ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 3, 2004

Forum Original Research Communication

Simultaneous Measurement of Oxygenation in Intracellular and Extracellular Compartments of Lung Microvascular Endothelial Cells

VIJAY KUMAR KUTALA, NARASIMHAM L. PARINANDI, RAMASAMY P. PANDIAN, and PERIANNAN KUPPUSAMY

ABSTRACT

A new technique is described for simultaneous determination of intra- and extracellular oxygen concentrations [partial pressure of O_2 (pO_2)] in bovine lung microvascular endothelial cells (BLMVECs) using electron paramagnetic resonance (EPR) oximetry. The measurements were performed in BLMVEC suspensions of a 20-µl volume containing 4,000 cells. The extracellular pO_2 was measured using a trityl EPR probe [triarylmethyl (TAM), 10 µ*M*], a tricarboxylate anion radical, that stays exclusively in the extracellular space. The intracellular oxygen was measured using a pre-internalized particulate spin probe, lithium 5,9,14,18, 23,27,32,36-octa-*n*-butoxy-naphthalocyanine (LiNc-BuO). Because there is a wide discrepancy in the reported values of cellular oxygenation by and large due to differences in the methods employed, we utilized the dual EPR probe technique to measure the oxygen gradient that apparently exists across the cell membrane. The intra- and extracellular pO_2 values were 139 ± 2.5 and 157 ± 3.6 mm Hg, respectively, for cells exposed to room air. A fairly smaller gradient of oxygen was observed in cells exposed to 7.5% oxygen (pO_2 = 57 mm Hg). In summary, this study confirms the feasibility of simultaneous and accurate measurements of intra- and extracellular pO_2 using LiNc-BuO and TAM EPR oximetry probes. *Antioxid. Redox Signal.* 6, 597–603.

INTRODUCTION

OXYGEN is an important modulator of cellular functions in both normal physiology and disease states. Cells respond to oxygen over a wide range of concentrations from anoxia to hyperoxia. Baseline metabolism and function typically occur in normoxic environments (30–90 mm Hg of O_2) and can modulate differentiated cell functions (15). Hyperoxic conditions often result in the generation of reactive oxygen species that have been implicated in cell injury via lipid peroxidation and cytokine expression (5). In lieu of such diversity in cellular responses to oxygen, the dynamics of tissue oxygenation, including the transport of oxygen and the possible existence of an oxygen gradient across the cell membrane, need to be measured accurately. Various methods such as manometry, photometry, mass spectrometry and polarography (Clark-type electrochemical) have been described to measure concentration and uptake of cellular oxygen (3, 4, 19, 25, 31). The microelectrode technique, despite being used widely, has several disadvantages. The electrode consumes oxygen during measurement, apparently causes systematic error under very low oxygen concentrations, requires insertion into the tissues, disturbs the local environment, and causes tissue damage (32, 33).

Although determining extracellular oxygen concentration in cell suspensions is straightforward, measurement of the intracellular partial pressure of O_2 (pO_2) is complicated. There are a few methods available to accomplish this, for

Center for Biomedical EPR Spectroscopy and Imaging, Davis Heart and Lung Research Institute, Department of Internal Medicine, The Ohio State University, Columbus, OH.

example, by insertion of an intracellular oxygen electrode into a single cell (33, 34) or by fluorescence quenching by O_2 following the cellular uptake of a fluorescent probe, pyrenebutyric or 2-nitroimidazole (2, 17). Electron paramagnetic resonance (EPR) spectroscopy, coupled with the use of oxygen-sensitive spin probes, has become a potential technique for accurate and precise determination of oxygen concentrations in a variety of biological samples, including tissues and cells (7, 13, 20, 27, 36). The technique, referred to as "EPR oximetry," uses soluble molecular spin probes for the determination of dissolved oxygen concentration and particulate spin probes for targeted determination of local oxygen tension (pO_2) in tissues and cells (10). The particulate probes have unique advantages over the other EPR oximetry probes: (i) They report pO_2, which is a better parameter in a heterogeneous cellular system; (ii) they do not consume oxygen; (iii) they provide higher resolution at lower pO_2; and (iv) they possess greater stability in cells and tissues, so that repeated measurements of oxygen tensions can be made for months without reintroduction of the probe. Hence, the particulate oximetry probe-coupled EPR spectroscopic determination of oxygen has advantages over the other methods of determination of oxygen in biological samples (3, 19, 25, 31). A variety of particulate probes that possess many of these desirable properties are useful in studies both *in vitro* and *in vivo* (10, 18). Recently, we synthesized and characterized the octa-*n*-butoxy-substituted naphthalocyanine neutral radical [lithium 5,9,14,18,23,27, 32,36-octa-*n*-butoxy-2,3-naphthalocyanine (LiNc-BuO)], which exhibits marked advantages, especially with respect to microwave power saturation, linear response to concentration of oxygen, dynamic measurement range, and higher spin density (22). We have demonstrated the application of this material by successfully internalizing it into lung microvascular endothelial cells in culture for measuring intracellular pO_2. The probe is capable of providing reliable measurements of intracellular pO_2 with 0.1 mm Hg resolution, and the measurements can be made in a single cell.

The aim of the present study was to demonstrate the accuracy and reliability of the EPR oximetry method for simultaneous measurement of intracellular pO_2 in bovine lung microvascular endothelial cells (BLMVECs) utilizing internalized particulates of LiNc-BuO and extracellular pO_2 using triarylmethyl (TAM). We have also measured the intracellular and extracellular pO_2 in these cells in the presence of metabolic inhibitors such as menadione (50 μ*M*) and potassium cyanide (100 μ*M*). As we have previously demonstrated that the LiNc-BuO enables very accurate and reliable measurement of pO_2 in cellular suspensions, we envisioned that the measurements will provide accurate values of intracellular pO_2 that have not been possible with the other techniques. Further, we extended such measurements to smaller sample volume (20 μl) with 4,000 cells. We observed an intracellular pO_2 of 139 mm Hg and extracellular pO_2 of 157 mm Hg, with an oxygen gradient of 18 mm Hg, under aerobic conditions. A gradient of 9 mm Hg of oxygen (extra- and intracellular pO_2 levels were 64 and 55 mm Hg, respectively) was observed when the cells were exposed to 7.5% oxygen. Menadione and potassium cyanide did not significantly affect the intra- and extracellular pO_2 levels.

MATERIALS AND METHODS

Reagents

LiNc-BuO was used as a probe for measuring intracellular oxygen concentration by EPR spectroscopy. LiNc-BuO belongs to the class of crystalline internal spin probe particulates that we have recently reported for measuring oxygen concentration in cellular suspensions and tissues (22). TAM was a gift from Nycomed Innovations (Malmö, Sweden). The EPR properties of TAM have been well characterized (1). Menadione and potassium cyanide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Stock solutions (1 m*M*) of menadione and potassium cyanide were prepared freshly in dimethyl sulfoxide and distilled water, respectively, and used immediately. Minimum essential medium (MEM), fetal bovine serum, and antibiotics were obtained from GIBCO-Invitrogen (Carlsbad, CA, U.S.A.).

BLMVECs

The BLMVECs used in this study were obtained from VEC Technologies, Inc. (Rensslaer, NY, U.S.A.). BLMVECs cultured in MEM were maintained in 75-mm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2/95% air and grown to contact-inhibited monolayers with a typical cobblestone morphology (23). Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh medium, and cultured in complete medium to 70% confluency for other studies. Cells from passages 10–14 were used in all the experiments.

Preparation of particulates for cell culture studies

Microcrystalline particulates of LiNc-BuO were suspended in complete MEM (10 mg/0.5 ml) and sonicated for 30-s pulses for 10 times on ice with a probe sonicator at a setting of 5. The particulate suspension was cooled for 1 min between two successive 30-s bursts of sonication. At the end of sonication, the suspension was placed on ice for exactly 2 min to allow the heavier particulates to settle down at the bottom of the tube, and the supernatant liquid was transferred to a separate tube for intracellular delivery. The solution contained fine particulates of LiNc-BuO with a particulate size of <2 μm. All the preparations were carried out under sterile conditions.

Internalization of particulates into endothelial cells

BLMVECs, at 70% confluence (10^4 cells/35-mm-diameter dish), in 1 ml of complete MEM were treated with 50 μl of LiNc-BuO particulate suspension that contained particulates of <2 μm prepared by the procedure described above. The cells were maintained at 37°C under a 95% air/5% CO_2 environment. At 6-h intervals, for 72 h, cells were examined under the light microscope for internalization of LiNc-BuO particulates. Upon confirming the particulate uptake by all the cells in a given dish after 48 h of exposure to particulates, the cells were washed 12 times with ice-cold MEM to remove unincorporated and extraneous particulates by gentle swirling and aspiration, scraped off into 1 ml of MEM, and

centrifuged at 1,000×*g* in a microcentrifuge for 10 min. The cell pellet was resuspended in MEM containing glucose (0.5 g/500 ml), at a density of 2 × 10^5 cells/ml, and used for EPR analysis. Cells after LiNc-BuO internalization and repeated washings were photographed under an inverted microscope while still adherent to the substratum of the 35-mm-diameter dish. Cell viability was assessed by light microscopy and Alamar Blue® assay according to the recommendations of the manufacturer (Biosource International, Camarillo, CA, U.S.A.). Cell separation by trypsinization was avoided to keep the cell morphology and function intact.

A 20-µl volume of the cell suspension containing 4,000 cells and 10 µ*M* TAM was drawn into a gas-permeable Teflon tube and subjected to EPR spectroscopy as described below. The measurements were also carried out with menadione (50 µ*M*) and potassium cyanide (100 µ*M*). Cell viability was assessed before and after the EPR measurements by the trypan blue exclusion method and found to be >95%.

EPR measurements

The EPR measurements were carried out using a Bruker X-band (9.8 GHz) spectrometer (Bruker Instruments, Karlsruhe, Germany) equipped with a TM_{110} cavity. EPR spectral acquisitions were performed using custom-developed data acquisition software (SPEX) that was capable of fully automated data acquisition and processing. Unless otherwise mentioned, the EPR linewidths reported are peak-to-peak widths (ΔB_{pp}) of the first derivative spectra.

Calibration of LiNc-BuO and TAM oximetry

The LiNc-BuO crystals were calibrated for EPR oximetry as described earlier (22). Measurements of the linewidth of LiNc-BuO after equilibration with a series of oxygen and nitrogen gas mixtures were performed. Calibration was performed over the oxygen concentration range (0–21%) with oxygen/nitrogen mixtures. A linear variation of linewidth was observed as a function of pO_2 in the entire range of 0–160 mm Hg. Similarly, the calibration of TAM (10 µ*M*) was also performed using different oxygen concentrations (0–21%). The oxygen-induced line-broadening (change in peak-to-peak width) of the signal was used to measure extracellular oxygen concentration. The line-shape of the EPR signal was precisely simulated using a Lorentzian function, and the Lorentzian width was used to establish the calibration curve.

Simultaneous measurements of LiNc-BuO and TAM line-shapes

LiNc-BuO and TAM were used as site-specific oximetry probes to measure intra- and extracellular oxygen concentration, respectively. Both LiNc-BuO and TAM give a single-line EPR spectrum, whose amplitude (intensity) and width depend on the amount/concentration of the probe and oxygen, respectively. Since the *g*-factors of LiNc-BuO (g = 2.0024) and TAM (g = 2.0030) are slightly different, their spectra do not overlap completely and show a composite feature where the two peaks can be separated by computer simulation (Fig. 1). The LiNc-BuO absorption profile is characterized by 100% Lorentzian (22), while the TAM signal can be approximated to be Lorentzian under conditions of oxygen-induced broadening. Thus, the deconvolution requires a simple two-component Lorentzian fitting to the measured spectrum. We validated the faithfulness of the deconvolution by performing the simulation under different combinations of oxygen broadening to the probes. The reproducibility was very good (R^2 > 0.99) for non-zero oxygen concentrations. The line-shape of TAM was non-Lorentzian under anoxic conditions.

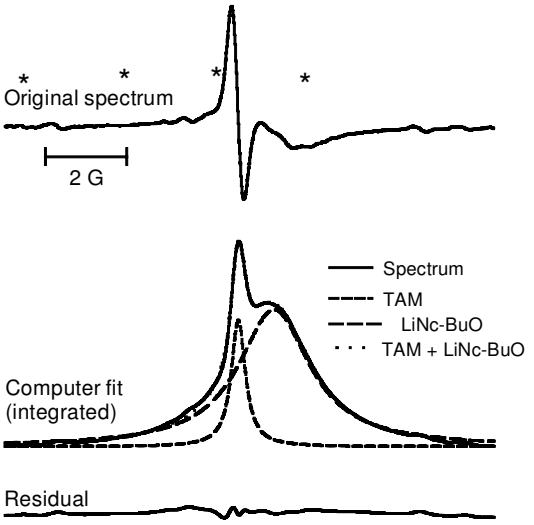

FIG. 1. EPR spectrum of a suspension of LiNc-BuO and TAM (10 µ*M*) in phosphate-buffered saline (pH 7.4) equilibrated with room air (20.9% oxygen). The original spectrum (**top**) is a composite of two components: a sharp peak from TAM (g = 2.0030) and a broad peak from LiNc-BuO (g = 2.0024). The additional peaks indicated by * on both sides of the spectrum are due to ^{13}C hyperfine from TAM (35). EPR data acquisition parameters were: modulation amplitude, 100 mG; microwave power, 1 mW; time constant, 80 ms; scan time, 15 s. The computer fit (**middle**) shows the decomposition of the original spectrum into two components, those of LiNc-BuO and TAM. The computer fit (sum of the two components) is superimposed onto the original spectrum. The residual (**bottom**) curve shows the difference between the original and computer fit (R^2 = 0.9977).

It should also be noted that there was no effect of TAM on the EPR spectrum of LiNc-BuO, and *vice versa,* when the two probes were suspended in the same medium having physical contact. This suggests that the two probes can be used together. However, in our experiments the probes were distributed in different regions (intra- and extracellular), and, hence, such a contact did not exist. The components were separated using PEAK FIT (SPSS, Chicago, IL, U.S.A.) software, and the intracellular and extracellular pO_2 values were determined from the calibration curves of LiNc-BuO and TAM.

Data analysis

All values are expressed as mean ± SD of four to six independent experiments. Analysis of variance and Student's *t* test were used for statistical analysis. Differences between groups were considered to be significant at $p < 0.05$.

RESULTS

Internalization of LiNc-BuO crystals into BLMVECs

The light microscopy, as shown in Fig. 2, clearly showed that within 18 h of treatment of BLMVECs with LiNc-BuO particulates (<2 μm) in complete MEM, nearly 95% of the cells internalized the particulates in a 35-mm-diameter dish. The internalized particulates showed no cytotoxicity up to 72 h as evidenced by light microscopy and Alamar Blue cytotoxicity assay. At the time of measurements, the viability of the cells that internalized LiNc-BuO was >95% as studied with the 0.4% trypan blue exclusion method.

The mean spin density of the LiNc-BuO particulates internalized in a single cell was calculated in comparison with a standard solution of TAM to be 6×10^{11} spins/cell. This sensitivity is greater than that offered by the X-band EPR spectrometer, which is typically better than 1×10^{10} spins. Thus, one can measure the EPR spectrum from a single cell that is internalized with the LiNc-BuO particulates.

Effect of molecular oxygen on the EPR spectrum of LiNc-BuO and TAM

Figure 3 shows the width of the probe as a function of pO_2 in the range 0–158 mm Hg. It is observed that the width increases linearly with pO_2 in the range 0–160 mm Hg. The slope of the curve, which reflects the oxygen sensitivity of the probe, is 8.5 mG/mm Hg. Thus the probe is capable of measuring oxygen tension to ~0.1 mm Hg resolution in the physiological range. Similarly, TAM exhibits a peak-to-peak width of 146 mG under the anoxic condition, and the spectrum is broadened in the presence of oxygen. The oxygen sensitivity of this radical is 0.36 mG/mm Hg. The line-shape of the EPR signal obtained with simultaneous use of intracellular LiNc-BuO and extracellular TAM was simulated precisely using two Lorentzian functions, and the Lorentzian width was used to measure pO_2 from the calibration curve.

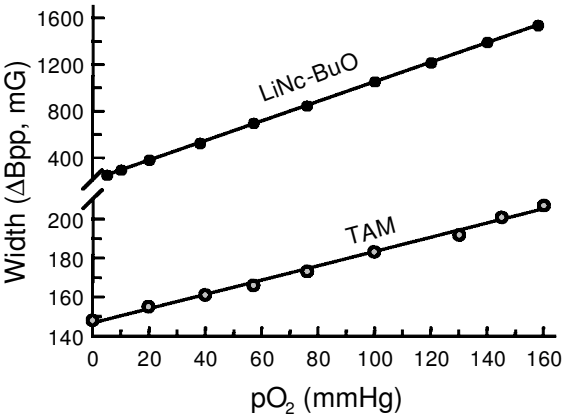

FIG. 3. Effect of oxygen concentration (pO_2) on the peak-to-peak EPR linewidth (ΔB_{pp}) of LiNc-BuO and TAM. Measurements were made independently on LiNc-BuO microcrystalline particulates suspended in saline and TAM (10 μ*M*) in phosphate-buffered saline equilibrated with mixtures of oxygen/nitrogen gases. The spectra were acquired as described in Fig. 1. The linewidth increases linearity with pO_2 in the range of 0–160 mm Hg, with an anoxic (0% oxygen) linewidth of 210 mG and slope (sensitivity) of 8.5 mG/mm Hg for LiNc-BuO and an anoxic linewidth of 148 mG and slope (sensitivity) of 0.36 mG/mm Hg for TAM.

Intra- and extracellular oxygen concentrations

The internalized BLMVECs (4,000 cells) mixed with TAM (10 μ*M*) in a 20-μl volume of aerated solution showed an oxygen gradient of 18 mm Hg, with an intracellular pO_2 of 139 mm Hg and extracellular pO_2 of 157 mm Hg (Fig. 4). A gradient of 9 mm Hg of oxygen was observed when cells

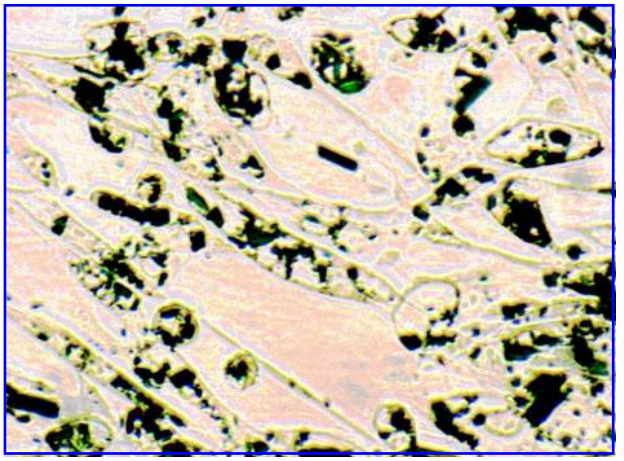

FIG. 2. Photomicrograph of BLMVECs showing internalization of the LiNc-BuO microparticulates. The LiNc-BuO particulates are seen as dark green crystals inside the cells.

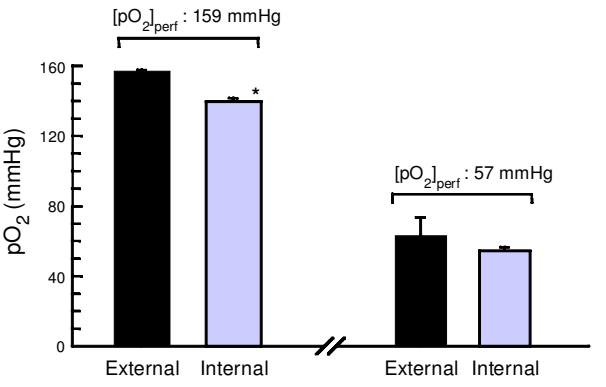

FIG. 4. Extracellular and intracellular measurement of pO_2 in BLMVECs. Intracellular pO_2 was measured using internalized LiNc-BuO particulates in BLMVECs. The extracellular pO_2 was measured simultaneously using 10 μ*M* TAM. Measurements were made at room air (20.9%, or pO_2 = 159 mm Hg) and at 7.5% (pO_2 = 57 mm Hg) oxygen. Values are mean ± SD of five experiments. *$p < 0.001$ versus extracellular pO_2.

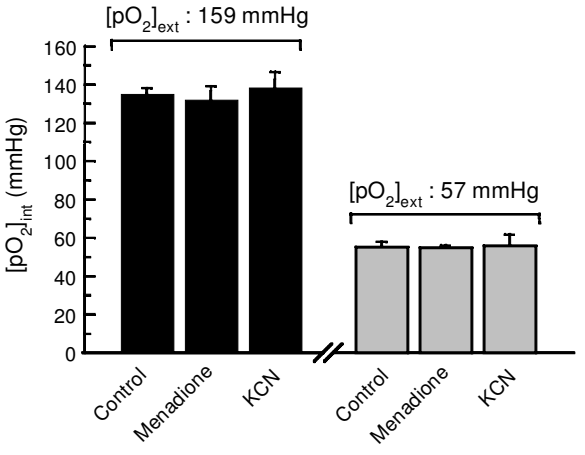

FIG. 5. Effect of menadione and cyanide on intracellular and extracellular pO_2 in BLMVECs. The pO_2 measurements were made in cells treated with menadione (50 μ*M*) and potassium cyanide (100 μ*M*). The measurements were performed as in Fig. 4. Values are mean ± SD of five experiments.

were exposed to 7.5% oxygen. Internalization of LiNc-BuO particulates into BLMVECs and the feasibility of accurate measurement of intracellular pO_2 were confirmed in cell lysates prepared by brief sonication (5 × 10 s) at 4°C. The pO_2 measured in the lysate was 158 mm Hg. This observation clearly indicated the internalization of particulate probe into BLMVECs and the existence of an oxygen gradient between intra- and extracellular compartments. There was no significant change in pO_2 and oxygen gradient in cells treated with menadione (50 μ*M*) or cyanide (100 μ*M*) (Fig. 5).

DISCUSSION

The oxygen gradient in physiological systems plays an important role in both maintaining homeostasis and inducing cellular responses. Therefore, an accurate and a reliable method to determine its concentration in cells and tissues is highly critical. Values of the oxygen gradient in cells measured by various methods reported so far in the literature vary widely in range from 1 to 40 μ*M* (6, 8, 14, 26, 28–30). This broad discrepancy apparently is due to technical difficulty associated with accurate measurement of intracellular oxygen concentration under physiological conditions. Using nitroxides and other agents, several new methods based on EPR oximetry have been developed to measure intracellular oxygen concentration in cells (9, 11–13, 24). The particulate probe-based EPR oximetry used in the present study has many advantages over the other oximetry probe-based EPR spectroscopy and other widely used methods to measure intracellular pO_2. Some of the distinct and advantageous features of LiNc-BuO paramagnetic spin particulates are: their ability to give rise to a single sharp and isotropic EPR spectrum characteristic with 100% Lorentzian shape; a linear variation of linewidth with pO_2 that is independent of particulate size; and, most importantly, their ability to internalize in cells. We have taken advantage of these favorable characteristics of LiNc-BuO particulates, successfully internalized them into in BLMVECs, and measured intracellular pO_2 using EPR spectroscopy.

The intra- and extracellular pO_2 values measured by this technique in BLMVECs were 139 and 157 mm Hg, respectively, at room air, with a gradient of 18 mm Hg. This technique also revealed the existence of a small oxygen gradient of 9 mm Hg at 7.5% (pO_2 = 57 mm Hg) oxygen (extra- and intracellular pO_2 values were 64 and 55 mm Hg, respectively). A similar finding was also observed by others (16). This suggests that the cells possess different gradients when exposed to different oxygen concentrations and that a smaller gradient exists at lower oxygen concentration. Santini *et al.* (24) used fusinite as an EPR oximetry probe to measure intracellular molecular oxygen in K56 (an erythroleukemic cell line) and A 431 (an epidermal carcinoma cell line) and demonstrated that menadione (200 μ*M*) increased both intra- and extracellular pO_2 by 10–15%. But in our study, menadione (50 μ*M*) did not alter intra- and extracellular pO_2 in BLMVECs. This may be due to different experimental conditions, dose, and different cell types used. Khan *et al.* (16) measured intra- and extracellular oxygen concentrations in Chinese hamster ovary cells by EPR oximetry using ^{15}N-perdeuterated Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) and lithium phthalocyanine as intra- and extracellular probes, respectively. The extra- and intracellular oxygen concentrations observed in this study were 162 (1 μ*M* oxygen is equal to 0.714 mm Hg in aqueous solution) and 129 mm Hg, respectively, at 150 mm Hg and 38.5 and 34.2 mm Hg, respectively, at 35 mm Hg of pO_2. Using this technique, they demonstrated that plasma membrane cholesterol is an important barrier in regulating the oxygen gradient across the cell membrane.

In our recent study, we used the LiNc-BuO particulate probe and measured the rate of oxygen consumption in mouse aortic endothelial cells in the presence of various stimulants and inhibitors of respiration (21) and also measured the pO_2 in normal and tumor tissues (22). In comparison with other oximetry probes, the unique advantage of LiNc-BuO is to prepare particulates of nanometer size without compromising its EPR behavior and oxygen-sensing abilities. The smaller particulates can be internalized in a variety of cells for different applications. It is possible to measure the pO_2 from a single cell internalized with LiNc-BuO.

The intracellular molecular oxygen is critical in determining the cytoplasmic chemical/physical environment of the cell. The use of a highly sensitive EPR probe like LiNc-BuO, capable of measuring intracellular pO_2 with greater sensitivity, offers advantages in biological EPR oximetry. The data presented in this study demonstrate that this novel EPR probe can be successfully employed for direct and efficient measurement of intracellular oxygen concentration with a sensitivity of 0.1 mm Hg in all cell types. The cells internalized with LiNc-BuO can be used as an important tool to monitor oxidative cellular functions and to study the cellular responses under pathophysiological and toxicological conditions.

SUMMARY AND CONCLUSIONS

The intracellular oxygen concentration in BLMVECs was measured by internalizing an oxygen-sensitive microparticulate spin probe (LiNc-BuO) using EPR oximetry. The method utilized a microparticulate spin probe (LiNc-BuO) with a high sensitivity for oxygen, enabling accurate measurement of intracellular pO_2 in BLMVECs. We also determined the extracellular oxygen concentration using another oxygen-sensitive oximetry probe, TAM, simultaneously. The effect of agents that can alter oxygen concentration such as menadione and potassium cyanide on oxygen gradient was also studied. The measurements were performed in a volume of 20 μl containing 4,000 cells (2×10^5 cells/ml) in a gas-permeable Teflon tube at room air and at 7.5% oxygen. The intracellular oxygen concentration in BLMVECs measured at room air by this technique was 194 μ*M,* and extracellular oxygen concentration was 220 μ*M,* with a gradient of 26 μ*M*; an oxygen gradient of 16 μ*M* was seen in cells exposed to 7.5% oxygen. There was no significant difference in extra- and intracellular oxygen concentration during treatment with menadione and potassium cyanide. In summary, we demonstrated that the measurements of intracellular oxygen concentration and oxygen gradient can be successfully performed using microparticulate-based EPR oximetry with a fewer number of cells.

ACKNOWLEDGMENTS

We acknowledge financial support from the NCI/NIH (grant CA 78886).

ABBREVIATIONS

BLMVEC, bovine lung microvascular endothelial cell; EPR, electron paramagnetic resonance; LiNc-BuO, lithium 5,9,14,18,23,27,32,36-octa-*n*-butoxy-2,3-naphthalocyanine; MEM, minimum essential medium; pO_2, partial pressure of oxygen; TAM, triarylmethyl.

REFERENCES

1. Ardenkjaer-Larsen JH, Laursen I, Leunbach I, Ehnholm G, Wistrand LG, Petersson JS, and Golman K. EPR and DNP properties of certain novel single electron contrast agents intended for oximetric imaging. *J Magn Reson* 133: 1–12, 1998.
2. Benson DM, Knopp JA, and Longmuir IS. Intracellular oxygen measurements of mouse liver cells using quantitative fluorescence video microscopy. *Biochim Biophys Acta* 591: 187–197, 1980.
3. Chapman KR, Liu FL, Watson RM, and Rebuck AS. Conjunctival oxygen tension and its relationship to arterial oxygen tension. *J Clin Monit* 2: 100–104, 1986.
4. Dahl BT, Kiaer T, and Lund B. Measurement of bone cell metabolism in vitro using mass spectrometry. *Calcif Tissue Int* 50: 290–292, 1992.
5. D'Angio CT and Finkelstein JN. Oxygen regulation of gene expression: a study in opposites. *Mol Genet Metab* 71: 371–380, 2000.
6. Grinberg OY, James PE, and Swartz HM. Are there significant gradients of pO_2 in cells? *Adv Exp Med Biol* 454: 415–423, 1998.
7. He G, Shankar RA, Chzhan M, Samouilov A, Kuppusamy P, and Zweier JL. Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging. *Proc Natl Acad Sci U S A* 96: 4586–4591, 1999.
8. Hu H, Sosnovsky G, and Swartz HM. Simultaneous measurements of the intra- and extra-cellular oxygen concentration in viable cells. *Biochim Biophys Acta* 1112: 161–166, 1992.
9. Hu HP, Sosnovsky G, Li SW, Rao NU, Morse PD 2nd, and Swartz HM. Development of nitroxides for selective localization inside cells. *Biochim Biophys Acta* 1014: 211–218, 1989.
10. Ilangovan G, Manivannan A, Li H, Yanagi H, Zweier JL, and Kuppusamy P. A naphthalocyanine-based EPR probe for localized measurements of tissue oxygenation. *Free Radic Biol Med* 32: 139–147, 2002.
11. Inoue M, Utsumi H, and Kirino Y. A comparative ESR study of some paramagnetic materials as probes for the noninvasive measurement of dissolved oxygen in biological systems. *Chem Pharm Bull (Tokyo)* 42: 2346–2348, 1994.
12. James PE, Grinberg OY, Michaels G, and Swartz HM. Intraphagosomal oxygen in stimulated macrophages. *J Cell Physiol* 163: 241–247, 1995.
13. James PE, Jackson SK, Grinberg OY, and Swartz HM. The effects of endotoxin on oxygen consumption of various cell types in vitro: an EPR oximetry study. *Free Radic Biol Med* 18: 641–647, 1995.
14. Jones DP and Kennedy FG. Intracellular O_2 gradients in cardiac myocytes. Lack of a role for myoglobin in facilitation of intracellular O_2 diffusion. *Biochem Biophys Res Commun* 105: 419–424, 1982.
15. Jungermann K and Kietzmann T. Oxygen: modulator of metabolic zonation and disease of the liver. *Hepatology* 31: 255–260, 2000.
16. Khan N, Shen J, Chang TY, Chang CC, Fung PC, Grinberg O, Demidenko E, and Swartz H. Plasma membrane cholesterol: a possible barrier to intracellular oxygen in normal and mutant CHO cells defective in cholesterol metabolism. *Biochemistry* 42: 23–29, 2003.
17. Koch CJ. Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. *Methods Enzymol* 352: 3–31, 2002.
18. Kuppusamy P, Afeworki M, Shankar RA, Coffin D, Krishna MC, Hahn SM, Mitchell JB, and Zweier JL. In vivo electron paramagnetic resonance imaging of tumor heterogeneity and oxygenation in a murine model. *Cancer Res* 58: 1562–1568, 1998.
19. Marshall RS, Koch CJ, and Rauth AM. Measurement of low levels of oxygen and their effect on respiration in cell suspensions maintained in an open system. *Radiat Res* 108: 91–101, 1986.
20. Morse PD 2nd and Swartz HM. Measurement of intracellular oxygen concentration using the spin label TEMPOL. *Magn Reson Med* 2: 114–127, 1985.

21. Pandian RP, Kutala VK, Parinandi N, and Kuppusamy P. Measurement of oxygen consumption in mouse aortic endothelial cells using a microparticulate oximetry probe. *Arch Biochem Biophys* 420: 169–175, 2003.
22. Pandian RP, Parinandi NL, Ilangovan G, Zweier JL, and Kuppusamy P. Novel particulate spin probe for targeted determination of oxygen in cells and tissues. *Free Radic Biol Med* 35: 1138–1148, 2003.
23. Roy S, Parinandi N, Zeigelstein R, Hu Q, Pei Y, Travers JB, and Natarajan V. Hyperoxia alters phorbol ester-induced phospholipase D activation in bovine lung microvascular endothelial cells. *Antioxid Redox Signal* 5: 217–228, 2003.
24. Santini MT, Morelli G, Fattorossi A, Malorni W, Rainaldi G, and Indovina L. The oxidizing agent menadione induces an increase in the intracellular molecular oxygen concentration in K562 and A431 cells: direct measurement using the new paramagnetic EPR probe fusinite. *Free Radic Biol Med* 20: 915–924, 1996.
25. Shibata M, Ichioka S, Ando J, and Kamiya A. Microvascular and interstitial PO(2) measurements in rat skeletal muscle by phosphorescence quenching. *J Appl Physiol* 91: 321–327, 2001.
26. Swartz HM. Measurements of intracellular concentrations of oxygen: experimental results and conceptual implications of an observed gradient between intracellular and extracellular concentrations of oxygen. *Adv Exp Med Biol* 345: 799–806, 1994.
27. Swartz HM, Bacic G, Friedman B, Goda F, Grinberg O, Hoopes PJ, Jiang J, Liu KJ, Nakashima T, O'Hara J, *et al.* Measurements of pO_2 in vivo, including human subjects, by electron paramagnetic resonance. *Adv Exp Med Biol* 361: 119–128, 1994.
28. Takahashi E, Sato K, Endoh H, Xu ZL, and Doi K. Direct observation of radial intracellular PO_2 gradients in a single cardiomyocyte of the rat. *Am J Physiol* 275: H225–H233, 1998.
29. Takahashi E, Endoh H, and Doi K. Intracellular gradients of O_2 supply to mitochondria in actively respiring single cardiomyocyte of rats. *Am J Physiol* 276: H718–H724, 1999.
30. Tamura M, Oshino N, Chance B, and Silver IA. Optical measurements of intracellular oxygen concentration of rat heart in vitro. *Arch Biochem Biophys* 191: 8–22, 1978.
31. Taylor J and Deutsch C. ^{19}F-nuclear magnetic resonance: measurements of $[O_2]$ and pH in biological systems. *Biophys J* 53: 227–233, 1988.
32. Vanderkooi JM, Erecinska M, and Silver IA. Oxygen in mammalian tissue: methods of measurement and affinities of various reactions. *Am J Physiol* 260: C1131–C1150, 1991.
33. Whalen WJ. Intracellular oxygen microelectrodes. *Adv Exp Med Biol* 37A: 17–22, 1973.
34. Whalen WJ, Riley J, and Nair P. A microelectrode for measuring intracellular PO_2. *J Appl Physiol* 23: 798–801, 1967.
35. Wind RA and Ardenkjaer-Larsen JH. ^{1}H DNP at 1.4 T of water doped with a triarylmethyl-based radical. *J Magn Reson* 141: 347–354, 1999.
36. Zweier JL, Chzhan M, Ewert U, Schneider G, and Kuppusamy P. Development of a highly sensitive probe for measuring oxygen in biological tissues. *J Magn Reson B* 105: 52–57, 1994.

Address reprint requests to:
Periannan Kuppusamy, Ph.D.
Center for Biomedical EPR Spectroscopy and Imaging
Davis Heart and Lung Research Institute
Department of Internal Medicine
The Ohio State University
420 West 12th Avenue, Room 114
Columbus, OH 43210

E-mail: kuppusamy-1@medctr.osu.edu

Received for publication July 8, 2003; accepted February 19, 2004.

This article has been cited by:

1. Yangping Liu, Yuguang Song, Francesco De Pascali, Xiaoping Liu, Frederick A. Villamena, Jay L. Zweier. 2012. Tetrathiatriarylmethyl radical with a single aromatic Hydrogen as a highly sensitive and specific superoxide probe. *Free Radical Biology and Medicine* . [CrossRef]
2. Benoît Driesschaert, Valérie Marchand, Philippe Levêque, Bernard Gallez, Jacqueline Marchand-Brynaert. 2012. A phosphonated triarylmethyl radical as a probe for measurement of pH by EPR. *Chemical Communications* . [CrossRef]
3. Yangping Liu, Yuguang Song, Antal Rockenbauer, Jian Sun, Craig Hemann, Frederick A. Villamena, Jay L. Zweier. 2011. Synthesis of Trityl Radical-Conjugated Disulfide Biradicals for Measurement of Thiol Concentration. *The Journal of Organic Chemistry* **76**:10, 3853-3860. [CrossRef]
4. Revital Halevy, Victor Tormyshev, Aharon Blank. 2010. Microimaging of Oxygen Concentration near Live Photosynthetic Cells by Electron Spin Resonance. *Biophysical Journal* **99**:3, 971-978. [CrossRef]
5. Minoru Miyake, Scott R. Burks, John Weaver, Pei Tsai, Wenlan Liu, David Bigio, Kenneth S. Bauer, Ke Jian Liu, Gerald M. Rosen, Joseph P.Y. Kao. 2010. Comparison of two nitroxide labile esters for delivering electron paramagnetic resonance probes into mouse brain. *Journal of Pharmaceutical Sciences* **99**:8, 3594-3600. [CrossRef]
6. Ramasamy P. Pandian, Guruguhan Meenakshisundaram, Anna Bratasz, Edward Eteshola, Stephen C. Lee, Periannan Kuppusamy. 2010. An implantable Teflon chip holding lithium naphthalocyanine microcrystals for secure, safe, and repeated measurements of pO2 in tissues. *Biomedical Microdevices* **12**:3, 381-387. [CrossRef]
7. Feng Wang, Zhenqing Li, Mahmood Khan, Kenichi Tamama, Periannan Kuppusamy, William R. Wagner, Chandan K. Sen, Jianjun Guan. 2010. Injectable, rapid gelling and highly flexible hydrogel composites as growth factor and cell carriers. *Acta Biomaterialia* **6**:6, 1978-1991. [CrossRef]
8. Guruguhan Meenakshisundaram, Edward Eteshola, Aharon Blank, Stephen C. Lee, Periannan Kuppusamy. 2010. A molecular paramagnetic spin-doped biopolymeric oxygen sensor. *Biosensors and Bioelectronics* **25**:10, 2283-2289. [CrossRef]
9. Guruguhan Meenakshisundaram, Ramasamy P. Pandian, Edward Eteshola, Stephen C. Lee, Periannan Kuppusamy. 2010. A paramagnetic implant containing lithium naphthalocyanine microcrystals for high-resolution biological oximetry. *Journal of Magnetic Resonance* **203**:1, 185-189. [CrossRef]
10. Jiangang Shen, Rohit Sood, John Weaver, Graham S Timmins, Aaron Schnell, Minoru Miyake, Joseph PY Kao, Gerald M Rosen, Ke Jian Liu. 2009. Direct visualization of mouse brain oxygen distribution by electron paramagnetic resonance imaging: application to focal cerebral ischemia. *Journal of Cerebral Blood Flow & Metabolism* **29**:10, 1695-1703. [CrossRef]
11. Guruguhan Meenakshisundaram, Edward Eteshola, Ramasamy P. Pandian, Anna Bratasz, Stephen C. Lee, Periannan Kuppusamy. 2009. Fabrication and physical evaluation of a polymer-encapsulated paramagnetic probe for biomedical oximetry. *Biomedical Microdevices* **11**:4, 773-782. [CrossRef]
12. Mahmood Khan , Iyyapu K. Mohan , Vijay K. Kutala , Sainath R. Kotha , Narasimham L. Parinandi , Robert L. Hamlin , Periannan Kuppusamy . 2009. Sulfaphenazole Protects Heart Against Ischemia–Reperfusion Injury and Cardiac Dysfunction by Overexpression of iNOS, Leading to Enhancement of Nitric Oxide Bioavailability and Tissue Oxygenation. *Antioxidants & Redox Signaling* **11**:4, 725-738. [Abstract] [Full Text PDF] [Full Text PDF with Links]
13. Edward Eteshola, Ramasamy P. Pandian, Stephen C. Lee, Periannan Kuppusamy. 2009. Polymer coating of paramagnetic particulates for in vivo oxygen-sensing applications. *Biomedical Microdevices* **11**:2, 379-387. [CrossRef]
14. Dhruv Sud, Mary-Ann Mycek. 2009. Calibration and validation of an optical sensor for intracellular oxygen measurements. *Journal of Biomedical Optics* **14**:2, 020506. [CrossRef]
15. Ramasamy P. Pandian, Michelle Dolgos, Camelia Marginean, Patrick M. Woodward, P. Chris Hammel, Periakaruppan T. Manoharan, Periannan Kuppusamy. 2009. Molecular packing and magnetic properties

of lithium naphthalocyanine crystals: hollow channels enabling permeability and paramagnetic sensitivity to molecular oxygen. *Journal of Materials Chemistry* **19**:24, 4138. [CrossRef]

16. Yuanmu Deng, Ramasamy P. Pandian, Rizwan Ahmad, Periannan Kuppusamy, Jay L. Zweier. 2006. Application of magnetic field over-modulation for improved EPR linewidth measurements using probes with Lorentzian lineshape. *Journal of Magnetic Resonance* **181**:2, 254-261. [CrossRef]
17. Jiangang Shen, Shimin Liu, Minoru Miyake, Wenlan Liu, Aaron Pritchard, Joseph P.Y. Kao, Gerald M. Rosen, Yao Tong, Ke Jian Liu. 2006. Use of 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl as an EPR oximetry probe: Potential for in vivo measurement of tissue oxygenation in mouse brain. *Magnetic Resonance in Medicine* **55**:6, 1433-1440. [CrossRef]
18. Atsuko Matsumoto, Shingo Matsumoto, Anastasia L. Sowers, Janusz W. Koscielniak, Nancy J. Trigg, Periannan Kuppusamy, James B. Mitchell, Sankaran Subramanian, Murali C. Krishna, Ken-ichiro Matsumoto. 2005. Absolute oxygen tension (pO2) in murine fatty and muscle tissue as determined by EPR. *Magnetic Resonance in Medicine* **54**:6, 1530-1535. [CrossRef]
19. Periannan Kuppusamy . 2004. EPR Spectroscopy in Biology and Medicine. *Antioxidants & Redox Signaling* **6**:3, 583-585. [Citation] [Full Text PDF] [Full Text PDF with Links]