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Forum Review

Using EPR to Measure a Critical but Often Unmeasured Component of Oxidative Damage: Oxygen

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ABSTRACT

Oxygen is a critical variable in oxidative damage. It can be a direct reactant in one or more of the pertinent reactions that result in oxidative damage. It also is an essential substrate for mitochondrial respiration and many other essential synthetic and degradative reactions. The level of oxygen can have a regulatory role, affecting the rate and direction of metabolic processes and physiological functions that are germane to the pathophysiological processes that are being studied. Its supply to tissue and to cells is therefore a critical parameter governing normal homeostasis. The level of oxygen at specific sites may affect cell signaling. It therefore seems clear that it can be very useful to measure oxygen when studying oxidative damage. In order for the measurements of oxygen to be most useful, it often is essential to measure the amount of oxygen at particular sites and under appropriate conditions. These needs require methodology that can make sensitive and localized measurements of oxygen. Electron paramagnetic resonance (EPR) oximetry is such a technique, plus it has the capability of making repeated measurements from the same site non-invasively. The principles and applications of EPR oximetry to viable systems, including cell suspensions and intact animals, are described in this paper. *Antioxid. Redox Signal.* 6, 677–686.

WHY DO WE NEED THE DIRECT MEASUREMENT OF OXYGEN FOR STUDIES OF OXIDATIVE PATHOPHYSIOLOGY?

ALTHOUGH THIS MAY SEEM like a superfluous question because of the obvious importance of oxygen in oxidative damage, the reality is that in most studies of oxidative processes, there are few if any direct measurements of oxygen. Perhaps this is because of a lack of familiarity with the techniques to measure pertinent levels of oxygen. It also often is due to simple oversight, perhaps because of the ubiquitous presence of oxygen. Regardless of the reason, it is indeed usually desirable and sometimes essential to make such measurements to understand and control studies of oxidative damage, for the following reasons: (1) In many of the pertinent reactions, oxygen is a direct reactant in one or more of the oxidative processes that are being studied. (2) Oxygen is an essential substrate for mitochondrial respiration and many other essential synthetic and degradative reactions. (3) The level of oxygen can have a regulatory role, affecting the rate and direction of metabolic processes and physiological functions that are germane to the pathophysiological processes that are being studied. Its supply to tissue and to cells is therefore a critical parameter governing normal homeostasis. (4) The level of oxygen at specific sites may affect cell signaling.

For a number of years several groups have developed and characterized techniques using electron paramagnetic resonance spectroscopy (EPR) (or equivalently electron spin resonance [ESR]) to measure oxygen using both spectroscopy and imaging [summarized in Swartz and Clarkson (20) and Swartz and Halpern (22)]. Our own experimental approach has focused on spectroscopy, and this will be the focus for the discussion in this paper.

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THE TYPES OF MEASUREMENTS OF OXYGEN THAT CAN BE MADE

In order for the measurements of oxygen to be most useful, it often is essential to measure the amount of oxygen *at particular sites and under appropriate conditions*. This is because: (1) Oxygen is involved in many different processes; (2) these processes often have quite different dependencies on the amount of oxygen; (3) the amount of oxygen can vary significantly over spatial dimensions smaller than the cell, especially within the cell; and (4) the delivery and utilization of oxygen are dynamic processes that can result in significant variations over periods as short as milliseconds.

Before describing the measurements of oxygen in more detail, however, it is useful to consider the physical properties of oxygen and how this relates to its measurement. Oxygen is quite lipophilic and therefore partitions significantly into the lipophilic compartments. In particular, it partitions into membranes by a factor of about 8 (18). Consequently, all other factors being equal, the concentration of oxygen ($[O_2]$) will be eightfold higher in membranes and other lipophilic regions of cells than in the aqueous compartment of the cell. Because of this, it is important to know the site where the oxygen measurement is being made and, equally importantly, the type of measurement parameter that is used in the measurement. In regard to the latter point some techniques, such as polarography and EPR oximetry based on particulates (see below for more on the types of EPR measurements), report on the partial pressure of oxygen (pO_2), while others, such as fluorescent quenching and EPR oximetry, which are based on soluble materials, report on concentration ($[O_2]$). Each type of measurement parameter is useful, as long as it is known and interpreted appropriately. A more complete description of various types of oxygen measurements has been recently published (21).

PRINCIPLES OF EPR OXIMETRY

There are a number of useful methods for measuring pO_2 or $[O_2]$ in viable systems, including *in vivo,* but all of these techniques also have some significant limitations. The most significant limitations often are in the ability to make repeated measurements, the degree of invasiveness required for the methods, or sensitivity/accuracy (14, 21, 26). For many applications, EPR oximetry can be a very effective approach because it has the capability of providing repetitive and sensitive measurements of oxygen non-invasively. Within the last several years, there has been a very significant amount of progress in EPR oximetry based on developments in several different laboratories [reviewed by Swartz and co-workers (19, 20, 22)]. This has resulted in the availability of instrumentation and paramagnetic materials capable of measuring pO_2 or $[O_2]$ in cells and tissues with an accuracy and sensitivity comparable or greater than those available by any other method. The balance of this article provides a detailed description of EPR oximetry. This includes descriptions of several different but related approaches using EPR spectroscopy in systems ranging from cells in culture to human subjects under clinical conditions. The use of spectroscopy is emphasized because we have found it to be especially useful, but there are a number of very productive applications of imaging, which are described in a recent review (22).

In principle, molecular oxygen affects all EPR spectra because it is paramagnetic, having two unpaired electrons in its ground state. Some paramagnetic materials, as described below, are especially useful for observing the effects of oxygen. Often the most useful technique to quantify the effects on the EPR spectra is to measure the changes in the linewidth that occur because of the interactions. With the relatively simple and well-defined molecules that often are used as soluble materials for measurements in solutions and imaging, the effects of oxygen can be followed equally well by measuring changes in the relaxation times of the unpaired electrons. With particulates, such as carbon-based materials and lithium phthalocyanine (LiPc), the effects may be more complex, and therefore the use of relaxation measurements with particulates may not be an optimal approach.

Data can be obtained from different types of systems by using different frequencies of EPR spectroscopy. For studies in cell suspensions or *ex vivo* samples, *e.g.,* isolated thin slices of tissue, blood, cerebrospinal fluid, or urine, where the sample size is limited and can be placed in a capillary or flat sample holder, 9.5 GHz ("X-band") generally is used because higher frequencies usually have higher sensitivity. When making measurements in larger samples and/or *in vivo,* it is necessary to use lower frequency (usually between 300 and 3,000 MHz). Lower frequencies have lower sensitivity but can penetrate more deeply. Using non-invasive methods, EPR spectroscopy at the higher end of this range can provide data at depths of a few millimeters, while at the lower end of the range measurements can be made deeper than 10 cm. The sensitivity tends to decrease with frequency within this range.

EPR specifically responds only to molecules with unpaired electrons, including free radicals, free electrons in some types of matrices, and some valence states of metal ions. The phenomenon that is observed in EPR is the transition (resonance absorption of energy) between the two energy states that can occur in an unpaired electron system placed in a magnetic field. The magnetic field separates the energy states associated with the two possible spin states of an unpaired electron (spin 1/2 system). The presence of other unpaired electron species can affect the EPR spectrum, by introducing a fluctuating magnetic field. Of particular importance to EPR oximetry is the fact that the ground state of molecular oxygen has two unpaired electrons. The magnetic field from the two unpaired electrons in oxygen will interact with other unpaired electron species, and the extent of this interaction will be a function of the amount of oxygen that is present. While this effect occurs with all paramagnetic materials, it is much larger in some materials, and these have been selected for use for "EPR oximetry." The EPR signal arising from these materials can be a sensitive reporter of the pO_2 or $[O_2]$ at that location. Typically, the spectral linewidth (the peak-to-peak splitting along the magnetic field axis) is measured and converted to pO_2 or $[O_2]$ using an appropriate calibration curve.

The choice of material depends on the system being investigated and the parameter to be measured. Both soluble and particulate oxygen-sensitive materials have been used. Each

type of preparation has some advantages and potential limitations. Both types share the virtue of relying on a physical interaction with oxygen that does not affect the local [O_2]. This differs from techniques such as polarography in which the oxygen is consumed in the process that is used to measure its concentration.

Soluble materials

There are a variety of oxygen-sensitive paramagnetic molecules [especially nitroxide and trityl (triphenyl derivatives) radicals] available that vary in structure and chemical properties [such as size, hydrophobicity, *etc.* (6)]. Most nitroxides exhibit a three-line hyperfine line splitting that is characteristic of the ^{14}N in the N-O· group, and a linewidth that shows some dependence on [O_2]. The linewidths in aqueous solutions of virtually all soluble paramagnetic molecules increase about 100 mG over the range of 0–20% O_2. The trityls have narrower lines in the absence of oxygen, and therefore the oxygen-induced broadening is measured more readily and accurately. The soluble materials report on [O_2] because their linewidth reflect changes in relaxation rates due to collisions with dissolved oxygen, and the rate of collisions is dependent on the solubility and/or diffusion of oxygen in the medium. Nitroxides for oximetry can be synthesized using ^{15}N and/or deuterium substitution, resulting in a narrow intrinsic linewidth and fewer lines in the EPR spectrum (Figs. 1 and 2). For example, Tempone (4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl) exhibits a three-line spectrum and a linewidth that depends on oxygen concentration; the linewidth of an individual line is about 360 mG in nitrogen to around 470 mG in air. Perdeuterated ^{15}N Tempone (^{15}N-PDT) has two spectral lines and a linewidth of about 165 mG in nitrogen and 300 mG in air. The ability to choose either a two- or three-line material is particularly important, as it allows the operator to use two probes simultaneously whose EPR lines do not overlap in the spectrum and can be monitored independently and simultaneously.

The triphenyl radicals have very narrow line widths in the absence of oxygen and also have a single line; both of these characteristics are especially favorable for imaging (6, 22). Many of the triphenyls are very stable in living systems.

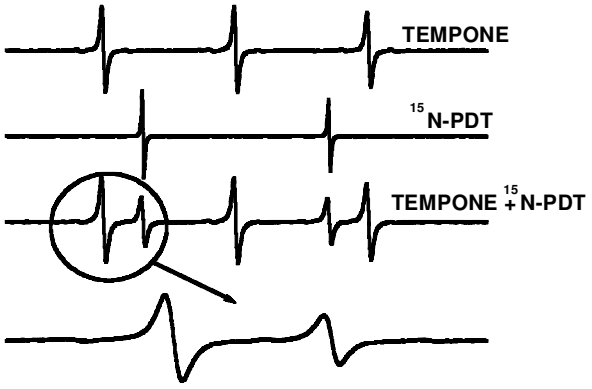

FIG. 2. EPR spectra of Tempone and ^{15}N-PDT illustrating the different hyperfine splittings (16.6 G and 23.3 G, respectively) and EPR lines arising from ^{14}N and ^{15}N (three and two lines, respectively). A combination of these two nitroxides can be used to measure oxygen in two different compartments if they are selectively localized.

Particulates

Certain particulate materials have unpaired electrons (especially carbonaceous systems such as coals and chars, and LiPc crystals) and often exhibit a single EPR line that can be quite sensitive to oxygen (2). They respond to pO_2 rather than to [O_2].

As shown in Fig. 3, the linewidth of LiPc responds linearly to the pO_2 over the entire range of biological interest. The carbon-based particulates often do not have a linear response at higher pO_2 levels, but unless the line gets so broad that it cannot be measured accurately, the use of an appropriate calibration curve compensates fully for the non-linearity.

The particulates can be prepared in various sizes, which can be a very useful way to achieve localization. In cell culture larger particles will stay extracellularly, while small particles (10 μm or less) often are phagocytized into the cell. *In vivo* the size of the particles can be made small to be able to enter cells, especially localizing selectively in macrophages (which results in a high degree of localization in the liver and

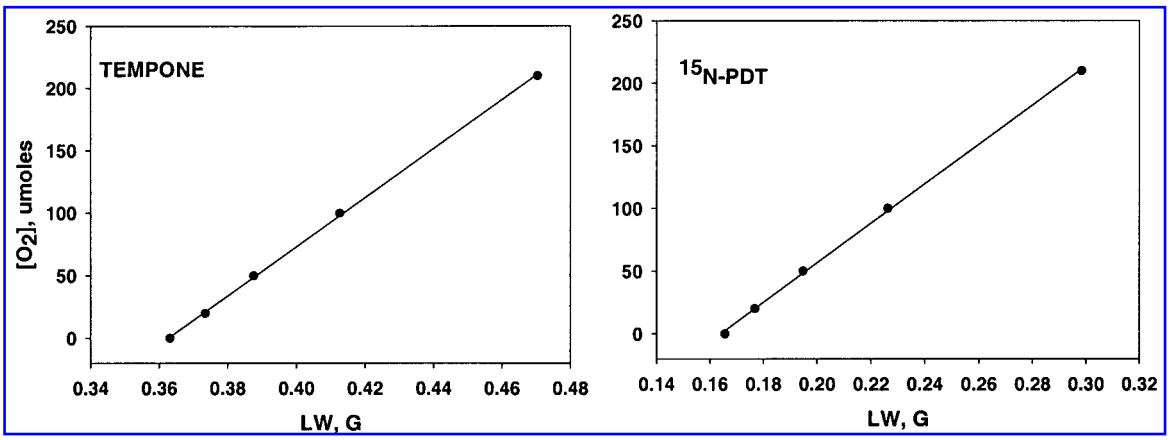

FIG. 1. Change in linewidths of TEMPONE and ^{15}N-PDT with different concentrations of perfused oxygen.

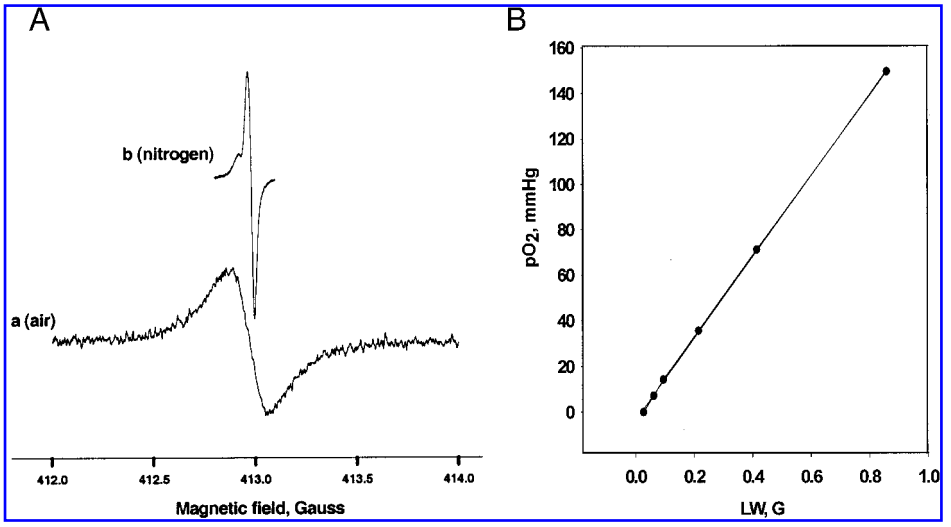

FIG. 3. A: Typical EPR spectra of LiPc at two different concentrations of perfused oxygen. B: Change in the linewidth of EPR signal from LiPc at different concentrations of perfused oxygen.

spleen), or large enough to remain extracellularly as a slurry, or the particles can be macroscopic aggregates that can be used for multisite oximetry. The latter technique provides multiple independent measurements of oxygen, with demonstrated feasibility of measurements from six surfaces and six particles (total of 36 independent measurements) in a 1-cm sphere (5).

Many of these particulate paramagnetic materials are very inert in biological systems as assayed in both cell cultures and *in vivo* (10, 28). Their paramagnetism is independent of local metabolic processes, the presence of other paramagnetic species, and pH. This inertness makes them especially useful for studies *in vivo* because once placed into tissues, they can be used to obtain repeated measurements of pO_2 from the same site. The measurements usually are done using a non-invasive method in which the measurements are made with a resonator on the surface of the skin (or with localized areas such as toes or a region of a small animal, measurements also can be made with a coil that fits around the site of interest). The measurements can be made as frequently as desired over a period of a year or more. The pO_2 that is measured is the average pO_2 in the tissues that are in immediate equilibrium with the surface of the paramagnetic particle(s) (24).

To summarize, the characteristics of *in vivo* EPR oximetry that is based on the use of particulate oxygen-sensitive materials include:

1. *Non-invasiveness.* Some approaches do require an initial placement of the paramagnetic materials into the tissues and in the future also could include approaches in which a resonator is placed at the site of measurement).
2. *Repeatability.* Measurements can be made as frequently as desired over a period of up to a year or more.
3. *Sensitivity.* Measurements can, at low pO_2, resolve differences of less than 1 torr.
4. *Accuracy.* Repeat measurements have a small variability and correlate closely with measurements of pO_2 by other methods.
5. *Provide localized measurements.* The spatial resolution is the same as the size of the paramagnetic particles, which can be as small as a single particle of less than 0.2 mm in diameter.
6. *Little or no effect of the range of chemical and physical conditions that are likely to be encountered in viable biological systems.* These include pH, oxidants, reductants, and the presence of other paramagnetic materials.
7. *Little or no toxicity.* The paramagnetic materials are very inert in biological systems as assayed in both cell cultures and *in vivo.*
8. *Capability of making several measurements simultaneously.* This is accomplished by inserting multiple discrete solid particles and applying a magnetic field gradient such that sites less than 1 mm apart can be resolved.
9. *Time resolution of seconds or less.* This is possible for following changes in pO_2.
10. *Respond to pO_2,* i.e., *rather than $[O_2]$.* This is in contrast to nitroxides and other oxygen-sensitive materials that may respond to the $[O_2]$ or a product of concentration and the rate of diffusion.

Examples of use of EPR oximetry for measurements with implications for studies of oxidative damage

The balance of this paper uses examples to illustrate some details of the methodology that is used to measure oxygen in viable systems and how this information is likely to be very important for studying oxidative damage. There are separate descriptions of the techniques for measurements in cell suspensions and *in vivo,* because they involve quite different procedures. The examples chosen to illustrate the method pro-

vide insights as to how EPR oximetry can facilitate understanding of oxidative damage.

MEASUREMENTS OF OXYGEN AT THE CELLULAR LEVEL

Typical procedures for measurement of oxygen concentrations with EPR

The key experimental aspect is to obtain selective measurements of oxygen in the intracellular and extracellular compartments (and, if needed, within particular intracellular compartments such as phagosomes, as illustrated in the example below) (8, 23). Selective measurements of the extracellular compartment can be obtained by using a material that is too large to enter cells, *e.g.,* particulates $\geq$20 μm, or by use of a charged material, *e.g.,* "Cat-1." Selective intracellular measurements can be achieved by several approaches using a particle that is small enough to be ingested by cells (which then may provide selective measurements within the phagosome). The measurements can be made by an hydrolysable ester that becomes converted intracellularly to a charged species that cannot pass out of the cell, or by a molecule that is distributed throughout the sample, with the spectra from molecules in the extracellular compartment broadened away by the presence of a charged paramagnetic metal ion that does not cross the cell membrane.

A typical procedure is to prepare 200-μl samples containing 2.5×10^7 cells/ml, 0.2 m*M* ^{15}N-PDT, 0.25 mg/ml LiPc, 10% dextran, and 50 m*M* gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) complex (the concentrations are the final concentrations in the sample). The linewidths of both the PDT and the LiPc are functions of the amount of oxygen (Figs. 1 and 3). Because of its solubility and moderate lipophilicity, the PDT distributes throughout the sample, while the size of the LiPc keeps it in the extracellular compartment. The addition of the positively charged Gd-DTPA complex broadens the extracellular ^{15}N-PDT signal, and the remaining signal provides a direct measurement of the average intracellular oxygen. At this concentration, the EPR signal arising from the Gd-DTPA complex is too broad to be observed under the experimental conditions. Because the unpaired electrons in LiPc are located deep within the crystal and hence physically shielded from the media, the Gd-DTPA complex does not affect the linewidth of LiPc, and the LiPc remains extracellular because of its size. Therefore, LiPc reports exclusively the average extracellular pO_2. The EPR signals of LiPc and ^{15}N-PDT do not overlap.

The cell suspension is mixed quickly but gently, and drawn into a gas-permeable Teflon tube with an inside diameter of 0.813 mm and a wall thickness of 0.038 ± 0.014 mm (Zeus Industries, Raritan, NJ, U.S.A.). The tube is folded into a W shape and inserted in a quartz tube open at both ends. Measurements are carried out at various perfused $[O_2]$ values, and the linewidths are calculated, for example, by spectral fitting, using the EWVoigt program (Scientific Software, Normal, IL, U.S.A.). For calibration, the cellular oxygen consumption is inhibited, *e.g.,* by 0.5 m*M* diphenyl iodonium chloride. It is assumed that in the absence of oxygen consumption, equilibrium exists for oxygen between the extra- and intracellular compartments and can be related to the oxygen tension of the perfused gas. A plot of the oxygen concentration in the medium against the linewidths provides the correlation equation, which is used to transform linewidths into oxygen concentrations.

The EPR measurements are carried out at with an X-Band (9 GHz) EPR spectrometer. The samples are kept at physiological temperature (37°C) under as good physiological conditions as possible (lowest feasible concentration of cells, physiological media, perfusion by oxygen through the gas-permeable sample holder). Typical EPR spectroscopic parameters are: microwave frequency, 9.35 GHz; center field, 3,320 G for ^{15}N-PDT and 3,338 G for LiPc; and modulation frequency of 25 KHz for LiPc and 100 KHz for ^{15}N-PDT. The $[O_2]$ of the perfused gas should be verified with an oxygen analyzer.

Measurement of respiration rates

The measurement of oxygen needs to be considered in the context of appropriate other physiological measurements. When studying cell samples, it very often is essential to know the rate of respiration (utilization of oxygen). Both the distribution and the availability of oxygen in cells will be affected by the rate of utilization. Also, oxygen utilization can be an important measure of cell function, providing insights as to the status of the cells. EPR oximetry is a very convenient method to measure oxygen consumption in small samples. In a typical experiment a 100-μl sample of cell suspension (2.5×10^6 cells/ml) is mixed with 10% dextran (to prevent settling of the cells during the measurement) and 0.2 m*M* ^{15}N-PDT. The cell suspension is placed in a glass capillary tube, which is sealed at both ends with Cytoseal® sealant to make it gas-tight, and then the sample is placed in the EPR resonator. Oxygen consumption by the cells results in a decrease in the oxygen concentration in the sealed tube, which causes a decrease in the linewidth of the ^{15}N-PDT. Spectra are recorded at 30-s intervals for 10 min, and the change in linewidths is transformed to $[O_2]$ using a calibration curve. Slopes of the decrease in $[O_2]$ versus time yield the oxygen consumption rates of the cells.

Examples of applications of measurements of oxygen at the cellular level: Dependency of superoxide production on intracellular oxygen and role of cholesterol on the gradient of oxygen between the intracellular and extracellular compartments

Although the finding remains somewhat controversial, there is a body of evidence that there can be significant gradients in $[O_2]$ values between the extracellular and intracellular compartments and among intracellular compartments (4, 13). This is in contrast to the usual assumption that the intracellular oxygen (except perhaps within the mitochondria) is the same as the extracellular oxygen. The latter is much easier to measure and/or to control by perfusing large amounts of gas

with a known $[O_2]$. But if significant gradients can occur then knowledge only of the $[O_2]$ being perfused would not be sufficient. The occurrence of significant gradients would have important implications for understanding oxidative processes in cells, because such processes are likely to be significantly affected by variations in the local $[O_2]$.

The importance of measuring $[O_2]$ levels and the occurrence of gradients between the extracellular and intracellular compartments is illustrated in studies of the oxygen dependence of superoxide production in macrophages, and their implication for an important reaction in oxidative biology, the production of superoxide, is illustrated in publications by James *et al.* (11, 12). As indicated in Table 1, the production of superoxide was consistent with the known oxygen dependence of NADPH-oxidase based on the intracellular $[O_2]$ (K_m of NADPH-oxidase is 30–40 μ*M*) (16), while it did not correlate with the measured value of extracellular oxygen. The information on the local $[O_2]$ levels also made it feasible to conclude that about 50% of the observed generation of superoxide occurred through another mechanism.

A key question, with important implications for studies of oxidative biology, is how cells maintain gradients of oxygen above those expected on the basis of simple kinetics with oxygen passively diffusing into cells and being consumed within cells. Although much remains to be understood about this process, there is recent evidence linking the concentration of cholesterol in the plasma membrane and the gradients in oxygen. Most of the cholesterol in cells is in the plasma membrane. The structure of the plasma membrane can be significantly altered by the amount of cholesterol and its distribution within the membrane. Using various Chinese hamster ovary (CHO) cell mutants with defined mutations in the metabolism of cholesterol and established techniques to alter the cholesterol content within a cell line, the relationship between cholesterol content and oxygen gradients has been measured recently (13). Using the oxygen measuring techniques described above and three different CHO cell lines (wild type, 25 RA, and M 19 CHO cells) (1), it was shown that the gradient was larger in cell lines with increased content of cholesterol in the plasma cell membrane. This result was supported by an additional increase in the oxygen gradients with the incorporation of additional cholesterol in the plasma membrane, and a decrease in the oxygen gradient when the cholesterol was depleted from the plasma membrane. The data are summarized in Fig. 4.

MEASUREMENTS OF OXYGEN USING EPR OXIMETRY *IN VIVO*

Experimental procedures for measuring oxygen in vivo *with particulates*

The particulates (LiPc, coals, chars, India ink) are especially useful for *in vivo* studies because with them one can make repeated measurements from the same site due to their stability and inertness in tissues. For many applications LiPc is especially useful, because in the absence of oxygen the line is very narrow and the linewidth increases proportionally with the amount of oxygen that is present (Fig. 3B). The other particulates are carbon-based. They tend to have much broader lines in the absence of oxygen, and in the presence of oxygen they broaden to a greater extent than LiPc. At very low levels of oxygen this can be advantageous, providing high sensitivity for small changes in the amount of oxygen. At higher concentrations (above 10–30 torr, depending on the material) the lines become so broad that they are difficult to measure and therefore are not suitable for measurements at high levels of oxygen. The oxygen-induced broadening of the EPR spectra of the carbon-based particulates departs from linearity at higher $[O_2]$ levels, but this is not a major problem because with appropriate calibration curves this still will provide accurate values of pO_2. The use of India ink has a singular advantage for studies in human subjects because it can be used without requiring extensive additional studies of its safety. This is because it already has been used extensively in human subjects as a marker for various medical procedures, *e.g.,* marking fields in radiation therapy and for following lymphatics during surgery, as well as for decoration on the skin (tattoos) (25).

The oxygen-sensitive material usually is implanted several days prior to the start of the rest of the experimental procedures, in order to ensure that the implantation procedure does not perturb the measurements. As noted above, these materials are quite inert in tissues, so that there is minimal reaction to their presence, but of course the process of physical im-

TABLE 1. MEASURED RATES OF SUPEROXIDE PRODUCTION IN RELATION TO EXTRACELLULAR AND PHAGOSOMAL $[O_2]$ VALUES

	$[O_2]$ (μM)		
Cell concentration (cells/ml)	*Extracellular*	*Phagosomal*	*Superoxide production (%)**
5×10^7	175	154.7	100
5×10^7	70	41.7	50
5×10^7	31	7.7	50
$2.5 \times 5 \times 10^7$	179	146.0	100
$2.5 \times 5 \times 10^7$	84	51.2	100
$2.5 \times 5 \times 10^7$	36	25.8	50

*Expressed as percent of maximum signal intensity obtain from 5×10^7 cells/ml, incubated in air (210 μ*M*). Reprinted with permission from The Journal of Leukocyte Biology.

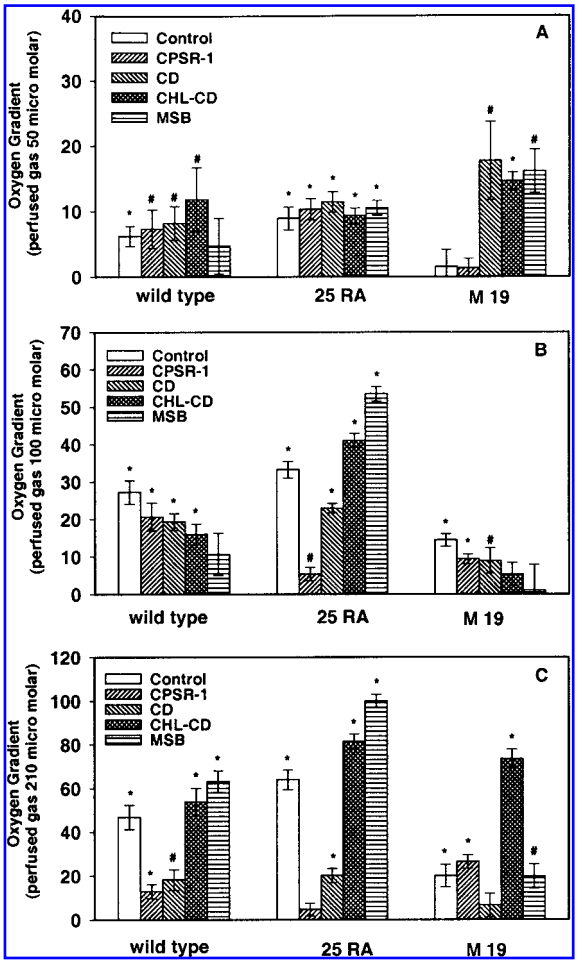

FIG. 4. Oxygen gradient (μ*M*) between extra- and intracellular compartments of cells at different perfused oxygen concentrations: (A) 50 μ*M* perfused gas, (B) 100 μ*M* perfused gas, and (C) 210 μ*M* perfused gas. Note the difference in the scale of the oxygen gradient plotted for each perfused gas concentration. For the occurrence of an oxygen gradient, $^{*}p \leq 0.001$; $^{\#}p < 0.05$. 25 RA, mutant cell line with increased cholesterol in plasma membrane; M 19, mutant cell line with decreased cholesterol in plasma membrane; CD, methyl-β-cyclodextrin (depletes cholesterol); CHL-CD, cholesterol-β-cyclodextrin complex (increases cholesterol); CPSR-1 is a dilipidated serum; MSB, cells treated with menadione, which increases the rate of oxygen consumption by the cells, potentially increasing the gradient. Reprinted with permission from the American Cancer Society.

plantation can cause temporary local damage. With other oximetric techniques, such as the Eppendorf oxygen electrode or the OxyLite fluorescent probe, there is a similar possibility of local damage, but with those techniques it is not usually feasible to wait for the several days that may be needed to allow the disturbance to subside.

In the experiments in rats the implantations usually are done under anesthesia. One week prior to the measurements of pO_2, the oxygen-sensitive paramagnetic materials, usually LiPc crystals, are placed via a 25-gauge needle directly into the brain at the desired depth through 1.0-mm drilled holes. The material usually is placed about 2.0 mm from the surface of the skull if the cortex is to be the site of measurement. With stereotactic placement it is possible selectively to place the material into other areas as well, and multiple placements can be made at different depths and/or on the side of the brain. For studies with acute perturbations of brain function, such as unilateral selective occlusion of the middle cerebral artery, it is especially useful to have at least bilateral implants. Then one can monitor on the "control" side changes that reflect effects occurring on all of the brain due to changes in blood pressure, intracerebral pressure, *etc.* Such information can be essential for understanding the changes observed on the experimental side.

In order to have meaningful and interpretable results with EPR oximetry, it is essential to maintain good physiological control during the measurements and to monitor appropriate physiological parameters. The latter are essential both for determining that an appropriate physiological state is achieved and to be able to relate the measured changes in tissue pO_2 to key physiological parameters that affect pO_2, such as blood pressure, heart rate, blood gases, and pH. The experimental details for one such study are included in the following discussion of the effects of anesthetics on cerebral pO_2.

Examples of applications of measurements of oxygen at the in vivo *level: Measurement of oxygen in the brain under various anesthetics and during ischemia-reperfusion injury*

These studies illustrate one of the most productive fields of application of EPR *in vivo* oximetry, which also is of direct pertinence to studies of oxidative pathophysiology and oxygen levels in the brain. These studies usually use LiPc because the pO_2 in the brain can be as high as 50 torr under normal conditions, and higher under some circumstances. The LiPc crystals equilibrate with local tissue pO_2 (PtO_2) in less than 30 s, and the response of the linewidth to changes in PtO_2 is stable for at least 30 days in brain. The high density of unpaired spins combined with a narrow intrinsic linewidth of LiPc allows measurements of PtO_2 in the brain using one to four crystals with a total diameter of ~200 μm.

By using EPR oximetry to follow cerebral PtO_2 under various anesthetics and breathing gases, we were able to demonstrate the profound effect that anesthesia can have on oxygen-dependent processes (7). The rats were randomly divided into five groups with five different anesthetics: ketamine/xylazine (100/10 mg/kg, i.m.), pentobarbital (80 mg/kg, i.p.), α-chloralose/urethane (50/1250 mg/kg, i.p.), halothane (1.5%, 1.5 minimal alveolar concentration [MAC]), and isoflurane (2.2%, 1.5 MAC). These doses/concentrations were chosen to produce comparable acute levels of anesthesia, based on the literature.

After an adequate level of anesthesia was achieved, the trachea was intubated using laryngoscopy and an "over-the-needle" 14-gauge catheter, and then positive pressure ventilation was started with continuous monitoring of inspiratory and expiratory pO_2 and partial pressure of CO_2 in arterial circulation ($PaCO_2$) and inhalation anesthetics. A polyethylene arterial catheter (PE-50) was placed in the left femoral artery for continuous monitoring of blood pressure and periodic blood gas measurements. This procedure was accomplished

TABLE 2. BRAIN PO_2 (MM HG) DURING EXPOSURE TO ANESTHETIC AGENTS AT VARIOUS DIFFERENT FiO_2

FiO_2	KT/XYLL	PB	CH/UT	HT	IF
0.21	3.5 ± 0.3	9.0 ± 2.1[k]	13.0 ± 2.9[k]	16.5 ± 0.8[k]	38.0 ± 4.5[k,p,c,h]
0.33	5.6 ± 0.9	13.9 ± 3.3[k]	18.2 ± 1.6[k]	22.6 ± 1.1[k]	44.6 ± 5.1[k,p,c,h,*]
0.50	7.0 ± 1.2[*]	19.5 ± 3.8[k,*,†]	20.9 ± 1.4[k,*]	28.9 ± 2.3[k,*,†]	53.0 ± 3.7[k,p,c,h,*,†]
1.00	8.8 ± 1.7[*]	—	27.5 ± 2.9[k,*,†]	31.8 ± 3.2[k,*,†]	56.3 ± 1.7[k,c,h,*]

Values are given as means ± SEM (n = 5 per group). KT/XYL, ketamine/xylazine; PB, pentobarbital; CH/UT, chloralose/urethane; HT, halothane; IF, isoflurane.

Superscripts indicate significant differences ($p < 0.05$) as compared with other groups: [k]ketamine/xylazine; [p]pentobarbital; [c]chloralose/urethane; [h]halothane. Compared with different FiO_2 in same group: [*]21%, [†]33%.

within 20–30 min after administration of the anesthetics. Rectal temperature was controlled at 37.0 ± 0.5°C via a heated pad. The fraction of inspired oxygen (FiO_2) was maintained at 0.33 while establishing vascular and airway access, and then the animals were allowed to stabilize for 30 min (the FiO_2 was maintained at 0.21), verified by two arterial blood gas analyses. Then the rats were exposed for 30 min each to FiO_2 of 0.33, 0.50, and 1.0. (Rats that received pentobarbital became too lightly anesthetized to get adequate data at a FiO_2 of 1.0.) In all rats, ventilation was controlled to maintain $PaCO_2$ between 35 and 40 mm Hg throughout. Fluid balance was maintained with 1.5 ml/h of saline (i.p.). Gross and microscopic examination (hematoxylin and eosin staining) of the tissue around the implanted LiPc confirmed that crystals were in the cerebral cortex and that there was no significant inflammatory infiltrate or necrosis around the LiPc.

Spectra of LiPc were obtained using an EPR spectrometer constructed in our laboratory with a low-frequency (1.2 GHz, "L-band") microwave bridge. The rat was placed in the magnet, and the head was positioned so that the brain was directly under the extended loop resonator, which was adjusted to obtain the maximum signal from the LiPc in the cerebral cortex. Typical settings for the spectrometer were: incident microwave power, 10 mW; magnetic field center, 425 G; scan range, 1 G; modulation frequency, 27 kHz. Modulation amplitude was set at less than one-third of the EPR linewidth. Scan time was 2 min, and three to five scans were usually averaged to achieve a better signal-to-noise ratio. The EPR linewidths were converted to pO_2 using a calibration curve determined for each batch of crystals.

Mean arterial blood pressure was continuously monitored by a pressure transducer (Biopac Systems, Holliston, MA, U.S.A.). Arterial blood (150 µl) was drawn into a glass capillary, and blood gases (pO_2 in arterial circulation, $PaCO_2$, and pH) were analyzed (Ciba-Corning Diagnostic Corp., Cambridge, MA, U.S.A.) at 30, 60, 90, and 120 min after anesthetic induction.

The results are summarized in Table 2. Note that although the pO_2 in the systemic circulation was quite similar in all of the animals, the pO_2 in the brain varied markedly with the type of anesthetic. Also, even with the animals breathing quite high pO_2, the differences persisted, and for ketamine/xylazine, the values were quite low even at the highest FiO_2. This has clear implications for studies of oxidative damage, where one might anticipate quite different rates of oxygen-dependent reactions and also probably quite different physiological parameters with such marked variations in local pO_2. EPR oximetry was a very effective way to make these measurements because the measurements were made noninvasively (any invasiveness associated with the implants occurred many days before the actual experiments), and could be made continuously over the entire experimental procedure.

The capabilities of EPR oximetry may be especially valuable in following the events in ischemia-reperfusion, providing data that should help to elucidate critical factors in the pathophysiology and the consequences of therapeutic interventions. We have approached this problem by placing oxygen-sensitive paramagnetic materials in the brain in both regions expected to become ischemic due to unilateral occlusion of the middle cerebral artery (MCAO) and in the contralateral regions in the other hemisphere (3). The latter provide direct measurements of changes in the pO_2 of the brain that are due to global factors such as increased intracranial pressure, while the materials in the potentially ischemic region show the direct effects of the intervention. Using LiPc that was placed within the brain several days prior to the MCAO, we then followed the time course of the pO_2 at three sites during a baseline period, during the period of occlusion, during the period of immediate reperfusion, and then again periodically for several days after the reperfusion. The long-term aim is to use this approach to relate changes in pO_2 to the occurrence of permanent and transient damage and to follow the effects of therapeutic interventions on these parameters. Figure 5 summarizes data from six rats that underwent reversible MCAO and had the cortical pO_2 followed until 24 h after the occlusion. The kinetics of the changes in pO_2 are quite complex, illustrating the value of being able to obtain serial measurements without perturbations from the technique used to measure the pO_2.

Simultaneous measurements of oxygen and nitric oxide (NO)

The concentrations of molecular oxygen and the product of its reduction, superoxide, also are closely linked to another important factor in oxidative damage, NO. In addition to potential direct reactions with NO, the amount of oxygen at critical sites is controlled in part through changes in the vascular system, and, as we now know, NO is an important regulator of vascular tone, functioning as a potent vasodilator when produced by endothelial cells lining the blood vessels. NO synthases release NO from the endothelial cells in response to a variety of stimuli that may be affected by oxygen, for example, agonists (such as bradykinin or acetylcholine), shear

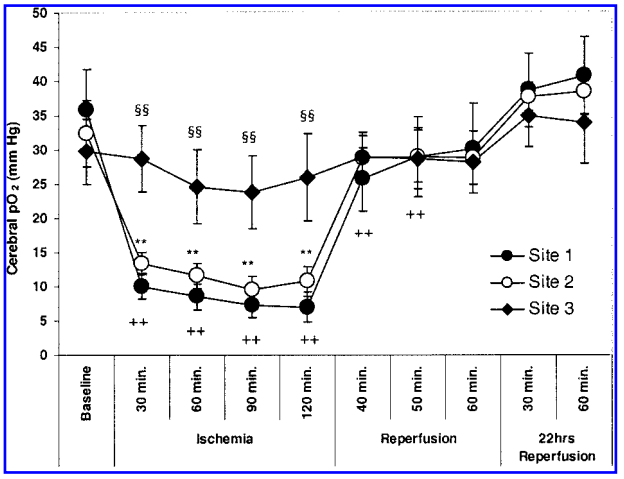

FIG. 5. Time course of mean cerebral pO_2 in three sites before, during, and after MCAO. Data are mean ± SE values ($n = 6$).**, $^{++}p < 0.01$ compared with baseline (paired t test); $^{§§}p < 0.01$ compared with site 1 and site 2 (one-way analysis of variance).

stress, and flow. Oxygen also is a critical co-factor for NO biosynthesis and is required at concentrations above 9 μ*M* for normal NO production (15, 27). Pathological conditions in which oxidative damage may have a role, including heart failure, vascular disease, diabetes, and stroke, also have been linked to defective oxygen supply or vascular dysfunction as a result of decreased NO production or availability (17).

Techniques that independently can monitor either oxygen or NO have already provided novel insights into some disease mechanisms. But it is often desirable (and important) to measure both NO and oxygen and to do this directly from the same site (in cells or in tissue). Dual electrodes have been developed and utilized simultaneously to monitor NO and pO_2, but these systems consume NO (or oxygen), and they are extremely brittle and therefore costly. They also cannot readily be used to follow changes over time in the same animal. The methods using EPR to measure NO are covered elsewhere in this issue. These can often be combined conveniently and effectively with EPR oximetry, as reviewed recently (9).

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ABBREVIATIONS

CHO, Chinese hamster ovary; EPR, electron paramagnetic resonance; FiO_2, fraction of inspired oxygen; LiPc, lithium phthalocyanine; Gd-DTPA, gadolinium diethylenetriaminepentaacetic acid; MAC, minimal alveolar concentration; MCAO, unilateral occlusion of the middle cerebral artery; NO, nitric oxide; $[O_2]$, concentration of oxygen; $PaCO_2$, partial pressure of carbon dioxide in arterial circulation; PDT, perdeuterated Tempone; pO_2, partial pressure of oxygen; PtO_2, partial pressure of oxygen within tissue; Tempone, 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl.

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