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Simultaneous measurements of the intra- and extra-cellular oxygen concentration in viable cells

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An EPR method that can measure the intra- and extra-cellular oxygen concentration [O_2] simultaneously in vitro has been developed using specially designed nitroxides. In the presence of $Fe(CN)_6^{3-}$ in the medium, intracellular [O_2] is measured by a neutral ^{15}N-nitroxide and extracellular [O_2] is measured by a negatively charged ^{14}N-nitroxide, since charged species do not enter cells and the EPR spectrum of a ^{15}N-nitroxide does not overlap with that of a ^{14}N-nitroxide. The method is based in part on the minimal broadening of negatively charged nitroxides by $Fe(CN)_6^{3-}$ and the very effective broadening of neutral nitroxides by the same paramagnetic ions. Results with this method confirm the existence of gradients in [O_2] between the extracellular and intracellular compartments in CHO cells and M5076 tumor cells, even without stimulation of cellular respiration by CCCP. The nature of the barrier that needs to be involved to account for the experimental results raises some significant questions.

Introduction

Oxygen is an important variable in many physiological and pathological processes, so the interpretation and use of many types of data from viable biological systems require accurate knowledge of the concentration of oxygen ([O_2]) both inside and outside of cells [1]. Although intracellular [O_2] is usually the important variable, much of the current understanding of the role of oxygen in cells is based on data obtained from measurement of extracellular [O_2] which then is used to infer to intracellular [O_2]. This approach is valid only if there are no differences between [O_2] inside and outside of the cells [1].

Electron paramagnetic resonance (EPR) oximetry using nitroxides has been developed recently [1–5] and used to study extracellular and intracellular [O_2] [1,5]. A significant gradient was found in Chinese hamster ovary (CHO) cells stimulated by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [1]. If confirmed this finding has significant practical and theoretical importance. The data on oxygen gradients were obtained using separate experiments with different methodology to measure intra- and extra-cellular [O_2].

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The potential disadvantage of the above approach is that different methods are used to determine intra- and extracellular [O_2], using two separate samples with somewhat different experimental conditions. Typically a freely diffusing nitroxide such as ^{15}N-4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl (^{15}N-PDT) and an extracellularly located broadening agent such as $Fe(CN)_6^{3-}$ have been used for the measurement of intracellular [O_2] and a membrane impermeable charged nitroxide such as 4-trimethylammonium 2,2,6,6-tetramethylpiperidine-1-oxyl (Cat_1) has been used for the measurement of extracellular [O_2] [1]. The uncertainties in measurement of [O_2] introduced by using different samples may obscure small differences in intra- and extra-cellular [O_2]; and the different treatments of the cells could affect the measurement, e.g., by affecting the cellular respiration rate and/or properties of the cell membranes. While by the use of suitable control experiments such effects can, in principle, be ruled out as the cause of the observed differences in intra- and extra-cellular [O_2], a more ideal method would be to measure intra- and extra-cellular [O_2] simultaneously in one experiment in one system.

In this report we present the development of such a method. Our approach differs from the previous method that was used by using a negatively charged nitroxide to measure extra-cellular [O_2]. Such nitroxides are only minimally broadened by concentrations of $Fe(CN)_6^{3-}$ which effectively broaden the EPR signal of

a neutral nitroxide. The method also uses ^{15}N substitution for the neutral nitroxide because the EPR spectrum of a ^{15}N-nitroxide does not overlap with that of a ^{14}N-nitroxide. The $Fe(CN)_6^{3-}$ acts not only as a broadening agent but also as an oxidizer of hydroxylamines (hydroxylamines are produced by bioreduction of nitroxides), maintaining the concentration of nitroxide in the cell suspension [1,6]. Using this method, we now have confirmed the existence of gradients in $[O_2]$ between the extra- and intra-cellular compartments in CHO cells and M5076 tumor cells.

Materials and Methods

Nitroxides

The chemical structures and the abbreviations of the nitroxides used in this study are shown in Fig. 1. ^{15}N-PDT and ^{15}N-Cat$_1$ were purchased from MSD Isotopes, St. Louis, MO; SL18 was synthesized as described in Ref. 7; and nitroxide I was newly synthesized and its synthesis will be reported elsewhere.

Growth and preparation of cells

The CHO cell line was a gift from Dr. L. Hopwood, Medical College of Wisconsin and has been maintained in our laboratory for several years. The CHO cells were grown to confluence at 37°C in McCoy's 5a media (from GIBCO, Grand Island, NY) and collected by trypsination. The cells ($2 \cdot 10^5$/ml) were then incubated at 37°C for 24 h in a Bellco spinner flask just prior to their use in these experiments.

The M5076 tumor is a reticulum cell sarcoma of macrophage origin which arose spontaneously in a CS7BL/6 mouse [8]. We obtained the cell line from Dr. R. Magin, College of Engineering University of Illinois and have maintained it in our laboratory for two years in female C57BL/6 mice through intraperineal injections. After injection of about 10^7 cells into a mouse, about $15 \cdot 10^7$ cells can be collected after two weeks. The cells were washed twice with RPMI-160 media (from GIBCO, Grand Island, NY) before their use in these experiments.

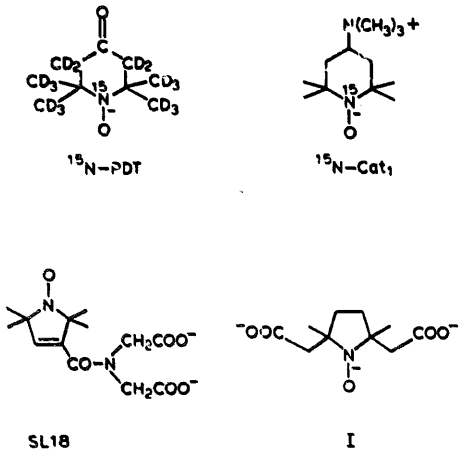

Fig. 1. Chemical structures and abbreviations used in this paper for the nitroxides.

Sample preparation for the EPR measurements

The nitroxides and $K_3[Fe(CN)_6]$ were dissolved in phosphate buffer solution at concentrations of 10 mM and 100 mM, respectively, (final pH = 7.3, osmolarity = 290 mmol/l) and added to the media to achieve the desired final concentrations; 20% dextran by weight also was added to the medium to retard the settling of the cells, by increasing the viscosity of the solution [1]. In each EPR measurement, the sample was placed into a gas permeable Teflon tube (Zeus Industries, Raritan, NJ), 1 mm in diameter, 10 cm long; the Teflon tube was then folded in half and placed in a quartz tube open on both ends. The quartz tube was placed in a vertical EPR cavity. The vertical setup was used to assure that the sample maintained a homogeneous cell distribution within the sensitive volume of the EPR resonator, for the time needed for measurements of $[O_2]$ (30 min).

The charge-dependent broadening effects of $Fe(CN)_6^{3-}$ on the EPR spectra of nitroxides ^{15}N-PDT, SL18, I, and ^{15}N-Cat$_1$ were studied by adding each nitroxide (final concentration of 100 μM) to 100 μl medium with and without 30 mM $Fe(CN)_6^{3-}$ and then recording the EPR spectra at 37°C in N_2 (Fig. 2). In other control experiments to determine the effects of the cells on the nitroxides, ^{15}N-PDT, SL18 or I (final concentration of 100 μM) were added to 100 μl medium containing 10^7 cells, 30 mM $Fe(CN)_6^{3-}$ and 20% dextran and the peak-height of the EPR signal of each nitroxide was recorded continuously over 30 min at 37°C in N_2.

For the measurement of intra- and extra-cellular $[O_2]$, a sample (100 μl) containing 10^7 cells, 300 μM ^{15}N-PDT, 200 μM SL18 (or I), 30 mM $Fe(CN)_6^{3-}$, and 20% dextran was prepared and EPR spectra were recorded at various $[O_2]$ and the O_2-sensitive EPR parameters (see Fig. 3) were measured. Calibration curves were obtained by adding 5 mM NaCN to the above system to inhibit cellular respiration in order to achieve equilibrium of oxygen between the extra- and intra-cellular compartments and then using the above procedures to establish the relationships between the O_2-sensitive EPR parameters and the perfused $[O_2]$. To study the intra- and extra-cellular $[O_2]$ during respiratory stimulation, CCCP was added to the above system. 5 μM CCCP with CHO cells and 1 μM with M5076 tumor cells.

Respiration rates at 37°C were obtained for both CHO cells and M5076 tumor cells with and without stimulation by CCCP, by measuring extra-cellular $[O_2]$ with ^{15}N-Cat$_1$ in a closed system over time, and finding

the slope of the resulting linear plot [1]. 10^6 cells were suspended in 100 μl medium containing 200 μM ^{15}N-Cat$_1$ and 20% dextran. Samples were drawn into glass capillary tubes which were then sealed at both ends and placed in quartz tubes. Spectra were recorded at 1-min intervals, ΔC (see Fig. 3) was measured for each spectrum and converted into an extra-cellular $[O_2]$, and the slope of extra-cellular $[O_2]$ vs. time was obtained to determine the respiration rate [1]. $[O_2]$ in the perfusing gas was measured with a microelectrode purchased from Microelectrode, Londonderry, NH and the readings were converted to $[O_2]$ μM) in water at 37°C. The oxygen parameter ΔC of ^{15}N-Cat$_1$ is more sensitive to changes in $[O_2]$ than that of ^{14}N-Cat$_1$, so ^{15}N-Cat$_1$ was used to measure the cellular respiration rates.

Instruments and EPR parameters

All spectra were taken at 37°C on a Varian E109E spectrometer. The usual instrumental parameters were:

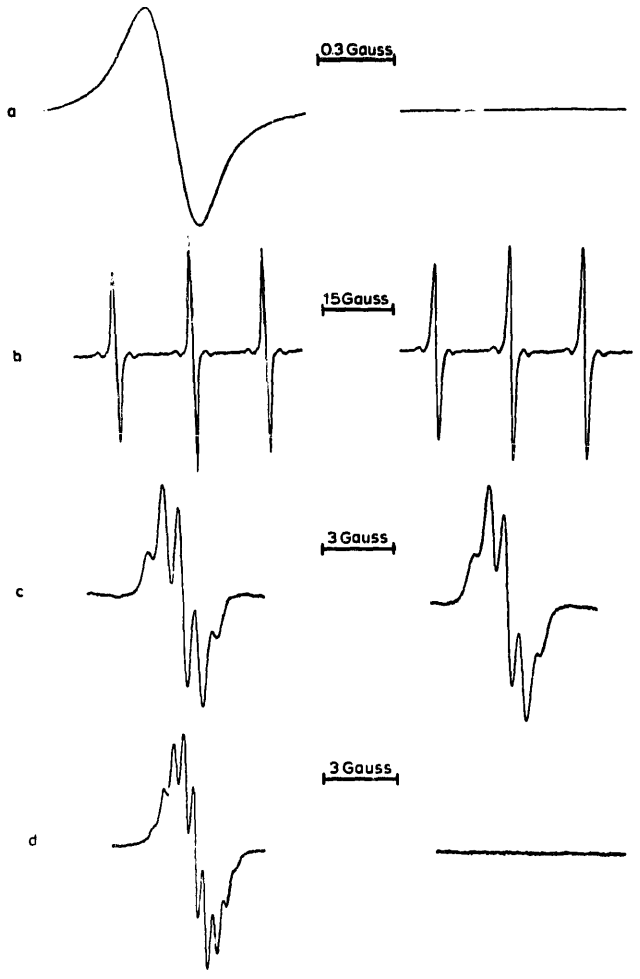

Fig. 2. Charge-dependent broadening effects of $Fe(CN)_6^{3-}$ on the EPR spectra of the nitroxides ^{15}N-PDT (a), SL18 (b), I (c) and ^{15}N-Cat$_1$ (d). The EPR spectra of the four nitroxides without 30 mM $Fe(CN)_6^{3-}$ are on the left and the spectra of these nitroxides with 30 mM $Fe(CN)_6^{3-}$ are on the right. The instrumental conditions were as described in Materials and Methods.

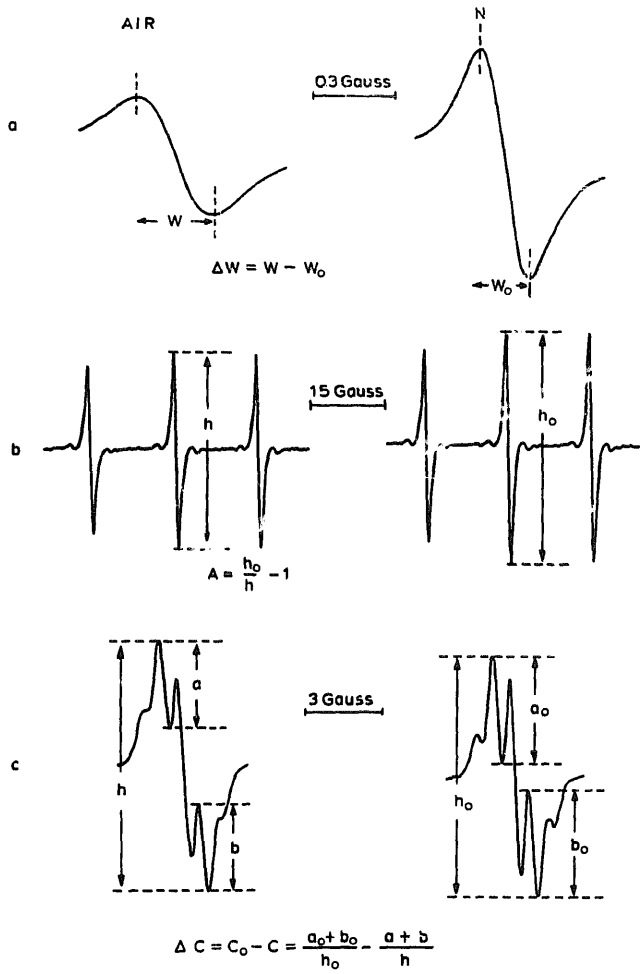

Fig. 3. Definitions of oxygen-sensitive EPR parameters. EPR spectra in air on left and in nitrogen on right (value in nitrogen indicated by subscript 'o'); (a) 100 mM ^{15}N-PDT in medium, (b) 100 mM SL18 in medium with 30 mM $Fe(CN)_6^{3-}$, (c) 100 mM I in medium with 30 mM $Fe(CN)_6^{3-}$. For ^{15}N-Cat$_1$ a parameter similar to that defined for I was used.

microwave frequency, 9.05 GHz; incident microwave power, 5 mW, center of the field, 3210 G; and field modulation 0.1 G. The temperature and concentrations of gases were controlled with a Varian gas flow variable temperature apparatus. EPR measurements included linewidth, superhyperfine splitting, peak-height, and in some cases, the change of the peak-height of the signal with time.

Results

Fig. 2 shows the differential broadening effects of 30 mM $Fe(CN)_6^{3-}$ on the EPR signals of 100 μM ^{15}N-PDT, SL18, and I. The 30 mM $Fe(CN)_6^{3-}$ totally broadened away the EPR signals of the neutral ^{15}N-PDT and positively charged ^{15}N-Cat$_1$ but had much less effect on the negatively charged SL18 and I. With nitroxide I, well resolved superhyperfine structure still could be observed when air was perfused through the sample.

To confirm that the concentration of SL18 and I remained constant in the presence of cells and therefore could be used to measure extra-cellular $[O_2]$, the peak-height change of the EPR signals of 200 μM SL18 or I in 100 μl medium containing 10^7 CHO cells and 30 mM $Fe(CN)_6^{3-}$ were recorded respectively. The experiments were carried out at 37°C in N_2 and no changes were found. The results were as expected since the doubly charged SL18 and I were not expected to cross the cell membrane and any small amounts of the nitroxides reduced outside cells were reoxidized by $Fe(CN)_6^{3-}$. Similar results were obtained with M5076 tumor cells.

As an experimental measure of the maintenance of a constant number of cells in the sensitive volume during the study, we followed the signal intensity of labeled cells over a 30-min period and found that this did not change significantly. The experiment was done in a vertical cavity with a system containing 30 mM $Fe(CN)_6^{3-}$, 300 μM ^{15}N-PDT, 20% dextran and 10^7 CHO cells in 100 μl medium. The vertical configuration was used to avoid possible artifacts from oxygen gradients caused by local high densities of cells which had settled to the bottom of the tube. In the vertical configuration an excessive rate of cell settling would have decreased the number of cells in the sensitive volume but, as the results of this experiment indicated, this was not the case.

Fig. 3 shows the definitions of the three EPR parameters used for the measurements of intra- or extracellular $[O_2]$. Fig. 4 shows the relation of those three EPR parameters to $[O_2]$. The systems used to obtain these calibration curves were 10^7 CHO cells suspended in 100 μl medium containing 300 μM ^{15}N-PDT, 200 μM SL18 or nitroxide I, 20% dextran, and 5 mM NaCN. NaCN inhibited the respiration of the CHO cells so that the $[O_2]$ in the medium was in equilibrium with that in the perfusing gas. Similar calibration curves also were obtained with M5076 tumor cells.

The differences between intra- and extra-cellular $[O_2]$, measured in the CHO cells at various perfused $[O_2]$, with and without the stimulation of the respiration rate by 5 μM CCCP are shown in Table I. The respiration rate of the unstimulated CHO cells was measured to be about $17 \cdot 10^8$ molecules per min per cell and it increased to $40 \cdot 10^8$ molecules per min per cell in the presence of 5 μM CCCP. Each value given in Table I is the average of the differences between intra- and extra-cellular $[O_2]$ at the given perfused $[O_2]$ measured on several samples by the simultaneous method. All the values for $[O_2]_e - [O_2]_i$ in Table I are significantly different from zero, except the first value for the normal CHO cells (Student's t-test). The size of the gradient in unstimulated cells was 4–10 μM and it increased to about 40 μM with stimulation of respiration by 5 μM CCCP.

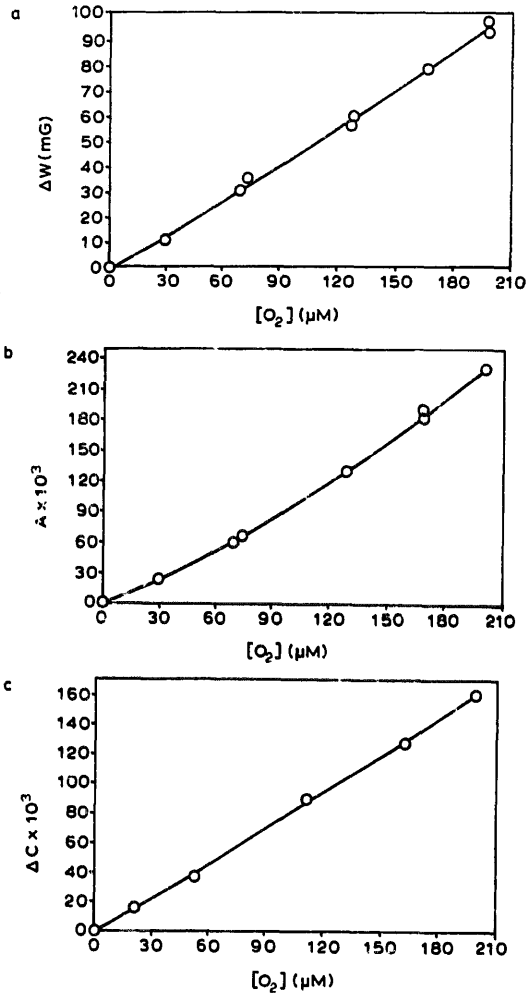

Fig. 4. Relationships between oxygen-sensitive EPR parameters and $[O_2]$ in CHO cells. See Fig. 3 for definitions of the parameters. 5 mM NaCN was added to the cell suspension to inhibit cellular respiration in order to achieve equilibrium of oxygen between the extra- and intra-cellular compartments.

The differences between intra- and extra-cellular $[O_2]$ at various perfused $[O_2]$, measured with the M5076 tumor cells under normal and stimulated conditions are shown in Table II. The respiration rate of the

TABLE I

Differences in $[O_2]_i$ and $[O_2]_e$ in CHO cells at various perfused $[O_2]$

Perfused $[O_2]$ (μM)	Average $[O_2]_e$ (μM)	Average $\Delta[O_2]_{e-i} \pm$ S.E. (number of expts.)
Unstimulated CHO cells		
200	139	4.5 ± 3.8 (6)
150	94	12.3 ± 5.3 (6)
100	47	9.2 ± 2.4 (6)
50	10	3.7 ± 0.6 (6)
CHO cells stimulated by 5 μM CCCP		
200	103	38.0 ± 13.0 (2)
150	62	44.0 ± 5.5 (2)
100	26	18.5 ± 9.5 (2)

TABLE II

Differences in $[O_2]_i$ and $[O_2]_e$ in M5076 tumor cells at various perfused $[O_2]$

Perfused $[O_2]$ (μM)	Average $[O_2]_e$ (μM)	Average $[O_2]_{e-i} \pm$ S.E. (number of expts.)
Unstimulated M5076 tumor cells		
200	137	12.3 ± 4.4 (12)
150	95	15.5 ± 3.1 (12)
100	53	15.1 ± 3.0 (12)
50	14	9.5 ± 3.0 (11)
M5076 tumor cells stimulated by 1 μM CCCP		
200	90	26.6 ± 3.9 (7)
150	48	26.3 ± 5.5 (7)
100	20	12.1 ± 2.8 (7)

M5076 tumor cells without stimulation was measured to be about $9 \cdot 10^8$ molecules per min per cell and that of the same cells stimulated by 1 μM CCCP was $30 \cdot 10^8$ molecules per min per cell. Each value shown in Table II is the average of the differences between intra- and extra-cellular $[O_2]$ at the given perfused $[O_2]$, measured on several samples by the simultaneous method. All the values for $[O_2]_e - [O_2]_i$ in Tables II are significantly different from zero (Student's *t*-test). The size of the gradient without stimulation was about 15 μM and it increased to about 26 μM with the stimulation of respiration by 1 μM CCCP.

Discussion

These results provide positive support for the two principal aims of this paper: confirmation of the existence of gradients between intracellular and extracellular $[O_2]$ in viable functioning cells and the demonstration of the feasibility of methods to measure intra-cellular and extra-cellular $[O_2]$ simultaneously.

The results in Table I and II clearly confirm the previous finding that under appropriate conditions, average $[O_2]$ inside the cells can be significantly less than the average $[O_2]$ outside of the cells [1]. Because of the importance and implications of this conclusion, which was reached initially on the basis of experiments using somewhat different methodology, the present results provide a valuable confirmation that such gradients can occur in cells with a high rate of respiration. Perhaps equally importantly, the improved methodology introduced in these experiments indicates that such a gradient can occur in cells without artificially stimulating their respiration. Consistent with the previous reports, respiratory stimulation of CHO cells resulted in a substantially larger difference between intra- and extra-cellular $[O_2]$ [1].

These studies also extend the finding of differences between intra- and extra-cellular $[O_2]$ to another cell line, M5076 tumor cells. This suggests that the occurrence of gradients may be a general phenomenon found in most or all cell systems under appropriate conditions. The responses of the two cell lines studied in the present experiments differ somewhat. There was a larger difference in the gradient between intra- and extra-cellular $[O_2]$ in the resting state for the M5076 tumor cells which was possibly due to their larger size (~ 12 μm in diameter vs. ~ 10 μm for CHO cells). This difference, however, did not increase as much in the M5076 tumor cells as in CHO cells although the selected concentrations of CCCP caused approximately the same degree of respiratory stimulation.

The confirmation of the observation that there can be a difference in $[O_2]$ between the intracellular and extracellular compartments of suspensions of cells makes it desirable to try to understand the nature of the process that allows this to occur. As has been shown previously, the existence of such gradients cannot be accounted for on the basis of simple diffusion of oxygen into the interior of oxygen consuming cells [1]. Apparently reasonable models of such diffusion indicate that gradients of the magnitude that were observed should not occur. Therefore, the results of these experiments imply that there must be some sort of a barrier to the free diffusion of oxygen. We have speculated previously that the most likely position for such a barrier would be the plasma membrane of the cell [1]. Recent studies by others have indicated that membranes can be effective barriers to the free transport of oxygen [9]. Our results indicate that the nature or the extent of the barrier may differ in different cell lines, suggesting that it may be useful to look at additional types of cells in order to seek cell lines which might have even larger apparent barriers to the diffusion of oxygen and which, therefore, could provide good experimental materials for understanding the nature of the barrier.

These results also indicate the feasibility of the methodological approach which measures intra- and extra-cellular $[O_2]$ simultaneously. In order to obtain well resolved EPR spectra from both compartments, the method uses similar charges on the nitroxide used to measure extra-cellular $[O_2]$ and the broadening agent which removes the resonances from the extracellular compartment of the neutral nitroxide which is located both intracellularly and extracellularly. This method also required the use of isotopic substitution of the neutral nitroxide in order to have its spectrum not overlap with that of the nitroxide localized in the extracellular compartment. The previously used methodology required the use of two separate experiments with somewhat different experimental conditions and used different nitroxides for the separate experiments measuring intra- and extra-cellular $[O_2]$. In addition to the conceptual and experimental desirability of making the measurements of intra- and extra-cellular $[O_2]$ at the same time and in the same

sample, the value of this approach also is indicated by its increased sensitivity to small differences; the new method detected differences in $[O_2]$ between the intra- and extra-cellular compartments in CHO cells without the need to stimulate respiration.

While the new methodology is an improvement over those used previously, it still has some drawbacks. It requires the use of relatively high concentrations of a paramagnetic metal ion as a broadening agent; upon prolonged exposure these substances can be toxic to cells (Swartz et al., unpublished data, also see Ref. 10). This approach also does not seem to be applicable to simultaneous measurements of intra- and extra-cellular $[O_2]$ in vivo. We currently are developing approaches that may overcome one or both of these limitations. These approaches measure extracellular $[O_2]$ by means of a charged nitroxide which is incorporated in a liposome [11] or by relatively large particles (greater than 10 μm in diameter) of the new oxygen sensitive materials, lithium phthalocyanine or fusinite [12]. The new approaches to measuring intracellular $[O_2]$ are the use of esters containing nitroxides which are designed such that the esters become hydrolyzed in the intracellular environment and thereby become trapped inside cells [13] or by the use of small particles of lithium phthalocyanine or fusinite which will become phagocytized by cells [12].

Acknowledgements

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References

1 Glockner, J.F., Swartz, H.M. and Pals, M.A. (1989) J. Cell. Physiol. 140, 505–511.
2 Subczynski, W.K. and Hyde, J.S. (1983) Biophys. J. 41, 283–286.
3 Belkin, S., Mehlhorn, R.J., Hideg, K., Hankovski, O. and Packer, L. (1987) Arch. Biochim. Biophys. 256, 232–243.
4 Backer, J.M., Budker, V.G., Eremenko, S.I. and Molin, Y.N. (1977) Biochim. Biophys. Acta 460, 152–156.
5 Morse P.D. II and Swartz, H.M. (1985) Mag. Reson. Med. 2, 114–127.
6 Chen, K. and Swartz, H.M. (1988) Biochim. Biophys. Acta 970, 270–277.
7 Sosnovsky, G., Rao, N.U.M., Li, S.W. and Swartz, H.M. (1989) J. Org. Chem. 54, 3667–3674.
8 Talmadge, J.E. and Hart, I.R. (1984) Cancer Res. 44, 2446–2451.
9 Subczynski, W.K., Hyde, J.S. and Kusumi, A. (1989) Proc. Natl. Acad. Sci. USA 86, 4474–4478.
10 Lai, C.-S., Froncisz, W. and Hopwood, L.E. (1987) Biophys. J. 52, 625–628.
11 Chan, H.C., Glockner, J.F., Swartz, H.M. (1989) Biochim. Biophys. Acta 1014: 141–144.
12 Swartz, H.M., Gast, P., Glockner, J.F., Hu, H., Ji, L.L., Liu, K.J., Nilges, M., Norby, S.W., Vahidi, N., Walczak, T., Wu, M. and Clarkson, R.B. (1991) in Oxidative Damage and Repair: Clinical, Biological and Medical Aspects, Pergamon Press, Oxford, UK.
13 Hu, H., Sosnovsky, G., Li, S.W., Rao, N.U.M., Morse P.D. II and Swartz, H.M. (1989) Biochim. Biophys. Acta 1014, 211–218.