[17] *In Vivo* Spin Trapping of Nitric Oxide by Heme: Electron Paramagnetic Resonance Detection *ex Vivo*

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Introduction

Nitrogen monoxide (nitric oxide, ·NO) is one of the 10 smallest molecules found in nature. Nitric oxide is a stable, neutrally charged, paramagnetic gas, with moderate water solubility (2 m*M* at 1 atm and 20°). The unpaired electron resides in its π^*_{2p} antibonding orbital and is thought to be localized on the N–O triple bond.[1,2] Unlike most radicals, ·NO does not dismute nor covalently dimerize; it readily diffuses through cell membranes.[3]

Nitric oxide can act as either an oxidant or a reductant. The reactivity of ·NO evolves from the interrelated redox couples it forms with the nitrosonium cation (NO^+) and the nitroxyl anion (NO^-), its conjugate acid being HNO (pK_a 4.7).[1,2] There are several important chemical properties of ·NO: (i) it forms adducts with nucleophiles such as amines, sulfite, and thiols, which in turn can slowly and spontaneously release ·NO by first-order kinetics, and (ii) it coordinates with transition metals such as manganese, copper, and iron [both Fe(II) and Fe(III)] in complexes. These general properties endow ·NO with tremendous versatility in biological systems, enabling it to act as either a toxic molecule or as a regulator molecule, the key factors being concentration and duration of release.

Nitric oxide has been used since the late 1960s as an electron paramagnetic resonance (EPR) probe to study oxygen binding sites in oxygen carriers and oxygen-metabolizing metalloenzymes; consequently, a considerable literature exists detailing its EPR spectral characteristics as a ligand in transition metal complexes. An excellent review by Henry includes data on the nitrosylated heme proteins, iron–sulfur proteins, nonheme and non-Fe-S iron proteins, multicopper proteins, and hemerythrin.[4]

Nitric oxide readily complexes with heme proteins, forming paramagnetic species (·NO–heme, $S = \frac{1}{2}$) that are observable at low temperatures

[1] Y. Henry, M. Lepoivre, J. Drapier, C. Ducrocq, J. Boucher, and A. Guissani, *FASEB J.* **7,** 1124 (1993).
[2] J. S. Stamler, D. J. Singel, and J. Loscalzo, *Science* **258,** 1898 (1992).
[3] H. Galla, *Angew. Chem., Int. Ed. Engl.* **32,** 378 (1993).
[4] Y. Henry, C. Ducrocq, J.-C. Drapier, D. Servent, C. Pellat, and A. Guissani, *Eur. Biophys. J.* **20,** 1 (1991).

by EPR. In red blood cells, ·NO will (1) form hemoglobin (Hb) complexes, HbNO·, or (2) be oxidized to nitrite and nitrate with formation of methemoglobin, Fe(III)-Hb. Under these conditions, the hemoglobin tetramer forms a mixture of nitrosylated valency hybrids that can be precisely characterized by EPR spectroscopy.[1,4] In arterial blood (O_2 saturation 94–99%), ·NO is almost quantitatively converted to Fe(III)-Hb and nitrate with little HbNO· formation.[5] In venous blood (O_2 saturation 36–85%) there is more HbNO· and less nitrate formed.[5] This allows blood to be sampled across a vascular bed to assess localized tissue ·NO release. Arterial samples serve as controls, and changes in HbNO· and nitrite concentration in venous samples can serve as indicators of changes in ·NO release. The purpose of this chapter is to detail a method by which ·NO release can be assessed *in vivo* by quantifying HbNO· and plasma nitrite concentrations in whole blood.

Methods

The purpose of our experiments is to assess ·NO release within the splanchnic region of the heat-stressed rat. We collect femoral artery and portal venous blood from hyperthermic rats so that it can be examined by EPR for the presence of HbNO·. Male Sprague-Dawley rats weighing 320–350 g are anesthetized with sodium pentobarbital (Nembutal). In these experiments it is essential to avoid anesthetics that generate ·NO as part of their mechanism of action. A midline laparotomy is performed, the portal vein isolated, and a catheter (silastic tubing over PE 10, Clay Adams, Parsippany, NJ), filled with heparinized saline (100 U/ml), is placed in the portal vein through a tributary vessel. Rats are also fitted with a femoral artery catheter for collecting arterial blood samples. Placing a length of Silastic tubing at the end of these catheters is extremely important for collecting blood samples *in vivo*. The more flexible Silastic tubing allows the catheter to accommodate animal movement. A stiffer form of tubing has a tendency to lay against the vessel, precluding blood collection. The distal end of each catheter is tunneled subcutaneously to the dorsal neck, exteriorized between the scapula, and capped with a stainless steel stylette. The midline incision is closed, and the animals are allowed to recover from surgery. On recovery, rats are heated, and blood samples are collected in sterile, 1-ml Monoject syringes[6] and immediately delivered into quartz EPR tubes (3 mm inner diameter). For selected samples the pH is lowered by

[5] A. Wennmalm, G. Benthin, and A.-S. Persson, *Br. J. Pharmacol.* **106,** 507 (1992).
[6] G. R. Buettner, B. D. Scott, R. E. Kerber, and A. Mügge, *Free Radical. Biol. Med.* **11,** 69 (1991).

the addition of acetic acid (final concentration ~100 mM). The samples are then quickly frozen and stored at 77 K until subsequent EPR analysis.

Electron Paramagnetic Resonance Analysis

Spectra are recorded with a Bruker ESP 300 EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with a standard TE cavity, ER4111VT variable temperature unit, an ER035M gaussmeter, and an EIP-625A microwave frequency counter. Signal averaging (multiple scans of the same sample) is used to improve the signal to noise ratio. Sample volume and geometry are kept constant to allow for comparisons of radical concentrations between samples. All data are collected at 100 K, using the variable temperature unit, with spectra reported as the normalized average of 20 scans. The variable temperature unit rather than a finger Dewar is used to minimize noise. At the very low EPR signal levels observed cavitation of liquid nitrogen in a finger Dewar produces far too much spectral noise for success during our lengthy signal-averaging approach. The EPR conditions are as follows: receiver gain 5.00×10^5, modulation frequency 100 kHz, modulation amplitude 4.0 gauss (G), microwave frequency 9.43 GHz, microwave power 10.0 mW, scan rate 6.2 G/sec over 600 G centered at approximately $g = 2.05$.

Properties of Technique

1. HbNO· stored at 77 K has an extremely long half-life, thus the EPR spectra can be obtained days or even weeks after sample preparation.

2. When using HbNO· formation to quantitate ·NO release, one must take note of the following reactions that can complicate matters: ·NO can oxidize HbO_2 to paramagnetic, EPR-detectable ($S = \frac{5}{2}$) methemoglobin Fe(III)-Hb, while ·NO is converted to nitrite and nitrate. Nitric oxide binds slowly (k_{on} ~10^3–10^4 M^{-1} sec^{-1}) and reversibly to Fe(III)-Hb to form diamagnetic nitrosylmethemoglobin, Fe(III)-HbNO·, which eventually autoreduces by a first-order reaction (k' ~10^{-3} sec^{-1}; $t_{1/2}$ ~12 min) to a paramagnetic species Fe(II)-HbNO·. Furthermore, nitrite oxidizes HbO_2 by an autocatalytic reaction. Finally, nitrite binds reversibly to methemoglobin to yield a mixture of EPR-detectable $S = \frac{5}{2}$ and $S = \frac{1}{2}$ complexes.[1]

3. A radical semiquinone species, $g = 2.005$, often overlaps the high-field line of the ·NO triplet.

4. A copper signal, $g = 2.06$, can also be observed in some situations. The copper signal most likely arises from the acute phase copper protein, ceruloplasmin.

5. Lowering of the pH with acetic acid (final concentration ~100 m*M*) accentuates the ·NO triplet feature of the spectrum.

6. The exact spectral shape of HbNO· varies from species to species. Thus, standards such as those shown in Fig. 1d,e must be established.

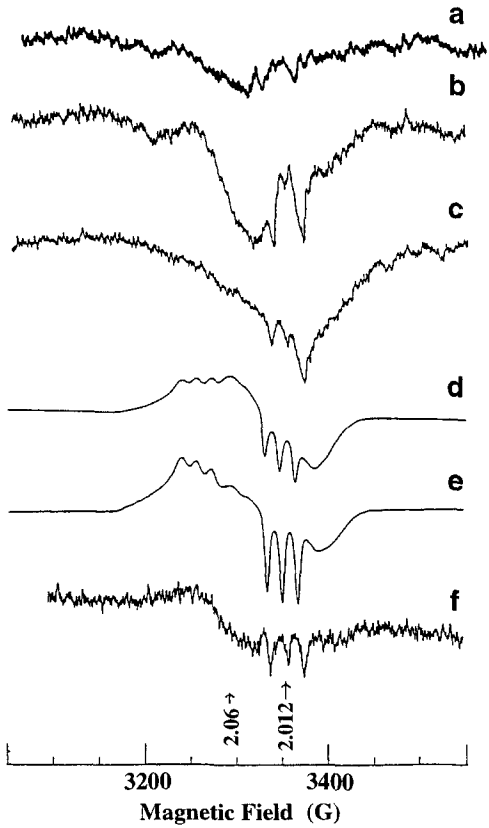

FIG. 1. Low-temperature (100 K) EPR spectra recorded from rat whole venous blood. (a) Femoral artery blood collected from a normothermic control rat. (b) Portal venous blood from heat-stressed rat collected 1 hr post-heat exposure. This spectrum consists of at least three species. The broad feature at $g = 2.06$ is consistent with the g value for copper. The region around $g = 2.012$ contains two species, ·NO–heme with $a^N = 17.56$ G and a semiquinone radical at $g = 2.005$ that overlaps the high-field line of ·NO–heme. (c) Sample from (b) treated with acetic acid to a final concentration of 90 m*M*. (d) Standard ·NO–heme generated by exposing whole blood to ·NO. (e) Sample from (c) treated with acetic acid to final concentration of 90 m*M*. (f) Sample from portal venous blood from a rat with core temperature higher than 42.5°. This animal died from heat stroke shortly after sample collection.

7. Nitric oxide release can be further quantified by assessing plasma nitrite concentration using the Griess reaction.

Results

We have found that HbNO· can be detected in portal venous blood of heat-stressed rats, Fig. 1. In addition to HbNO· at least two other species can be observed: one with a broad feature having an effective g value of 2.06 which is consistent with the copper-binding, acute phase protein ceruloplasmin; the second is a single narrow line $\Delta H_{pp} \approx 10$ G with $g = 2.005$ that is most likely a semiquinone radical.[7] The heat-induced HbNO· signal displays the classic nitrogen triplet hyperfine structure with $a^N = 17.5$ G centered at $g = 2.012$. This feature is consistent with a five-coordinate heme complex of a ferrous–·NO–heme species derived from rat hemoglobin.

In conclusion, we have found that this EPR approach has given us a window into the study of the role of nitric oxide in the potentially catastrophic effects of heat stress. We have been able to demonstrate the local release of ·NO in the splanchnic region during prolonged hyperthermia.[7] This has lead us to hypothesize that hyperthermia-induced release of ·NO leads to splanchnic vascular dysfunction. This phenomenon in turn leads to severe hypotension and cardiovascular shock that are characteristic of prolonged heat exposure.

Acknowledgment

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[7] D. M. Hall, G. R. Buettner, R. D. Matthes, and C. V. Gisolfi, *J. Appl. Physiol.* **77,** 548 (1994).