

In vivo Detection of Nitric Oxide and NO_x Species Using *ex vivo* Electron Paramagnetic Resonance Spectroscopy¹

David M. Hall,^{*2} Garry R. Buettner,[†] and Carl V. Gisolfi[‡]

With the technical assistance of Ronald D. Matthes

^{*}*Mallinckrodt Institute of Radiology, Section of Cancer Biology, Washington University School of Medicine, St. Louis, Missouri 63108; †The Free Radical Research Institute, The University of Iowa, Iowa City, Iowa 52242; and ‡The Department of Exercise Science, The University of Iowa, Iowa City, Iowa 52242*

Nitric oxide is a paramagnetic molecule that exhibits high affinity for heme proteins, forming a mixture of nitrosylated valency hybrids that can be precisely characterized using low-temperature electron paramagnetic resonance spectroscopy. In red blood cells, nitric oxide will form paramagnetic complexes with deoxyhemoglobin or be oxidized to nitrite and nitrate by oxyhemoglobin with formation of paramagnetic methemoglobin. We have developed a technique to monitor temporal changes in local nitric oxide production *in vivo*, by sampling arterial and venous blood across a tissue bed and measuring changes in hemoglobin–nitric oxide and methemoglobin concentration *ex vivo* using electron paramagnetic resonance spectroscopy. Changes in local nitric oxide concentration are further clarified by quantifying plasma nitrite/nitrate levels. With these techniques, we have observed that whole body hyperthermia increases portal venous concentrations of hemoglobin–nitric oxide and organic radicals that are consistent with enhanced nitric oxide synthase activity, cytotoxic leukocyte activity, and cellular oxidative stress in splanchnic tissues. © 1997 Academic Press

INTRODUCTION

Nitrogen monoxide (nitric oxide, NO[•]) is a stable, neutrally charged, paramagnetic molecule that readily diffuses through cell membranes (1). Unlike most free radicals, it neither dismutates nor dimerizes. NO[•] can act as an oxidant or as a reductant, critical conditions being level of production and local concentration. The reactivity of NO[•] is related to the redox couples it forms with the nitrosium cation (NO⁺) and the nitroxyl anion (NO⁻) (it's conjugate acid being HNO pK_a = 4.7) (2). NO[•] also combines with superoxide anion (O₂⁻) at near diffusion controlled rates (4.3 × 10⁹ M⁻¹ s⁻¹) (3), generating the powerful oxidant peroxynitrite (O=NOOH/O=NOO⁻, pK_a = 6.8) (4). NO[•] can form adducts with nucleophiles such as amines, sulfite, and thiols, which in turn can release NO[•], suggesting that NO[•] “sinks” exist in living cells. Alternatively, NO[•] can coordinate with the transition metals manganese, copper, or iron (both ferric and ferrous complexes) (2, 5).

NO[•] exhibits high affinity for heme proteins, forming a mixture of nitrosylated valency hybrids (heme–NO[•], S = 1/2) that can be precisely characterized using low-temperature electron paramagnetic resonance (EPR) spectroscopy (5). In red blood cells, NO[•] will form paramagnetic complexes with deoxyhemoglobin (Hb–NO[•]) or

¹ This article is part of the special section devoted to novel microanalytical methods for the detection and quantification of NO and NO_x species.

² To whom correspondence should be addressed.

be oxidized to nitrite and nitrate by oxyhemoglobin with formation of paramagnetic methemoglobin (Fe(III)-Hb), $S = 5/2$). Respective yields depend on molecular oxygen concentration. In arterial blood (O_2 saturation 94–99%), NO^{\bullet} is converted to Fe(III)-Hb and nitrate with little Hb- NO^{\bullet} being formed. Quantitatively more Hb- NO^{\bullet} and less nitrate is produced in venous blood (O_2 saturation 36–85%) (6). NO^{\bullet} from activated leukocytes can also nitrosylate cellular proteins and metals, yielding paramagnetic iron-nitrosyl complexes that are biomarkers of cytotoxic white cell activity (7). Henry's review (8) includes data on the EPR spectral characteristics of nitrosylated hemo-proteins, iron-sulfur proteins, nonheme and nonFe-S iron proteins, multicopper proteins, and hemerythrin.

Collectively, these data suggest that endogenous heme proteins can be viewed as spin traps to investigate temporal changes in NO^{\bullet} production in living systems. Indeed, Lancaster's kinetic models (9), detailing diffusion characteristics of NO^{\bullet} *in vivo*, predict that alterations in vascular Hb- NO^{\bullet} concentration are indicative of shifts in local tissue NO^{\bullet} release. We have developed a technique to monitor temporal changes in vascular Hb- NO^{\bullet} levels *in vivo*, by sampling arterial and venous blood across a tissue bed and measuring Hb- NO^{\bullet} and Fe(III)-Hb concentrations *ex vivo* using low-temperature EPR and a signal averaging approach (10). Arterial samples serve as controls, and changes in venous Hb- NO^{\bullet} and Fe(III)-Hb provide indices of local NO^{\bullet} production. NO^{\bullet} levels can be further quantified by measuring plasma nitrite/nitrate concentration using the Greiss reaction. With these techniques, we have observed that whole body hyperthermia increases portal venous concentrations of Hb- NO^{\bullet} and organic radicals that are consistent with enhanced nitric oxide synthase (NOS) activity, cytotoxic leukocyte activity, and cellular oxidative stress in splanchnic tissues (10).

METHODS

Male Sprague-Dawley rats weighing 320–350 g were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.). An incision was made in the inguinal region of the right hind-quarter, and animals were fitted with a femoral artery cannula (PE 50 Clay Adams, Parsippany, NJ) filled with heparinized saline (100 U/ml) for collecting arterial blood. A midline laparotomy was performed, the portal vein isolated, and a second cannula (PE 10, Clay Adams, Parsippany, NJ, inserted 0.5 mm into a 1.5 mm length of silastic tubing) filled with heparinized saline (100 U/ml), was placed in the portal vein through a tributary vessel ≈ 2 mm from the liver, and secured with Vetbond tissue glue. Tributary vessels were chosen as cannulation sites to avoid occluding portal blood flow, both during surgery and subsequent sample collection. With unrestrained animals, silastic tubing is superior to PE tubing in that the flexible silastic moves with a vessel as the animal changes position, more easily conforming to shifts in vessel orientation. A stiffer form of tubing has a tendency to hold its position, frequently occluding blood flow and precluding sample collection. Using this technique, we have achieved a better than 75% success rate in collecting portal venous blood from conscious, unrestrained animals under a variety of experimental conditions.

The distal ends of both cannulae were tunneled subcutaneously to the dorsal neck, exposed through an incision between the scapulae, and capped with stainless steel

stylettes. Incisions were closed, and animals were allowed to recover from surgery. Upon recovery, rats were heated in an environmental chamber and blood samples were collected in sterile, 1 ml Monoject syringes (11). The 200 μ l aliquots were delivered to quartz EPR tubes (3 mm inner diameter), cooled under liquid nitrogen, and stored at -80°C pending EPR analysis. To enhance semiquinone radical dismutation, pH was lowered to ≈ 4.6 in selected samples by addition of acetic acid, final concentration ≈ 100 mM.

EPR ANALYSIS

An electron possesses a magnetic moment by virtue of its spin. In the presence of an applied magnetic field, this magnetic moment will adopt one of two allowed orientations corresponding to the two spin states of the electron. Transitions between spin states (resonance) can be induced if oscillating electromagnetic radiation of appropriate frequency (ν) is applied perpendicular to the external magnetic field (B_0). EPR detects unpaired electrons in a sample by their absorption of microwave irradiation when the sample is placed in a strong magnetic field.

An EPR absorption spectrum, which is expressed graphically as the first derivative of the sample absorption line, is characterized by four main parameters: intensity, g value, linewidth, and multiplet structure.

The integrated area under a specific deflection is directly proportional to the concentration of the unpaired spins giving rise to that deflection. If physical conditions and sample volumes are kept constant, comparisons in radical concentration can be made between biological samples by measuring the peak-to-peak intensities (line height) of the derivative deflection. We have chosen a sample volume (200 μ l) large enough to completely fill the active area of the spectrometer cavity, allowing us to compare radical concentrations between blood samples.

The g value defines the position of a resonance deflection in an EPR spectrum and identifies a particular class of radical species. It is a measure of the local magnetic field experienced by the unpaired electron during resonance and is defined by the equation $g = 0.714487\nu/B_0$, where ν represents the microwave frequency used to induce resonance and B_0 is the applied (or external) magnetic field at resonance. The g value contains information about electronic structure, as well as the symmetry of the spin system, and the g value associated with each class of radical remains consistent from experiment to experiment as manipulations of ν are offset by changes in B_0 .

The linewidth of an EPR resonance deflection is less well understood and is comprised of two types of contributions: spin-lattice relaxation time and inhomogeneous broadening.

The multiplet structure of a spectrum consists primarily of hyperfine interactions where the hyperfine structure arises from the interaction of unpaired electron spins with nuclear spins. g values plus multiplet structure often allow precise identification of radical molecules.

EPR Conditions

EPR spectra were recorded in these experiments with a Bruker ESP 300 EPR spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with an ER035M gaussmeter, an ER4111VT variable temperature unit, and an EIP-625A microwave

frequency counter. Signal averaging (addition of multiple scans of the same sample) was used to improve the signal-to-noise ratio. All data were collected at 100 K with spectra reported as the normalized average of 20 scans. EPR conditions: receiver gain 5.00×10^5 , modulation frequency 100 kHz, modulation amplitude 4.0 G, microwave frequency 9.43 GHz, microwave power 10.0 mW, scan rate 500 G/42 s.

PROPERTIES OF THE TECHNIQUE

1. Hb-NO[•] and methemoglobin in whole blood cooled to -80°C have extremely long half-lives, on the order of days or even weeks. Also, samples are easily transported on dry ice and can be briefly thawed and refrozen without losing appreciable concentrations of either radical.

2. When using these techniques to quantitate NO[•] release, the following reactions may complicate data interpretation (2, 5):

(a) NO[•] can oxidize Hb-O₂ to paramagnetic Fe(III)-Hb;

(b) NO[•] binds slowly ($k_{\text{on}} \approx 10^3\text{--}10^4 \text{ M}^{-1} \text{ s}^{-1}$) and reversibly to Fe(III)-Hb to form diamagnetic nitrosyl-methemoglobin (Fe(III)-Hb-NO[•]), which autoreduces by a first-order reaction ($k' \approx 10^{-3} \text{ s}^{-1}$; $t_{1/2} \approx 12 \text{ min}$) to a paramagnetic, EPR-detectable Fe(II)-Hb-NO[•] species. The ratio of rates of NO[•] uptake and release for Fe(II)-Hb is 5 to 6 orders of magnitude greater than that of O₂ (10^{-5} s^{-1} for NO[•] and 20 s^{-1} for O₂), predicting that reduced methemoglobin is a more effective NO[•] scavenger that can also rapidly release NO[•].

(c) Nitrite autocatalytically oxidizes Hb-O₂. Nitrite can also reversibly bind to methemoglobin to yield a mixture of EPR-detectable $S = 5/2$ and $S = 1/2$ complexes. These data suggest that local acid/base conditions and oxygen concentration are important considerations in EPR data interpretation.

3. A free radical semiquinone ($g = 2.005$) and a $g = 2.03$ species that is associated with activated cytotoxic leukocytes often overlap the high field line of the NO[•] triplet in venous blood samples. Lowering sample pH removes these radicals and accentuates the NO[•]-triplet feature of the spectrum. Interestingly, a similar decrease in pH of authentic Hb-NO[•] also accentuates the NO[•] triplet feature (Fig. 1).

4. The precise spectral shape of Hb-NO[•] varies from hybrid to hybrid, therefore standards such as those shown in Fig. 1 must be established.

5. Signals associated with the paramagnetic species observed in these experiments are often very weak, with deflections occurring at or slightly above the spectrometer noise level. To improve the spectral signal-to-noise ratio and more clearly define the shape of our spectra, we utilized a signal averaging approach (addition of multiple scans of the same sample), with all spectra reported as the average of 20 scans. EPR spectra were collected at 77 K, by scanning samples immersed in liquid nitrogen, or at 100 K, using a variable temperature system which maintains the sample at a preset temperature by passing cold nitrogen gas past the sample in the spectrometer cavity. Both methods provided identifiable radical signals, but the signal-to-noise ratio markedly improved using the variable temperature accessory.

RESULTS AND DISCUSSION

We have found that Hb-NO[•] and nitrosyl complexes can be detected in portal venous blood and splanchnic tissue biopsies (small intestine, liver) from heat-stressed

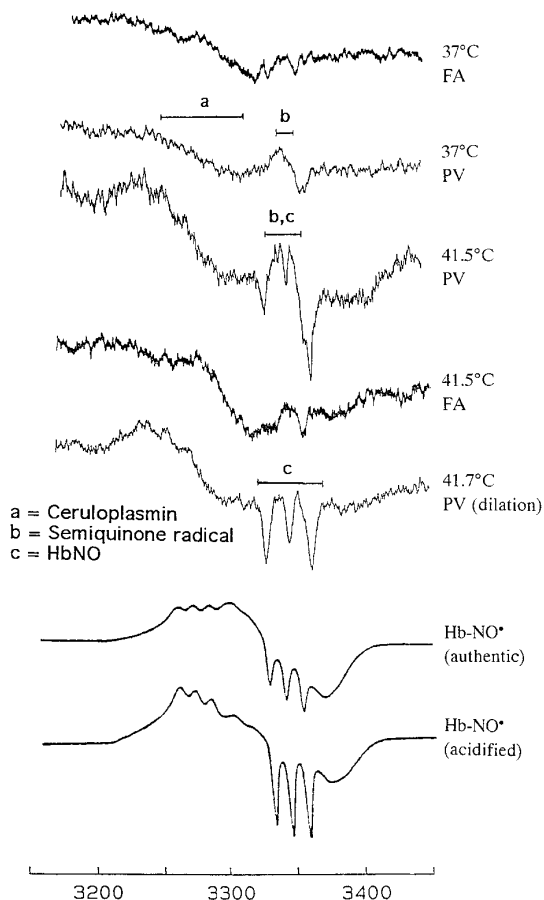


FIG. 1. Low-temperature (100 K) EPR spectra collected from rat whole blood. Rats were heated unanesthetized and unrestrained and blood samples were drawn at the indicated colonic temperature ($^{\circ}\text{C}$). Spectra collected from hyperthermic rats contain at least three species. The broad feature at $g = 2.06$ (a) represents ceruloplasmin. The region around $g = 2.012$ contains two species: semiquinone radical (b) and Hb-NO[•] (c). Authentic Hb-NO[•] was made by briefly bubbling NO[•] gas into a container of whole venous blood collected from a donor rat. The container was first purged of ambient air with argon gas. PV, portal venous blood; FA, femoral artery blood.

rats. The hyperthermia-induced Hb-NO[•] signal displays the classic nitrogen triplet hyperfine structure, $a^{\text{N}} = 17.5$ G and centered at $g = 2.012$. We have identified this species as a five-coordinate heme complex of a ferrous-heme-NO[•] moiety derived from rat hemoglobin (10). An identifiable Hb-NO[•] signal is not evident in corresponding arterial samples suggesting that this is a local phenomenon that occurs within the splanchnic region (Fig. 1). Supporting this interpretation, portal venous Hb-NO[•] signal intensity progressively increases with elevations in colonic temperature (10, 12), until the splanchnic vascular bed dilates (12), independent of sympathetic nerve activity and circulating catecholamines (13).

We and others have observed that similar radical signals are evident in blood and tissues collected from rats following endotoxin challenge, septic shock, and hemorrhagic shock (14, 15). These data suggest that loss of splanchnic vascular resistance precipitates shock in the heat-stressed rat. They further suggest that increased NOS activity at high colonic temperature contributes to this phenomenon. The constitutive NOS antagonist nitro-L-arginine methyl ester (L-NAME) dose-dependently decreases intensity of this signal, establishing that NO[•] is derived from enzymatic sources under these conditions (12). L-NAME treatment significantly decreases total heating time and peak colonic temperature before shock however, indicating that intact NOS activity is essential for normal thermotolerance (12).

In addition to Hb-NO[•], at least two other paramagnetic species can be observed in these experiments: (1) a broad spectral feature, characteristic of transition metals, having an effective *g* value of 2.06, that is consistent with the acute phase protein, ceruloplasmin, and (2) a more narrow feature, characteristic of a free radical, $\Delta H_{pp} \approx 10$ G at *g* = 2.005, that is consistent with semiquinone radical (10). These data suggest that whole body hyperthermia promotes hepatic hypoxia and cellular oxidative stress in splanchnic tissues. Experiments using the xanthine oxidase inhibitor allopurinol in conjunction with EPR quantitation of portal venous radical profile suggest that xanthine oxidase is an important source of reactive oxygen species that contribute to cardiac deficiency and multi-organ tissue injury during hyperthermia (16).

In conclusion, we have found that this EPR approach has given us a window into the study of the respective roles of NO[•] and reactive oxygen species in the pathophysiology of whole body hyperthermia. Data collected with this technique have led us to hypothesize that NO[•] and reactive oxygen species produced in splanchnic tissues contribute to the cardiovascular deficiency, consumptive coagulopathy, and multi-organ failure characteristic of heat stroke. We speculate that splanchnic oxidant generation contributes to the pathophysiology of multiple shock syndromes.

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