Mitochondrial Nitric Oxide

A mitochondrial role for catabolism of nitric oxide in cardiomyocytes not involving oxymyoglobin

Jim Peterson, Anthony J. Kanai, and Linda L. Pearce

Department of Chemistry, Carnegie Mellon University, Pittsburgh 15213; Renal Electrolyte Division, University of Pittsburgh School of Medicine, Pittsburgh 15261; Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania 15260

Submitted 1 August 2003; accepted in final form 12 August 2003

Mitochondrial Nitric Oxide

A mitochondrial role for catabolism of nitric oxide in cardiomyocytes not involving oxymyoglobin. Am J Physiol Heart Circ Physiol 286: H55–H58, 2004; 10.1152/ajpheart.00744.2003.—The maximal concentration of nitric oxide (NO) developing in cultured cells following stimulation of endogenous NO synthases was shown to be submicromolar by NO-selective microelectrode measurements. In electron paramagnetic resonance experiments with isolated and finely divided pericardium, NO was found to react with oxymyoglobin to form metmyoglobin provided that NO was supplied at concentrations in excess of a few micromolar. However, at NO concentrations achievable by endogenous sources, this reaction did not take place to any measurable extent. Oxidative conversion of NO to nitrite ion by cytochrome c oxidase appears to be the most plausible route for cellular catabolism of NO.

Experimental Methods

Pericardial tissue preparations. Finely divided pericardial tissue suitable for the electron paramagnetic resonance (EPR) measurements was prepared from 8- to 10-wk-old wild-type rat or mouse hearts. Animals were treated humanely in accordance with the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals employing a University of Pittsburgh Institutional Animal Care and Use Committee-approved protocol. Briefly, after the animals were induced into deep anesthesia with pentobarbital sodium (50 mg/kg), the thoracic cavity was opened and the heart rapidly excised, cannulated at the aorta, and then retrogradely perfused (37°C, 120 mmHg) for 5 min with a solution of (in mM) 144 NaCl, 5.4 KCl, 0.4 NaH2PO4, 10 HEPES, 1 mM MgCl2, and 1.8 mM CaCl2; pH 7.4. Removal of the blood in this fashion ensured that any EPR signals subsequently measured could be safely attributed to myoglobin rather than interfering hemoglobin species. The ventricular tissue was then removed, and the remaining pericardium was minced in two passes at right angles to each other, with the use of a McIlwain motorized tissue chopper set to chop at 150 μm intervals. Because cardiomyocytes can be considered roughly cylindrical with a length of ~75 μM and a diameter of ~15 μM, this treatment ensured that a high proportion of intact cardiomyocytes remained in the samples. The minced tissue was suspended in buffer (perfusion solution), and then 0.2-ml aliquots were introduced into 4-mm OD quartz EPR tubes. After the addition of any further reagents (if required by a particular experimental procedure) with the use of a long Teflon “needle” with agitation to ensure mixing, the sample was quickly frozen (~20 s including additions/mixing) by immersion in liquid nitrogen and then stored at 77 K. Subsequently, samples were transferred to the EPR cryostat for measurement without thawing.

Microsensor methods. Porphyrinic microelectrodes (0.1–15 μM tip diameter, 1 nM NO detection limit, 1-ms response time) were prepared from carbon strands (1–5 fibers, 5 μM diameter each, AMOCO) as previously described (7). Monomeric tetrakis(3-methoxy-4-hydroxyphenyl)nickel(II)-porphyrin (TMHPPNi, Frontier Scientific) was dissolved in 0.1 M NaOH and deposited as a polymeric film on the carbon fiber by cyclic voltammetry (~0.2 to +1.0 V, 20 cycles, EG&G 283 Potentiostat). Natron (Sigma) was then applied to the microelectrodes by dipping in a 1% ethanolic solution. Measurements were performed using a three-electrode system consisting of a working microsensor, a saturated calomel reference, and a platinum counter electrode. Microelectrodes were characterized by differential pulse voltammetry to determine the effective redox potentials of NO and nitrite. Quantitations by chronoamperometry were performed at a constant overpotential of 50 mV to determine sensitivities and detection limits. High purity (≥99.9%) NO standards were prepared for accurate calibration as previously described (9). The microelectrode was mounted on an ultramicromanipulator (0.2 μm resolution), enabling the tip to be placed on the cell surface for NO measurements.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
measured the time-dependent NO release originally developed by Malinski and Taha (11), we have clearly demonstrated the conversion of cardiomyocytes or bladder epithelial cells in which inducible nitric oxide synthase (iNOS) was stimulated to produce endogenous NO sources were always observed, except for endothelial cells, the NO conversion was detected for a few seconds at shear stress. Maximal NO concentrations that developed following stimulation of endogenous NO sources were always submicromolar. Moreover, except for endothelial cells, the elevated NO concentration was detected for a few seconds at most; 1 s in the particular case of cardiomyocytes. These events cannot be measured with the currently available commercial electrodes that typically have response times of at least several seconds. The NO microsensors we construct have response times on the order of 1 ms and, when properly calibrated, output the true NO concentration at the tip as a function of time, with ~0.01 s resolution. In experiments with cardiomyocytes or bladder epithelial cells in which inducible NOS has been upregulated, we measure constant NO production at submicromolar levels. Where NO donors are used, we generally find the prevailing NO concentrations to be one to two orders of magnitude higher than the maxima documented in Fig. 1.

An elegant study by Flögel et al. (5) employing 1H NMR detection of myoglobin species in Langendorff-perfused mouse hearts has unequivocally demonstrated the conversion of MbO2 to Mb+ in response to delivery of NO. In Fig. 2 we have replotted some of the relevant data originally presented by these earlier authors. There are two issues we want to raise in relation to these results. First, conversion of MbO2 to Mb+ was convincingly detected only at NO concentrations in excess of 1 µM. Our own results concerning endogenous NO production (Fig. 1) strongly suggest that >1 µM is higher than normal physiological levels. Second, Flögel et al. (5) commented that their failure to observe production of Mb+ at low (<1 µM) NO delivery was due to the metmyoglobin reductase system present in myocytes reconvert Mb+ to deoxymyoglobin (Mb) faster than the time scale of the measurements. This is a qualitatively reasonable explanation but does not stand up to closer scrutiny of the data. At delivery of −7 µM NO, [MbO2] = [Mb+] = constant (Fig. 2) and also, the rate of MbO2 conversion to Mb+ exactly equals the reverse reaction rate, a steady-state approximation. Therefore, using the argument of the earlier authors, we may write

\[ k_{2ox}(\text{constant}) \cdot [\text{NO}] = k_{2red}(\text{constant}) \cdot b_3 \]  

(2)

where \( k_{2ox} \) is the second-order rate constant governing the reaction between MbO2 and NO \( \cdot 10^8 \text{ M}^{-1} \text{s}^{-1} \) at 37°C (6) and \( k_{2red} \) is the second-order rate constant governing the reduction of Mb+ by cytochrome \( b_5 \cdot 2 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) at 37°C (10). Solving for \([b_3]\), the appropriate component of the metmyoglobin reductase system (10), we find \([b_3]\) = 0.35 M. That is, for the reaction shown in Eq. 1 along with reduction of Mb+ to Mb by cytochrome \( b_5 \) to be a valid and complete explanation for the observed interconversion of MbO2 and Mb+ encompassed by Eq 2, cytochrome \( b_5 \) must be present at 0.35 M, or some three orders of magnitude greater than the total myoglobin concentration! This is clearly an untenable position.

In fact, the data of Fig. 2 can be readily explained by postulating the existence of at least two NO removal pathways. At low NO levels (<1 µM) there appears to be a rapid process by which NO is removed and that does not involve myoglobin. The sites at which NO is preferentially catabolized effectively
and establish the fraction of exogenously added NO that the cardiomyocytes effectively experience.

Topical addition of norepinephrine to cardiomyocytes leads to the endogenous generation of about 0.7 μM NO for <1 s (8) (see also Fig. 1). Addition of 1 μM norepinephrine to EPR samples before freezing did not lead to any measurable increase in Mb⁺ content (Fig. 4, middle trace). Addition of 20 μM NO-saturated buffer (10% vol/vol) before freezing did not lead to any measurable increase in Mb⁺ content either (Fig. 4, top trace). Whereas the concentration was nominally ~20 μM in NO immediately following its addition (Fig. 4, top trace), the maximal effective NO concentration experienced by the tissue would have been 1–2 μM (estimating >90% of the exogenous NO was lost by diffusion as described above in relation to Fig. 3). Therefore, at effective NO concentrations of <2 μM, there is no evidence in these spectra for formation of Mb⁺. Also, because the timescale of the EPR measurements is 45 times shorter than that of the earlier NMR experiments (5), it follows that the results of Fig. 4 strongly suggest that the failure of the NMR experiments to detect Mb⁺ formation at <1 μM NO delivery was not due to its rapid reduction to Mb by the specific reductase system. It is comforting that our current data and that of the earlier authors are actually in very good agreement, only the interpretations differ.

If reaction of NO with MbO₂ is an insignificant pathway for catabolism of NO at levels attained by stimulation of endogenous NOS, the question remains as to how the rapid removal of NO from cells such as cardiomyocytes and neurons evident in Fig. 1 is achieved. These, of course, are mitochondria-rich cell types, whereas endothelial cells with lower mitochondrial content, eliminate NO less efficiently. The hydrophobic NO mol-

become saturated at high NO levels (>1 μM), and so the secondary process involving stoichiometric reaction with MbO₂ becomes significant under such conditions. To further test the validity of this idea, we undertook a series of EPR experiments on finely divided murine pericardial tissue (see EXPERIMENTAL METHODS). The advantage of this approach is that samples can be subjected to elevated NO levels and then cryogenically preserved within 20 s as described under EXPERIMENTAL METHODS. Sample momentarily exposed to 190 μM NO (solid trace); control not exposed to NO (broken trace).

Fig. 3. Reaction of MbO₂ with high (nonphysiological) levels of NO in minced rat heart pericardium. X-band electron paramagnetic resonance (EPR) spectra recorded at 15 K. Samples were prepared at 22°C and then cryogenically preserved within 20 s as described under EXPERIMENTAL METHODS. Sample momentarily exposed to 190 μM NO (solid trace); control not exposed to NO (broken trace).
ecule will tend to partition into membranes rather than remain in the aqueous cytosol. Shiva et al. (15) have demonstrated a nonenzymatic means of NO conversion to nitrite ion (NO$_2^-$) associated with mitochondrial membranes, but this process is two orders of magnitude too slow to explain the elimination times we observe. Many groups have shown that NO can regulate mitochondrial function by partial inhibition of complex IV (cytochrome c oxidase) (2, 3, 13). Torres et al. (16) have shown that NO reacts with complex IV to produce NO$_2^-$ under a variety of conditions. We have shown that endogenously produced NO is quantitatively converted to NO$_2^-$ under certain conditions. In short, whereas the details of the mechanism remain an area of study (12, 14), there is a good deal of circumstantial evidence implicating complex IV as the in vivo site of NO catabolism.

**REFERENCES**