation of nitric oxide synthases (NOS)] NO have been well established to attenuate respiration; yet, endogenous generation of NO and its exact role in the competitive binding to CcO, when the pO₂ drops below 10 mmHg, has not been completely elucidated.

NO is synthesized by three isoforms of NOS in cells: *neuronal* NOS, *inducible* nitric oxide synthase, and *endothelial* nitric oxide synthase (eNOS; Ignarro 2000). Among these isoforms, eNOS has been shown to play a prominent role in the regulation of cellular respiration due to its high abundance (Ilangovan et al. 2004a; Loke et al. 1999; Palacios-Callender et al. 2004). Moreover, eNOS is activated by heat shock protein 90 (Hsp90) by forming an Hsp90/eNOS complex, and hence, eNOS activation through S-1179 phosphorylation is facilitated (Averna et al. 2008; Harris et al. 2008; Presley et al. 2008; Pritchard et al. 2001; Song et al. 2002; Xu et al. 2007). A mild heat shock, known as hyperthermia, can increase such an association between Hsp90 and eNOS and lead to an enhanced production of NO. Moreover, hyperthermia has been demonstrated as a protective mechanism against oxidative stress, ischemia–reperfusion injury, and lethal shock (Latchman 2001). Therefore, we have developed a hypothesis that such a protection may be partially due to an upregulation of NO in cells subjected to hyperthermia and its regulatory role in respiration (Ilangovan et al. 2004a; Latchman 2001). Similarly in hypoxia exposed cells, Hsp90/eNOS activation attenuated respiration (Presley et al. 2008).

In the present work, we study the mechanism of endogenous NO-induced inhibition of cellular respiration with relevance to pO₂. ETC inhibition, induced by NO in NOS-stimulated cells, significantly occurs at higher pO₂ values by irreversible inhibition of ETC complexes; yet, the pO₂-dependent reversible inhibition, which is convincingly demonstrated for exogenously added NO, is not observed. We propose that inhibition of ETC by NO through competitive binding is diminished at low pO₂ ranges due to a lack of excess NO to engage in competitive binding with CcO of complex IV (Mason et al. 2006). This mechanism is supported by studying two different systems, in which the NOS function is altered by activation or inhibition. A quantitative electron paramagnetic resonance (EPR) oximetry approach is utilized to measure different phases of cellular respiration (Ilangovan et al. 2004a; Presley et al. 2006). EPR oximetry is capable of yielding high-resolution O₂ data similar to data obtained in high-resolution respirometry. The advantage of using this

method is that only a micro-volume (~20–40 µl) of sample is necessary to acquire measurements. Moreover, EPR oximetry has been proven to be a technique that is accurate and highly sensitive (Froncisz et al. 1985; O'Hara et al. 2005; Presley et al. 2006; Swartz 2004). From these measurements, we have found that the inhibitory role of NO is predominant at high pO₂ values; however, during endogenous NO generation, it is reduced or ineffective at lower pO₂ ranges (<10 mmHg). Our results reveal that endogenous NO regulates cellular respiration at low pO₂ ranges, only by modifying the ETC proteins and not by competitive binding at CcO.

Materials and methods

Materials

4, 5-Diaminofluorescein diacetate (DAF-2DA) was purchased from Alexis Biochemicals (San Diego, CA, USA). Dilithium phthalocyanine (Li₂Pc), acetonitrile, cocktail protease inhibitor, cytochrome c, nitro-L-arginine methyl ester (L-NAME), cytochrome c from equine heart, nicotinamide adenine dinucleotide reduced (NADH), succinate, sodium azide (NaN₃), geldanamycin (GA), *S*-nitroso-*N*-acetylpenicillamine (SNAP), bradykinin (Bk), and calcium ionophore III (Ca²⁺ ionophore A23187) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from Gibco Invitrogen Life Technologies (Carlsbad, CA, USA). NO and argon gases were obtained from Praxair (Danbury, CT, USA). Tetrabutyl ammonium perchlorate was purchased from ICN Biochemicals (Aurora, OH, USA). The monoclonal antibodies for Western blot were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Methods

Cell culture Bovine aortic endothelial cells (BAECs) were obtained from Cell Systems (Kirkland, WA, USA). BAECs were cultured in CS-C Complete medium (Cell Systems). The growth medium contained JetFuel formulated with growth factor, 10% serum, and antibiotic (gentamycin sulfate). The cell viability was determined by a NucleoCounter system (New Brunswick Scientific, Edison, NJ, USA), comprised of the NucleoCounter automatic cell counter, the NucleoCassette, a cell preparation lysing buffer and a stabilizing buffer, and NucleoView software (Ilangovan et al. 2004a, 2006).

Experimental protocol for drug treatments For each drug treatment, the appropriate concentrations of bradykinin (1 µM), L-NAME (0.5 mM), Ca²⁺ ionophore (1 µM),

SNAP (1 mM), or GA (10 μ M) were added to the cell culture and incubated for 30 min (or 20 min for SNAP). The cells were trypsinized and isolated for respiration measurements. For the exogenous addition of NO, either 1 mM of NO-saturated phosphate buffer solution (PBS) or 50 μ M SNAP was directly added to the cell suspension immediately before measurements. At the time of measurements, cell suspensions containing 1 mg/ml of glucose in PBS were incubated at 37°C in a water bath for 10 min and then drawn into the glass capillary tubes for oxygen measurements. The cell densities in this study are expressed as the viable cell counts per milliliter, estimated after the addition of the drug. The cell viability in each case was $\geq 97\%$.

Heat shock treatment One day before experiments, the cells were placed in a 42°C incubator for 2 h to induce heat shock. Following this, they were returned to 37°C overnight. A temperature of 42°C was preferred because it has been reported that Hsp90 is more specific at this temperature, and higher temperatures will primarily induce Hsp70 (Ilangovan et al. 2004a).

Western blot Cells were washed twice with ice-cold PBS, trypsinized and centrifuged at 1,500 rpm for 5 min. The cell pellet was homogenized in ice-cold radio immunoprecipitation assay (RIPA) buffer (1 $\times$ Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.004% sodium azide, 1 $\times$ protease inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) for 45 min in ice. The protein concentrations of the supernatants were measured by the bicinchoninic acid method and normalized to 20 μ g per sample. The samples were resolved on 4–12% Bis-Tris polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane at 45 V for 2 h. After blocking with 5% nonfat milk, blots were probed with a rabbit anti-Hsp90 or anti-eNOS antibody (1:1,000 dilution). Goat anti-rabbit horseradish peroxidase-conjugated antibody was used as the secondary antibody, and blots were developed with enhanced chemiluminescence.

Immunoprecipitation The total cell lysates were prepared as described in the Western blot procedure and incubated with either anti-eNOS or anti-Hsp90 polyclonal antibody overnight at 4°C while rotating. To immunoprecipitate eNOS or Hsp90, the protein A/G agarose was added to the lysates and rotated at 4°C for 2 h. The immunoprecipitates were centrifuged at 10,000 rpm for 30 s at 4°C. The supernatant was carefully aspirated and discarded. The pellet was washed with 500 μ l of RIPA buffer three times and centrifuged as described. After the final wash, the supernatant was removed, and the pellet was suspended in 40 μ l of

sample buffer. The samples were boiled at 98°C for 8 min and subjected to electrophoresis. The PVDF membrane was immunoblotted with anti-Hsp90 or anti-eNOS to determine the amount of association of Hsp90-eNOS.

Measurement of cellular respiration The oxygen measurements in the present study were performed using EPR oximetry (Ilangovan et al. 2004a, b; Presley et al. 2006). EPR oximetry is capable of determining pO₂ with a resolution of submicromolar concentrations in small volumes (10–20 μ l; Presley et al. 2006). Lithium phthalocyanine (LiPc) was used as the paramagnetic oxygen sensing probe (Ilangovan et al. 2001). The EPR line width vs. pO₂ calibration curve was constructed using known ratios of premixed O₂ and N₂ gases. The slope of the calibration curve was 5.8 mG/mmHg. Although this calibration curve was constructed using gas mixtures, we have previously demonstrated that this curve is applicable in aqueous solutions as well (Ilangovan et al. 2001). Thus, by measuring the EPR line width, the pO₂ in the solution can be obtained at any given time.

The EPR respiration studies have been carried out using an X-band (9.7 GHz) EPR spectrometer, fitted with a TM₁₁₀ microwave cavity. A 50- μ l microcapillary tube was used to hold the cells in the EPR cavity. In a typical experiment, the cell suspension of the required cell density was saturated with room air (pO₂ $\approx$ 160 mmHg). The cell suspension was incubated for 10 min in a 37°C water bath. Following this, 20 μ g LiPc microcrystals were added to the cells and sampled into 50 μ l capillary tubes. The tube was then closed off at both ends using tube sealing clay (Chase Scientific Glass, Rockwood, TN, USA). While sealing, care was taken to ensure that there were no air gaps present inside the tube, since such a gap may act as an additional O₂ source. The tube was placed inside the microwave cavity, and EPR spectral acquisitions of the LiPc were immediately started. Due to the fact that the cells do not internalize the LiPc microcrystals, the acquired data are extracellular pO₂ measurements. During measurements, the modulation amplitude was adjusted to always be less than one third of the line width to avoid modulation-induced broadening. From the EPR line width, the pO₂ in the cell suspension was determined using the standard curve. Due to the stationary nature of the sample, the cells and the LiPc particles may settle down to the bottom of the capillary tube. We have previously addressed this concern by rotating the capillary tube 180° within seconds of each acquisition. We found that there was no difference in the rotated sample and the stationary sample (Presley et al. 2006).

O₂ kinetics Quantitative EPR oximetry was performed using the recently described procedure (Presley et al.

2006). Briefly, there are three phases of cellular respiration that can be analyzed from a single run of pO_2 vs. time using EPR oximetry: pO_2 -dependent, pO_2 -independent, and a steady-state respiration. These levels of cellular respiration were obtained by adopting the following equation:

$$V_{O_2}/V_{O_2 \max} = ((pO_2\theta) - p_0)/(p50 + (pO_2\theta - p_0)) \quad (1)$$

From this equation, the $V_{O_2 \max}$, p_0 , and $p50$ values were acquired (Presley et al. 2006). The $V_{O_2 \max}$ is defined as the maximum oxygen consumption rate at coupled conditions, p_0 is the equilibrium pO_2 , θ is the volume under diffusion control, and $p50$ is the concentration at which the $V_{O_2 \max}$ is reduced to 50%. This half maximum value is analogous to the K_m value in enzymatic reactions and provides an indication of the oxygen affinity. Specifically, $p50$ is the inverse of the mitochondrial oxygen affinity to CcO in complex IV of the ETC (Presley et al. 2006). Since LiPc measures the extracellular pO_2 around each cell, the p_0 provides an indication of the potential intracellular O_2 content.

Preparation of aqueous NO solution NO solution was prepared in a fume hood as previously described (Liu et al. 2005). Briefly, NO gas was scrubbed of higher nitrogen oxides by passage through a U-tube containing NaOH pellets, followed by a 1 M de-aerated (bubbled with 100% Argon) KOH solution in a custom-designed apparatus using only glass or stainless steel tubing and fittings. The purified NO was collected by saturating a de-aerated phosphate buffer solution (0.2 M potassium phosphate, pH 7.4) contained in a glass-sampling flask with a septum (Kimble/Kontes, Vineland, NJ, USA).

Measurements of NO by Clark-type NO electrode The electrochemical system for measuring NO included a Clark-type NO electrode (ISO-NOP from World Precision Instruments, Sarasota, FL, USA), a four-port water-jacketed electrochemical chamber (NOCHM-4 from World Precision Instruments), a Haake DC10-P5/U circulating bath, a magnetic stirrer, and an Apollo 4000 free radical analyzer (World Precision Instruments). The NO electrode was inserted into the water-jacketed chamber containing 2 ml PBS for NO measurements in the presence of BAECs or buffer alone. The solution was rapidly stirred at a constant speed with a magnetic stirrer bar, controlled by the stirrer. The temperature of the chamber was maintained at 37°C. To prevent O_2 in the atmosphere from dissolving into the solution or to inhibit NO in the solution from volatilizing into the atmosphere (in some cases), the chamber was sealed with a cap that was pushed down to the solution until the solution flowed into the holes of the cap. The holes of the cap were also sealed off to ensure no gas exchange.

EPR spin trapping The magnitude of NO generation was studied using Fe-MGD, a spin trap that specifically detects the presence of NO. The control and HS cells were collected from the culture dishes by trypsinization. The viability was measured, and the cells were re-suspended in PBS containing 1 mg/ml of glucose. The required volume of sample was taken out from the bulk for each condition studied. Fe-MGD of 1 mM was added to the sample and incubated at 37°C for 15 min. The entire sample was immediately transferred into an EPR flat cell to be used for measurements.

Fluorescence microscopy NO production in BAECs was analyzed using fluorescence microscopic imaging with an inverted light Nikon TE2000-U microscope. DAF-2DA, a green fluorescence NO-specific probe, was used. The cells were suspended in serum-free medium, and a 10 μ M concentration of DAF-2DA was added directly to the medium of the control and heat-shocked cells. They were incubated at 37°C for 20 min and washed twice with PBS. The fluorescence microscopy measurements were immediately performed. *MetaMorph* software was used to calculate the average fluorescence intensity of individual cells.

Glucose uptake by BAEC cells Glucose uptake was determined by flow cytometry as described before (Dai et al. 2007). Cells were incubated overnight with culture media containing a fluorescent, non-cleavable glucose analogue 2-(*n*-97-nitrobenz-2-oxa-1,3 diazol-4-yl)amino-2-deoxyglucose (2-NBDG, Invitrogen, 0.5 mg is dissolved in 15 ml of medium). The cells were trypsinised, washed, and suspended in minimum essential medium. The relative fluorescence intensity was measured using BD FACScalibur Flowcytometer, and the data were analyzed using WinMDI.

Mitochondrial membrane potential measurements Rhodamine123 (Rho123, Molecular Probes, Eugene, OR, USA) was used to measure mitochondrial potential using the procedure described before (Papandreou et al. 2006). Cells were trypsinised, and viable cells were counted using NucleoCounter system (Ilangovan et al. 2004a; Ilangovan et al. 2006).

Mitochondrial ETC enzymatic activities The BAECs were homogenized in ice-cold 3 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer (pH 7.2), 0.5 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.25 M sucrose, and 2.5% cocktail protease inhibitor. The mitochondrial ETC complex activities were acquired by analyzing the supernatant using a Varian UV-Visible spectrophotometer Cary 50. For complex IV activity, the enzyme activity of CcO was assayed by measuring

cytochrome c oxidation; where the assay mixture contained 50 mM NaK/PO₄ buffer and 50 μ M reduced cytochrome c (pH=7.4). The cytochrome c was reduced by adding a few microcrystals of sodium ascorbate. For 0.5% O₂ CcO measurements, the supernatant was bubbled with 99.5% N₂ and 0.5% O₂ gas mixture, capped and measured as described above. The activity of CcO was obtained by evaluating the decrease in absorption at 550 nm using first-order kinetics. Complex I activity was determined by measuring the decrease in absorbance at 340 nm of NADH initiated by ubiquinone 1 (Q₁). The assay mixture included 20 mM potassium phosphate buffer, pH8.0, 2 mM NaN₃, phospholipid (0.15 mg/ml), 0.1 mM Q₁, and 0.15 mM NADH. To determine complexes II/III activity, the increase in absorbance at 550 nm was measured. The activity of succinate–cytochrome c reductase was assayed by assessing ferricytochrome c reduction. The assay mixture contained 50 mM phosphate buffer, pH7.4, 0.3 mM ethylenediaminetetraacetic acid, 50 μ M KCN, 19.8 mM succinate, and 50 μ M ferricytochrome c (Han et al. 2007).

Curve fit and data analysis Data are presented as means $\pm$ SE. Statistical analysis was performed using Student's *t* test and one-way analysis of variance. The general acceptance level of significance was $P<0.05$. The EPR spectra, collected during the cellular respiration measurements, were analyzed as formerly described (Presley et al. 2006). The correlation coefficient of 0.98 was set as the standard of acceptance of the results. The pO₂ data conversion, differentiation, and curve fit were carried out as described before (Presley et al. 2006).

Results

Effect of exogenous NO on the respiration of BAECs Three sets of experiments, namely, addition of NO suspension, addition of NO releasing agent, and incubation with an NO-releasing agent, were carried out. In the first set of experiments, the effect of addition of NO suspension on respiration was studied. 1 mM NO-saturated solution was added to the cell suspension immediately before sampling into the EPR microtube, and respiration was measured. Although in cells the NO generation is in the order of nanomolar (maximum about 50 nM), the addition of a higher concentration was necessary because NO reacts rapidly with O₂ (Edmunds et al. 2003). Knowing that the half life of NO is very short, we measured the change in NO concentration in the cell suspension with time using the Clark electrode, and the results are summarized in Fig. 1a. As seen, the NO concentration rapidly decreased, and after 1 min, only 0.2 μ M NO was measured (although it peaked

to 80 μ M within a few seconds, Fig. 1a, inset). In addition, there was not much change in the NO with time that followed. Thus, cell suspensions were prepared by adding cells in NO-added respiratory medium, 2 min later than NO addition, ensuring that cells are exposed only to low NO concentration, about 2 μ M.

The oxygen consumption of BAECs was measured for control and NO-added cells by EPR oximetry. EPR spectra were obtained at 30-s intervals for a period of nearly 2 h for 4×10^6 cells/ml for both NO-treated and control cells. The EPR spectrum narrowed down with time due to the consumption of oxygen by BAECs within the sealed microtube (Fig. 1b). The EPR line width of each obtained spectrum was converted into pO₂ data, i.e., rate vs. oxygen concentration, by using a standard calibration curve and was plotted with respect to time as shown in Fig. 1c (Ilangoan et al. 2004b). The pO₂ data for control and NO-treated cells, show three different regions: a linear portion, a curved portion, and a constant region. To guarantee that cell settling would not cause any serious artifacts, experiments were carried out by continuously rotating the microtube in between acquisitions. The initial pO₂ was close to 160 mmHg, in the same order of room air saturated solution, indicating that NO addition did not deplete the O₂; perhaps, the microtube was sealed a few minutes after the NO addition. The data shown in Fig. 1c were further transformed into dpO₂/dt (i.e., rate of oxygen consumption) vs. pO₂ data as shown in Fig. 1d. In this figure, it is clear that the rate of oxygen consumption remains constant in higher pO₂ ranges, for both control and NO treated cells, and decreases at lower pO₂ ranges (<10 mmHg). However, the steady-state oxygen consumption rate ($V_{O_2 \text{ max}}$, observed >10 mmHg) was lower in the case of cells that were treated with NO solution (Fig. 1d). A complete analysis of the data was performed as described in the experimental section, and the relevant parameters have been summarized in Table 1. The steady-state oxygen consumption rate of the control is 3.66 ± 0.32 mmHg/min/ 5×10^6 cells (5.37 ± 0.46 μ M/min/ 5×10^6 cells), which is comparable to the value of 13.1 ± 1.3 μ M/min/ 10×10^6 cells for porcine thoracic aortic endothelial cells at similar experimental conditions, using polarographic electrode techniques (Clementi et al. 1999). The cells treated with NO showed a reduced rate of respiration (2.15 mmHg/min/ 5×10^6 cells; Table 1) compared to the control. There was no significant difference in p₀ (0.70 ± 0.03 mmHg for the NO-treated cells and 0.71 ± 0.07 mmHg for control; Table 1). This also shows that there was no substantial NO-induced EPR line broadening as an artifact, at such a low NO concentration. However, the p50 (see the experimental details for the description of this parameter) was significantly increased for NO-added cells (5.90 ± 0.12 and 2.70 ± 0.29 mmHg for NO-treated and control cells, respectively, Table 1). It is

Fig. 1 Effect of exogenous NO on BAEC respiration. **a** NO concentration as a function of time, measured the Clarke electrode. NO of 1 mM was spiked in PBS or PBS containing 1 mill/ml BAECs as indicated in the figure. Although 1 mM NO was added, only around 0.2 μM was measured (*inset*). **b** EPR spectra obtained for NO treated cells at 0 and 80 min. EPR spectra were obtained for both NO-treated and control cells, and the EPR line width data was converted into $p\text{O}_2$ data. **c** A plot of $p\text{O}_2$ with respect to time for both control and 1 mM

NO saturated cells (4 mill/ml). **d** The derivative of $p\text{O}_2$ vs. time was obtained, and the oxygen consumption rate was determined for both cellular conditions. As seen, the NO-treated cells have a lower consumption rate in comparison to the control cells. The *broken lines* are an actual fit of the measured data to Eq. 1 (experimental section). A distinct rightward shift is observed for the NO added cells (in comparison to the control) at low $p\text{O}_2$ (*inset*), implying an increase in the p_{50} value

Table 1 Effect of endogenous and exogenous NO on mitochondrial respiratory chain

Condition	$V_{\text{O}_2 \text{ max}}$ (mmHg/min/ 5×10^6 cells)	p_0 (mmHg)	p_{50} (mmHg)	Mitochondrial affinity (mmHg^{-1})	n
Control	3.66 ± 0.32	0.71 ± 0.07	2.70 ± 0.29	0.41 ± 0.04	10
1 mM NO added ^a	$2.15 \pm 0.05^{**}$	0.70 ± 0.03	$5.90 \pm 0.12^{**}$	0.17 ± 0.02	3
SNAP ^b	$3.02 \pm 0.12^*$	0.83 ± 0.03	2.83 ± 0.12	0.35 ± 0.01	3
Bradykinin ^b	$2.80 \pm 0.05^*$	0.69 ± 0.06	2.88 ± 0.15	0.35 ± 0.02	3
Calcium Ionophore ^b	$2.76 \pm 0.28^*$	0.91 ± 0.09	2.34 ± 0.20	0.44 ± 0.04	5
L-NAME ^b	$3.85 \pm 0.17^*$	0.82 ± 0.03	2.38 ± 0.18	0.43 ± 0.03	4
HS (2 h)	$1.85 \pm 0.17^{**}$	0.44 ± 0.06	2.18 ± 0.21	0.45 ± 0.06	7
Geldanamycin	$3.86 \pm 0.40^*$	0.35 ± 0.03	2.92 ± 0.43	0.37 ± 0.04	5

^a Added immediately before measurements

^b Pre-incubated with these drugs

* $p < 0.05$, ** $p < 0.001$

Fig. 2 Effect of a direct addition of SNAP (NO donor) on BAEC respiration. **a** NO concentration as a function of time, measured by Clark electrode, in buffer and cells (5 mill/ml) containing medium upon addition of 50 μM SNAP. About 8 μM NO release was observed, which slowly increased to 10 μM in 80 min. **b** A plot of pO₂ with respect to time for both control and 50 μM SNAP added cells. **c** The derivative of pO₂ vs. time was obtained, and the oxygen

consumption rate was determined for both cellular conditions. As seen, the SNAP-treated cells have a lower consumption rate in comparison to the control cells. The *broken lines* are an actual fit of the measured data to Eq. 1 (experimental section). There is a distinct rightward shift in the SNAP saturated plot (in comparison to the control), implying an increase in the p50 value (*inset*)

clear in Fig. 1c and Table 1 that the respiration is attenuated in the NO-treated cells. Moreover, there is a distinct increase in p50 in comparison to the control (inset in Fig. 1c and Table 1). The increase in the p50 value is an indication that the externally added NO inhibits respiration at a low pO₂ by reversible, competitive binding at CcO of complex IV, as previously proposed (Brown 2001). These results are consistent with previously measured parameters using polarographic techniques (Clementi et al. 1999; Koivisto et al. 1997).

In the second set of experiments, SNAP was used as an NO donor. Unlike the previous case where NO solution was added, the addition of SNAP will enable a controlled release of NO. The release of NO by SNAP was measured by the Clarke electrode, and the results are shown in Fig. 2a. Addition of 50 μM of SNAP steadily released about 8 μM of NO. Even in the presence of cells, almost

the same level of NO is released (Fig. 2a). The respiration data were obtained from EPR oximetry in the absence and in the presence of 50 μM SNAP. Figure 2 demonstrates EPR results for control and SNAP-added cells. These results show a similar trend as seen in Fig. 1b and c. When treated with SNAP, the pO₂ declined to ~15 mmHg, followed by an increase. The increase in the low pO₂ range is due to NO-induced line broadening (Chamulitrat 2001). The data from Fig. 2b were converted into rate and plotted vs. pO₂ (Fig. 2c). Unlike the control, the addition of SNAP strongly attenuated the respiration ($V_{O_2 \text{ max}} = 1.575 \text{ mmHg/min}/5 \times 10^6 \text{ cells}$) and increased the p50 to 7.295 mmHg (Fig. 2c, inset).

In the third set of experiments, the cells were incubated with 1 mM of SNAP for 20 min. SNAP was withdrawn, and regular medium was added before trypsinizing the cells for respiration measurements. Although the cells were

exposed to a constant flux of NO for 20 min, NO was not present at the time of measurements. For SNAP pre-incubated cells, pO_2 vs. time data (Fig. 4b) were converted into dpO_2/dt , and the corresponding rate vs. pO_2 data are shown in Fig. 4c. Unlike the insets in Fig. 1d and 2c, the decrease in the consumption rate at a low pO_2 was not different from the control for SNAP incubated cells (Fig. 4c, inset). The data were analyzed as described above and the results are summarized in Table 1. The p_0 for the pre-incubated SNAP cells was 0.83 ± 0.03 mmHg (control = 0.71 ± 0.07 mmHg, $n=10$), and the p_{50} was 2.83 ± 0.12 and 2.70 ± 0.29 mmHg for SNAP-incubated and control cells, respectively (Table 1). Similar to the NO-added cells (Figs. 1 and 2), the SNAP incubated cells also showed a reduced $V_{O_2 \max}$. However, there was no significant difference in the p_{50} in comparison to the control (Table 1). On this basis, it is clear that there is an increase in p_{50} only when NO (or NO releasing agents such as SNAP) is directly added, demonstrating that excess NO in the respiration medium is required for reversible inhibition of CcO.

In order to determine the effect of added NO on the activities of the ETC complexes, we measured the activity of ETC complexes I–IV in control, 1 mM NO added, 50 μ M SNAP added, and SNAP pre-incubated cells, by complex-specific assays using UV spectrophotometry and correlated the results obtained in respiration measurements. Activity measurements were carried out in two different pO_2 values, namely, 21% and 0.5% O_2 , representing a high and low pO_2 region. There is a significant attenuation in the activity of both NO-treated and SNAP-added BAEC lysates, in complexes II–IV (Fig. 3a, b). Especially, a considerable reduction in the complex IV activity was observed at 0.5% pO_2 . This confirms that, at this pO_2 , NO attenuates respiration by directly binding at CcO of complex IV. However, for lysates of SNAP-incubated cells, the complex IV activity measured at 0.5% O_2 was not significantly different from the control (Fig. 4c). This shows that the observed increase in p_{50} for NO-added cells (Table 1) is indeed due to the additional reversible inhibition of complex IV by the excess NO present in the solution.

Effect of endogenous NO on BAEC respiration Bradykinin (Bk) and calcium ionophore III were used as the NOS stimulators; whereas L-NAME was used as a NOS inhibitor. It is known that Bk activates eNOS in endothelial cells by way of the B_2 receptors and decreases mitochondrial respiration (Loke et al. 1999). Likewise, Ca^{2+} ionophore III enhances the production of NO by calmodulin-dependent binding, thus lowering the respiration rate. The cells were incubated with either Bk (1 μ M) or calcium ionophore III (1 μ M) for 30 min, pelleted and subjected to respiration measurements. The EPR data were acquired for

Fig. 3 Mitochondrial ETC complex activities for exogenous NO treated cells. **a** The activity of complexes I–IV for control and NO-treated cells. Complexes II–IV reveal a reduction in activity; yet, complex I shows an increase. For complex IV, there is a significant reduction in the activity at normal, as well as low O_2 conditions. **b** The activity of complexes I–IV for control and cells treated with 50 μ M SNAP immediately before measurements. Complexes II–IV reveal a reduction in activity; yet, complex I shows an increase. For complex IV, there is a significant reduction in the activity at normal, as well as low O_2 conditions

both control and NOS stimulators as previously described. The EPR line width data were converted into pO_2 and plotted as pO_2 vs. time. Similarly, EPR data was acquired for L-NAME treated cells. From the pO_2 vs. time data, dpO_2/dt was obtained and plotted with respect to pO_2 for both NOS stimulators and the inhibitor (Fig. 5a, b). The respiration data obtained from these results are summarized in Table 1. As shown in Fig. 5a and Table 1, both Bk and Ca^{2+} ionophore III-treated cells had reduced respiration rates (2.80 ± 0.05 mmHg/min/ 5×10^6 cells ($n=3$) and 2.76 ± 0.28 mmHg/min/ 5×10^6 cells ($n=5$), respectively) compared to the control (3.66 ± 0.32 mmHg/min/ 5×10^6 cells). Interestingly, the mitochondrial affinity of oxygen, determined from p_{50} , does not show any significant difference among the control, Bk, and Ca^{2+} ionophore-treated cells (Fig. 5a and Table 1). This indicates that a significant attenuation of respiration was

Fig. 4 Effect of pre-incubation of BAECs with SNAP on respiration. EPR data were acquired for cells (2.5 mill/ml) pre-incubated with 1 mM of SNAP, as well as control. **a** A plot of pO₂ vs. time for control and SNAP-treated BAECs. **b** The derivative of **a** was taken to obtain dpO₂/dt vs. pO₂. The broken lines are an actual fit of the measured data to Eq. 1 (experimental section). Although a decrease in the respiration rate is seen in the pre-incubated SNAP cells, there is no significant difference as the pO₂ reduces to a lower value (inset). **c** Complexes I–IV activity assays. There is a clear attenuation in the activity of each complex in the presence of SNAP treatment. At a low O₂ content, there is no difference in the complex IV activity

observed at a higher pO₂ (>10 mmHg), although NO-induced inhibition by competitive binding at CcO was not present at a low pO₂, below 10 mmHg. To ensure that the observed effect was associated with NO, experiments were also performed where BAECs were treated with Bk and L-NAME. As expected, the L-NAME reversed the reduction in the respiration rate caused by Bk, resulting in a value similar to the control (Fig. 5c).

The activity of the ETC complexes in NOS-activated cells (Fig. 5d) was determined as described above for the cells that were treated with exogenous NO. The NOS activation by both Bk and Ca²⁺ ionophore moderately reduced the activity of complexes I and II/III, similar to the SNAP-treated cells. Interestingly, complex IV activity, measured at 21% pO₂, showed decreased values for Bk and Ca²⁺ ionophore-treated cells, compared to the control (Fig. 5d). However, there was no significant difference at 0.5% pO₂ in comparison to the control. This illustrates once again that the activation of NOS as an endogenous source

could potentially inhibit cellular respiration via irreversible inhibition of complexes I–IV; however, any additional reversible inhibition of complex IV by competitive NO binding at CcO is not present. This supports the idea of why there is no change in the p50 value in these NOS-activated cells in comparison to the control.

Further studies were carried out to determine whether the attenuated respiration in NOS-activated cells (treated with Ca-ionophore and Bk) was due to any toxicity effects of these agents or due to increased glycolysis. Control and drug-treated cells were stained with Rho123 and analyzed through flow cytometry. Rho123 selectively stains the intact mitochondria (Papandreou et al. 2006). In both control and drug-treated cells, more than 99% positive staining was observed (Fig. 6a), indicating that incubation of BAECs with these agents is not toxic. Likewise glucose uptake in these cells was determined using 2-NBDG. Once again, the mean fluorescence of the histograms obtained from the flow cytometry (Fig. 6b) is the same for both

Fig. 5 Activation of NOS on cellular respiration. To demonstrate the respiratory effect of NOS, the cells were treated with NOS stimulators and inhibitors. **a** The rate of O_2 consumption vs. pO_2 was plotted for bradykinin (1 μM) and Ca^{2+} ionophore (1 μM), NOS stimulators. For the NOS-stimulated cells, it is an obvious attenuation in the respiration rate. However, there is no considerable difference as the pO_2 decreases (inset). **b** Upon the treatment of L-NAME (0.5 mM), the cells

demonstrated a similar respiration rate as the control cells. This observation was also seen at a lower pO_2 range (inset). **c** For the Bk-treated cells, a distinct reduction in the maximum O_2 rate was seen. However, Bk+L-NAME treatment reverses this behavior. **d** The activity of complexes I–IV is measured for the NOS stimulators, as well as control. Complex IV shows only a slight decrease in activity in the presence of NOS

control and drug-treated cells, indicating that the glucose uptake is the same for both control and drug-treated cells. Thus, the decreased respiration measured with NOS-stimulated cells is most likely and exclusively due to excess NO and its derivatives. More importantly, in NO-incubated cells, there was no competitive binding of NO at CcO.

Effect of heat-shock-induced NO on cellular respiration Heat shock has been shown to activate eNOS through the Hsp90/Akt pathway. In the present work, Hsp90-enhanced eNOS activity was used as another system where endogenous NO and its regulation of respiration in BAECs can be studied. The cells were heat shocked at 42°C for 2 h and returned to 37°C for 24 h. Following this, these cells were isolated and

used for respiration measurements, as well as Western blot analysis (in order to prove that such an increased association between eNOS and Hsp90 is present). Figure 7a and b shows Western blots of eNOS and Hsp90, respectively, for control and heat-shocked (for 2 h) cells. The quantitative plots show that upon heat shock, eNOS is overexpressed nearly threefold compared to the control. Similarly, Hsp90 expression increased about three times the expression of the control. Furthermore, the association between Hsp90 and eNOS was found to be higher in heat-shocked cells, as confirmed by co-immunoprecipitation of each other using the procedure described recently (Fig. 7c, d; Harris et al. 2003; Ilangoan et al. 2004a).

Fig. 6 Flow cytometric analysis of mitochondrial integrity and glucose uptake in NOS activated BAEC. **a** Quadrant plots of Rho123 stained control, Bk (1 μ M), CaII ionophore (1 μ M) treated BAECs. In all these cases, more than 99% of the cells were positively stained for Rho123, indicating that mitochondria are intact in these cells. **b** Histograms of 2-NBDG-stained control and BK and CaII ionophore-treated cells. Dotted circles in the insets show the fraction of total gated cells. In all the cases, more than 90% of the cells were gated to be plotted in the histograms. The mean fluorescence intensity is indicated on each histogram

Both EPR spin trapping and fluorescence microscopic imaging were used to infer whether the increased association between Hsp90 and eNOS results in an increased production of NO. As shown in Fig. 8d, the NO-FeMGD EPR signal showed an increased amplitude for heat-shocked cells compared to the control. When the Hsp90 antagonist, GA, was used, the amplitude was slightly reduced. This indicated that the increase, observed in the heat-shocked cells, is due to the Hsp90 association. This effect was further confirmed by using DAF-2DA staining, an NO and NO-derivative specific dye, for fluorescence microscopic imaging. Figure 8a and b shows the fluorescence images of control and heat shock cells stained with DAF-2DA. Based on the intensity measurements, the heat-shock-treated cells had an average intensity of 124 ± 5.8 au ($n=33$), whereas the control had an average intensity of

69.7 ± 2.5 au ($n=31$), demonstrating nearly a twofold increase (Fig. 8c). Rho123 staining was used to determine whether heat shock exerts any toxicity to mitochondria in BAECs and subsequently attenuate respiration. Rho123-stained control and heat-shocked cells showed more than 99% positive staining (Fig. 8e, f), indicating that the mitochondrial intactness is the same both in control and heat-shocked cells.

Respiration measurements were carried out for both control and heat-shocked BAECs to determine whether the enhanced production of NO, due to the interaction of Hsp90 and eNOS, reduce the respiration rate. The respiration curves obtained from EPR oximetry experiments are shown in Fig. 8a and b. Compared to the control, the overall O₂ consumption was observed to be slower for heat-shocked cells (Fig. 9a). This attenuation is likely due to an

Fig. 7 Western blot analysis of eNOS and Hsp90 in BAECs. **a** Western blot analysis of eNOS in the control and heat-shocked (HS) BAECs at 2 h. Quantitative plots are the average of three independent experiments ($n=3$), and the error bars are standard errors. In comparison to the control, there is an increase in the HS state. **b** Western blot analysis of Hsp90 in the control and HS BAECs at 2 h. There is a twofold increase in 2 h HS, implying a higher amount of protein present. β -Actin was used as the loading control for both **a** and **b**. **c** IP:eNOS/IB:Hsp90 in the control, 2 h HS. In the presence of HS, there is a twofold increase in association between eNOS and Hsp90. **d** IP:Hsp90/IB:eNOS in the control and 2 h HS. A threefold increase is shown in the presence of HS in comparison to the control cells

increased production of NO upon exposure to heat shock. This effect is diminished upon the addition of GA, as shown in Fig. 9a and b, confirming that Hsp90-enhanced NO production is responsible for the decline in the respiration rate. Figure 9b and Table 1 clearly reveal approximately a twofold decrease in the respiration of the heat-shock-treated cells in comparison to the control. Moreover, there was no significant change in the p50, once

again confirming that the reversible CcO inhibition by NO is not present.

Finally, the glucose uptake in control and heat-shocked cells were determined using 2-NBDG (Fig. 10b). The histogram obtained for heat-shocked cells showed different characteristics compared to the control. There were two envelopes, one matching with unstained cells and another one matching with 2-NBDG-stained control cells (Fig. 10b), indicating heterogeneous cell populations in terms of glucose uptake in heat-shocked cells. However, both envelopes show mean fluorescence intensity lesser than the control cells (middle panel of Fig. 10b). We further determined Glut-1 and Glut-2, the glucose transporters in

Fig. 8 Analysis of NO production. **a** DAF-2DA-stained control cells ($\times 20$ magnification). **b** Two hours heat-shocked cells ($\times 20$ magnification). **c** Quantitative fluorescence image intensity [arbitrary units (au)] averaged from intensities from individual cells ($n=33$). **d** Fe-MGD spectra with control, 2 h heat shock treatment and 10 μ M of GA, following 2 h of heat shock treatment. The amplitude is enhanced in the presence of heat shock, implying a boost in NO production. Upon the treatment with GA (10 μ M, added before heat shock), the spectrum slightly reduces to a similar spectrum as seen in the case of the control cells. **e**, **f** Quadrant plots of Rho123-stained control and heat-shocked cell. More than 99% cells were intact even in heat-shocked cells

Fig. 9 Attenuation of respiration by heat shock. **a** pO₂ vs. time data for 2 h HS, control and GA (10 μM) following 2 h of HS. **b** The rate of the control and GA-treated cells demonstrate no significant difference. However, the HS cells show approximately a twofold decrease in the respiration rate

BAEC (Presley et al. 2008). In the case of heat-shocked cells, the Glut-1 was found to be lesser than control, correlating with the NBDG uptake. These results together indicate that increased glycolysis is not responsible for the decreased respiration in heat-shocked cells. Thus, the decreased respiration is due to adaptation of these cells, caused by NO, to consume less oxygen.

Discussion

The important outcome of the present study is that endogenously generated NO, by activation of NOS, may not compete with O₂ binding at CcO at below 10 mmHg in order to inhibit mitochondrial oxygen consumption. The regulation of cellular respiration by NO, through the interaction at CcO in the mitochondrial ETC, has been found to be an important factor in many pathophysiological conditions such as ischemia, hyperthermia, etc. (Basu et al.

2008; Ilango et al. 2004a; Zhao et al. 2005). A number of studies have been carried out to unravel the mechanistic details of how NO affects the CcO activity at normal and pathologic conditions (Cleeter et al. 1994; Cooper and Davies 2000). Cleeter et al. reported reversible inhibition

Fig. 10 Flow cytometric analysis of glucose uptake in heat shocked BAEC. **a** Western blots of Glut-1 and Glut-2. The error bars in quantitative plots are SEM from three independent measurements ($n=3$). **b** Histograms of 2-NBDG incubated control and heat-shocked BAECs. The circles in the dotted plots (insets) show fraction of total gated cells, sampled for plotting histograms. While in control, more than 90% of cell could be sampled in the case of heat-shocked cells, only less than 35% of gated cells could be sampled. The mean fluorescence intensity is marked on each histogram

In order to gain further insights into the role of endogenously generated NO on the mitochondrial respiration, three different conditions were used in the present

Fig. 11 Simplified schematics of heat stress activated Hsp90/NOS and NO-induced inhibition of the ETC in the mitochondria. Heat stress activates the Hsp90/NOS complex and increases the NO generation. NO competes for the binding site in the cytochrome c oxidase of complex IV. This competitive binding depends upon the prevailing pO_2 . On the other hand, NO reacts with O_2^- , also generated in the ETC and the $ONOO^-$, which can irreversibly bind or nitrate some of the ETC complexes and attenuate the respiration. It seems that when NO is exogenously added, the complex IV inhibition is significant while the endogenous NO generation leads to $ONOO^-$ -induced process

oxygen affinity at this site (Table 1), as reported recently by Mason et al. that binding at the heme a₃ site depends on the pO₂ (Mason et al. 2006). On the other hand, once the pre-treated SNAP cells were isolated and re-suspended in the SNAP-free respiratory medium, the Cu_B bound NO is known to exert its effect on the CcO activity, in the form of reduced oxygen consumption at a higher pO₂ in comparison to the control ($V_{O_2 \text{ max}}$, Table 1). However, there was no increase in the p50 as the pO₂ decreased because there was no free NO to compete at the heme a₃ site, resulting in a comparable oxygen affinity as control cells at a low pO₂. This was indeed observed when the CcO activity was measured at a low pO₂ (0.5%). The CcO activity was observed to significantly reduce for the case where SNAP or NO was directly added (Fig. 3). However, there was no difference in CcO activity between the control and SNAP-incubated lysates (Fig. 4c). Overall, these results illustrate that NO can inhibit the ETC by binding at complex IV if NO is present during respiration (Fig. 11).

The acquired results from exogenously added NO can be applied to the endogenous activation of NOS and its dependence on the pO₂. Previously reported studies have found that the activation of the NOS enzyme during oxygen consumption measurements in endothelial cells (Loke et al. 1999; Palacios-Callender et al. 2004) and cardiac myocytes (Dai et al. 2001) reduced the $V_{O_2 \text{ max}}$ and increased p50. However, the results obtained in the present studies for BAECs pre-incubated with NOS stimulators or inhibitors exemplify different results. In the case of the cells pre-incubated with NOS stimulators such as bradykinin and Ca²⁺ ionophore, the $V_{O_2 \text{ max}}$ values were reduced as reported before. However, the p50 remained unchanged, showing that NO-induced inhibition of O₂ consumption is different from instances where NO is added before measurements.

To clarify this observation, there are potentially two scenarios. As explained in the case of cells pre-incubated with SNAP and NOS stimulators, NO irreversibly binds to the reduced Cu_B site and remains even after removal from the medium containing stimulators; showing a reduced interference. Since there is no excess of NO to compete with O₂ at low pO₂, there is no increase in p50. Secondly, as the pO₂ decreases, the NO production also declines due to the requirement of oxygen as a substrate for NO generation from NOS. Thus, at a very low pO₂, the flux of the NO may be greatly reduced, resulting in a progressive withdrawal of the competitive reversible inhibition at heme a₃ site. Moreover, as the oxygen declines, many additional physiological events also occur. In the case of ischemia, tissue pH decreases and promotes the reduction of nitrite to NO in the absence of oxygen. As a result, the activity of NOS is reduced. This may potentially lead to an ineffective inhibition of CcO at low oxygen levels (Davidson and Duchon 2006; Moncada and Erusalimsky 2002; Zweier

et al. 1999). It has also been reported that as the [O₂] decreases, superoxide may form (Palacios-Callender et al. 2004).

The observation with NOS stimulators or inhibitors was further confirmed with an additional system, where NOS was activated by Hsp90. Previously, we have used cardiac H9c2 cells to demonstrate that heat shock decreases the V_{O_2} rate (Ilangovan et al. 2004a). Knowing that nitric oxide is enhanced under heat-stressed conditions, the effect of heat shock on respiration has shown similar effects. Upon heat shock treatment, Hsp90 increasingly associates with NOS and activates NOS to generate NO (Fig. 7). As expected, heat shock causes a decrease in respiration in BAECs (Fig. 9 and Table 1). However, this behavior was reversed by the addition of GA, an Hsp90 inhibitor (Fig. 9). In the case of heat-shocked cells that were pre-incubated with GA, the respiration rate showed a $V_{O_2 \text{ max}}$ comparable to control, indicating that the formation of the Hsp90/eNOS complex and subsequent increased NO production is prevented in these cells. Hence, the expected post-treatment effect observed with the heat-shocked cells is not observed in this study.

Overall, the present work highlights very interesting facts about the behavior of NO on mitochondrial respiration. If NO is present in the system, it prefers to bind at the Cu_B site of the reduced form of CcO irrespective of the pO₂. At a low pO₂, it competes with O₂ for the heme a₃ site for reversible binding, and hence, the affinity for the O₂ is reduced (Fig. 11). On the other hand, if the cells are exposed to a higher NO flux and removed, the attenuation of oxygen consumption occurs due to irreversible binding at the reduced Cu_B site of CcO. Furthermore, there is no competitive binding at the heme site, and the affinity of oxygen does not change. This finding is especially important in terms of the regulation of oxygen consumption in various pathological conditions. For example, an ischemia/reperfusion model of the heart has demonstrated that sudden bursts of NO can be measured, and then the heart returns to its normal state (Zhao et al. 2005). The oxygen consumption was proven to be lower in that condition, but it is unclear what mechanism causes such a reduction, especially at a low prevailing pO₂ during ischemia (Zhao et al. 2005). The present results imply that the decrease in oxygen consumption could be attributed to irreversible binding at Cu_B, instead of competitive binding at the heme a₃ site. It also implies that NO generated during ischemia may not interfere with the ETC of myocytes, which are deprived of O₂ in such a condition. Further studies with ex vivo organs may be required to prove this hypothesis.

Acknowledgement We acknowledge support from the National Institutes of Health Grant R21 EB004658, R01-HL-078796 and F31 GM078772-01. We would like to thank Drs. Yeong Renn-Chen and Bin Liu for their assistance with the ETC complexes activity measurements.

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