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BINDING OF NITRIC OXIDE TO INTACT HUMAN ERYTHROCYTES AS MONITORED BY ELECTRON PARAMAGNETIC RESONANCE

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Summary: Human blood was diluted in isotonic saline at pH 7.4 and deoxygenated. After gentle exposure to nitric oxide gas (NO), the red blood cells (erythrocytes) remained intact. Increasing the cell volume fraction allowed detection of strong electron paramagnetic (EPR) signals, even at ambient temperature (293 K). These spectra were compared to those recorded at 77 K. With maximal NO exposure, a relatively featureless and stable spectrum was recorded. Reduced exposure produced a spectrum, which gradually transformed into the final one, with more structure. The spectral features with unfrozen samples reflected the degree of resolution of the hyperfine triplet component observed at 77 K. This hyperfine coupling was independent of the temperature. The spectra reveal rearrangement of the NO ligand between the subunits of hemoglobin. At subsaturation levels, binding to the α subunit, with its iron penta-coordinated in the T-state, dominates. © 1994 Academic Press, Inc.

Nitric oxide, or nitrogen monoxide (NO), was used by biophysicists as soon as electron paramagnetic spectroscopy (EPR) became available to the study of hemoproteins (1). This gas has been used as a strong ligand, rendering silent ferrous hemoproteins detectable by EPR, due to the unpaired electron of NO (2-11). A renaissance in the interest for the NO took place in the early 1980's, when unraveling of its physiological role began and many reviews have described this development (see e.g. 11-14).

The main biological target for NO is the hemoprotein guanylate cyclase, but affinity for this ligand is also demonstrated by other metalloproteins. Formation of thiol adducts has also been suggested to occur. Depending on the oxygen concentration, the lifetime of free NO is in the order of seconds (11,13). Free NO can diffuse within tissues and through membranes. In contact with blood, NO will rapidly interact with the hemoglobin (Hb) within the red blood cell (RBC; erythrocyte). Hence, Hb can inhibit the biological effect of NO. With oxygenated blood, NO can oxidize hemoglobin, which can normally be ezymatically reduced inside the intact RBC.

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Vascular and neuronal activities are regulated by NO synthetized in the endothelia and neurons, respectively. Macrophage stimulation can give rise to a NO burst. The production of NO *in vivo* can be determined by EPR using deoxyhemoglobin as a trap (reviewed in 11,14). Hence, during septic shock characteristic HbNO spectra were detected with venous, as well as arterial blood. After injection of cytokines into rats, HbNO spectra were observed in the blood. Furthermore, HbNO can be detected in rats during pregnancy. HbNO has also been detected in patients after nitrogycerin intake or NO inhalation. In the basal physiological state, no EPR spectrum from HbNO has been detected.

Isolated Hb has been used earlier to study EPR spectra after exposure to NO, and the spectra reported from HbNO using frozen samples. The human RBC contains about 5 mM of Hb, as well as its BPG effector (in addition to about 2 mM glutathione). It is demonstrated here that this allows detection of strong characteristic EPR signals from HbNO in small samples, even at *room temperature*. The present experiments were performed *in vitro* with deoxygenated fresh RBCs, which remained morphologically intact after flushing with NO gas in an isotonic medium at controlled pH. The room temperature spectra were then compared to those obtained with liquid nitrogen (77 K). In this communication some qualitative observations with RBCs are presented, which support earlier reported EPR and optical studies employing hemoglobin solutions (4-7).

Materials and Methods

Chemicals. Nitrogen monoxide gas, with a purity of > 99%, and pure nitrogen and argon gas were bought from Aga Gas AB, Stockholm. Inert gases could be purified using a column (Sorbal 500, Alphagaz). The NO gas was further purified by bubbling through a concentrated alkaline solution. 2,3-Bisphospho-D-glyceric acid (BPG), Grade I, and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, were obtained from Sigma.

Blood samples. Blood (group 0) was obtained by finger puncture. Usually, about 200 μl of blood was collected using a standard capillary tube and mixed directly with 5 ml medium, containing 137 mM NaCl buffered at pH 7.4 with 20 mM Hepes. Plasma components were removed by centrifugation (maximally at about 750 g) and washing three times with 5 ml medium. Samples, containg either 5 ml diluted blood (at about 1.6% hematocrit, hct) or washed RBCs, were freed from oxygen by bubbling with inert gas for more than 30 min. These samples, dark red in colour, were subsequently maintained under inert gas. The NO gas was introduced into the supensions through polyethylene tubing (1.6 mm i. d.). The tubing was removed, usually after 5 bubbles ("puffs"). The suspensions were finally bubbled for about 5 min with nitrogen. The pH value in the sample was usually almost constant and was not allowed to drop below 7.3. The morphology of the RBCs was inspected by ligh microscopy, using a hemacytometer with albumin added (about 0.02% w/v) to the samples to avoid surface effects (15).

Electron paramagnetic resonance. After centrifugation, the suspensions of RBCs (at about 50% hct) were filled into blood capillaries (1 mm i.d. from Vitrex, Modulohm, Denmark). These RBCs were packed gently (< 100% hct) by a short centrifugation. The

sample was the placed in a 3 mm EPR quartz tube with a septum and flushed for a few minutes with purified argon through a syringe. EPR recording could be performed about 10 min after initiation of NO exposure. X-band spectrometers (Varian Model E-9 and Bruker Model ESP 300) were employed, using 100-kHz field modulation (usually 0.5 mT amplitude) and 10 mW microwave power. A sweep range of 40 mT (around g~2) was normally used. In order to detect any signals at g >2 the field was also varied in the range 0 - 400 mT. A response time of 1 s was normally employed, with a variable sweep time. The cavity was equipped with a cold-finger dewar vessel. Samples were measured either at room temperature (about 20°C) or frozen in liquid nitrogen. At both temperatures a signal with a rather strong component at g~4 was detected, originating from an impurity in the glass capillary tubes used.

Results and Discussion

Although NO is very reactiveve it was possible to perform experiments *in vitro* with suspended RBCs without changing their morphology. Using careful deoxygenation, the pH in the medium remained almost constant at a physiological value (~7.4) after NO treatment. Strong EPR signals from HbNO with g-value components around g~2 could be recorded even at room temperature (293 K; Fig. 1, upper spectra). Depending on the conditions employed, the shape of the EPR spectra slowly changed with time (within minutes) at this temperature. Therefore, samples were also quenched and comparison with spectra obtained at 77 K was performed (Fig. 1, lower). No signal at g~6, due to formation of free methemoglobin, was detected within any of the EPR spectra at 77 K. The weak NO complex with methemoglobin is diamagnetic (9). Valency hybrid species have been studied utilizing EPR (16). Exposure to NO gas should result primarily in fully reduced tetramers, with only low concentrations of the $(\alpha^{2+}\beta^{3+})_2$ (16).

No major differences in the EPR spectra recorded with NO-treated RBCs, suspended in diluted plasma or after carefully washing were observed. Untreated whole blood did not show any signals. Adding 5 mM BPG to the medium had no effect on the spectra with washed RBCs, indicating a low permiability or the presence of endogenous effector.

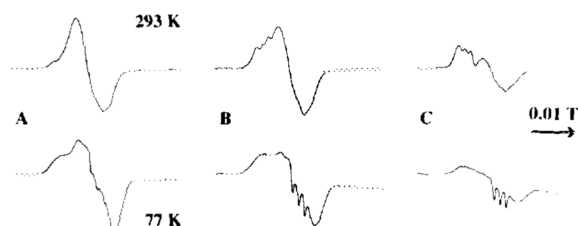

Figure 1. EPR spectra from human erythrocytes exposed to NO gas at two different levels. These spectra were either recorded at room temperature (upper spectra) or in liquid nitrogen (lower spectra). **A**) High-dose spectra; **B**) The initial low-dose spectra; **C**) The final low-dose spectra (waiting for >30 min, after which the sample was frozen).

Two conditions were of importance for the nature of the spectra, i.e., the level of NO exposure and period elapsed after the initiation of the exposure. Prolonged exposure was usually avoided in order to prevent damage to the RBCs. Three main types of spectra were observed (Fig. 1 A-C). The longest NO exposure resulted in a rather symmetrical and featureless spectrum centred at g=2.026 (Fig. 1A). Only the three shoulders at the high-field side (g~2.07) were discernible. This type of spectrum was stable at room temperature for at least 1 h, in agreement with the stability of fully liganded Hb, which has a lifetime ($t_{1/2}$) of several hours in the R-state (11, 14). At 77 K, the spectrum barely demonstrated any hyperfine triplet (Fig.1A) and was similar to that assigned to saturated $(\alpha^{2+}\beta^{2+})_2$ hemoglobin (7,16).

When the NO exposure time was reduced by about half, resolution of the shoulders was improved (Fig. 1B) and at 77 K a better resolution of hyperfine triplet, centred at g=2.009 and with a coupling of about 1.5 mT, was observed. With a second-derivative recording, this triplet could be revealed even at room temperature (Fig. 2). This type of spectrum gradually changed at room temperature and within less than one hour stabilized as a more highly resolved spectrum, both at 293 K and 77 K (Fig. 1C). The separation between the three shoulders centred at g=2.07 was about 1.5 mT, i.e., approximatly the same as the hyperfine splitting at 2.009. No major change in the integrated intensities, as measured with frozen samples, was observed during this period.

This observed time-dependence is probably the result of an internal rearrangement between the subunits of hemoglobin, at subsaturating levels of NO (5-7). The α and β subunits bind NO at equal rates, but the affinity of the α subunit is higher. The final

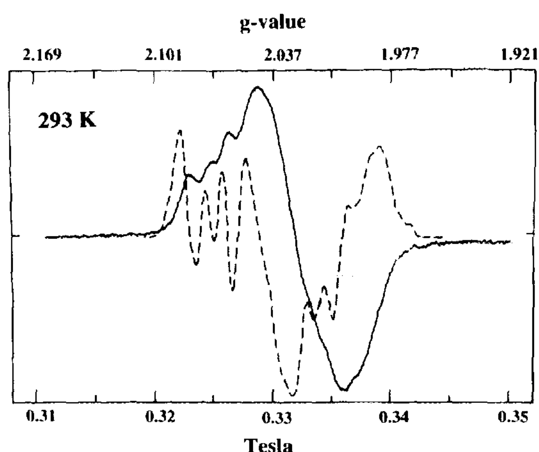

Figure 2. The initial low-dose EPR spectrum recorded at room temperature. The second derivative spectrum (broken line) was recorded with 3-point moving averaging.

spectrum achieved is characteristic of hemoglobin in the T-state, where the NO ligand ends up bound primarily to the α subunit. In the presence of BPG, the Fe^{2+} of this subunit is penta-coordinated. Hence, the ^{14}N hyperfine triplet observable at low saturating levels of NO reflects binding to the α subunits. This triplet becomes obscured by the increasing intensity around g=2.00 from the β units at an elevated degree of saturation. The viscosity inside the RBC is around 4 mPa·s (15), which probably should result in the spectra being of a powder type even at room temperature. The hyperfine coupling due to the NO ligand remains about the same (1.5 mT) at 77 and 293 K. After thawing of samples, causing hemolyzed RBCs, the spectra recorded at room temperature were identical with the corresponding spectra obtained before freezing.

By quenching of a sample, non-equilibrium states might occur. However, the EPR spectra from RBCs at room temperature confirm the NO-binding properties of hemoglobin, studied earlier using frozen hemoglobin solutions. Although freezing the sample confers an inherently higher sensitivity, the noise and baseline problems are usually more severe in the presence of liquid nitrogen. Moreover, the low temperature spectra are more sensitive to the recording conditions, due to various relaxation phenomena (10).

The present study demonstrates the possibility of monitoring the interaction of NO with intact RBCs at room temperature. The RBCs were exposed here to bursts of gas. A gentler experiment could be carried out by anaerobically injecting an aqueous saturated (2-3 mM) solution of NO (13, 14). Release of NO from labile chemicals (e.g. vasodilators) can also be followed (16). In principle, it should be possible to use EPR to study by the interaction of nitric oxide with blood at physiological hematocrit and in movement.

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