In Vivo Detection of Radicals in Biological Reactions

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Introduction

Noninvasive approaches to the detection of free radicals (ROS) are indicated in Table 1. The most sensitive of these approaches is the optical method in which emission light from the tissue, either intrinsically or extrinsically with lucigenin as an amplifier (1). The sensitivities of $10^8$ centers/sec from the exposed surface of an organ are obtained in these studies from the liver. Other studies from the heart (2) and the brain (5 Nioka and DS Smith, personal communication) give less of a signal and are quite difficult to obtain.

The chemiluminescence method is versatile and response speed is good, but it requires tissue exposure because the wave-lengths of emission of the detectable radicals are in the visible and the near red regions. The far red light is not detectable. The approximately 1 μ single oxygen emission has been extremely difficult to detect in tissues and still challenges some investigators. Yet, water droplets entrained in the human breast contain radical species as well, presumably a singlet O_2 dimer of sufficient stability to allow detection after exhalation.

The optical method is very useful for quantitative measurements. The fact that catalase combines with low concentrations of H_2O_2 to give a green Compound I enables determination of the rate of its decomposition, which depends upon the H_2O_2 concentration and upon the added hydrogen donor (i.e., alcohol, formate, etc.). This method is unique because it quantitatively measures an intracellular oxygen stress compound, i.e., H_2O_2. Furthermore, the localization of the catalase in the peroxisomes is well established and thus the site at which H_2O_2 is measured is known. The H_2O_2 in tissue extracts can be equally or better detected by yeast peroxidase that combines with H_2O_2 to form directly a red compound that is detected spectrophotometrically. The sensitivity of the in vivo and in vitro methods is approximately $10^{13}$ molecules, still one of the more sensitive methods for ROS detection.

Electron paramagnetic resonance (EPR) (3) has a high sensitivity and a good response speed and has been cited in this symposium as a detector of NO, which has an unpaired electron. Extrinsic probes for electron spin resonance (ESR) such as carbon center radicals, and numerous spin traps, or indeed free radicals, are sensitive to their environment (i.e., oxygen in case of carbon and TEMPO). The sensitivity is in the region of $10^{13}$ spins.

Two- and three-dimensional imaging by EPR is under way for a small object such as the perfused heart. It is unlikely that the human body will be imaged; nevertheless, the technique may provide quantitation of the localization of radical species in the perfused organ.

Nuclear magnetic resonance (NMR) employs intrinsic probes for phosphorus spectroscopy, such as ATP, pH, PCr/Pi, and energetically related signals that are crucial to the estimates of survivability of the bioenergetic apparatus under hyperoxic ROS stress. ATP deficit in particular gives a measure of the loss of cell function due to bioenergetic disability.

While no extrinsic probes for NMR studies of ROS have been developed yet, deoxyhemoglobin affects the water structure and has been used in some forms of functional imaging. Furthermore, myoglobin itself gives a detectable NMR signal in vivo and can also be localized. No doubt, with the increasing importance of free radicals and free radical studies, radical-sensitive NMR probes based on paramagnetic effects may be developed in the near future. However, sensitivity will be low, approximately $10^{16}$ molecules.

In this article, the authors’ principal interest which in the past has been focused in the lung) has shifted to the liver because of the technical difficulties of experiments in the lung and the probability that most fundamental principles of free radical damage can readily be demonstrated in other organs, particularly the liver. The liver has the great advantage that spectroscopy of catalase allows quantitative assay of cytosolic H_2O_2. Also, transfections may be made on the liver that will afford additional fundamental insight on the effect of ROS on the bioenergetic capability of the liver, and in fact, lead to an improvement in the resistance of the liver to ischemic stress and reperfusion.
Results

The complexities of free radical generation and utilization in the liver are illustrated by Figure 1. The peroxisomal catalase has access to cytosolic H₂O₂ while there is a diffusion gradient between mitochondrial H₂O₂ generation and the peroxisomes. Nevertheless, the location of catalase is known and the concentration of H₂O₂ may be estimated through methanol titration with significant precision.

Quantitation of H₂O₂ Generation in the Perfused Liver and Its Correlation with the Glutathione Redox State

A combination of biochemical and optical probes can be measured and gives a clear picture of ROS stress in hyperoxia. The typical protocol for combined measurement of glutathione, NADH and H₂O₂ is indicated in Figure 2 where radical generation due to high pressure oxygen causes NADH oxidation and, at the same time, glutathione depletion. Before this event, the titration of catalase H₂O₂ takes small amounts of methanol, indicative of low H₂O₂. After the event, the methanol titration is more prolonged, i.e., higher methanol concentrations are required to deplete catalase H₂O₂. Quantitation of H₂O₂ under these conditions gives values in the nanomolar region. Based upon the equilibrium of SOD and its reactant and product, the O₂⁻ concentration is in the range of 10⁻¹² M. Thus, the substances that could compete effectively with SOD for O₂⁻ must have not only a high reaction rate, but must be essentially irreversible. Figure 2 lacks a chemiluminescence trace, but it would be expected to show increased light emission with increasing oxygen stress.

Chemiluminescence Responses

The experimental method for chemiluminescence is illustrated in Figure 3. The perfused or intact organ is placed in an essentially dark chamber and a larger area photomultiplier cathode, which receives chemiluminescent photons from as large a solid angle as possible, is placed within a centimeter of the perfused organ. Under these conditions, the perturbation of the ROS in the intact liver can be readily monitored by single photon counting of chemiluminescence.

Figure 4 illustrates the “titration” of the antioxidant capability of the perfused liver by successive perturbations with t-butyl peroxide. The data clearly show that low levels of t-butyl peroxide are readily withstood by the antioxidant reserves of the liver. As the concentration of t-butyl hydroperoxide and the integrated ROS increases, the chemiluminescence increases. Thus the balance of antioxidant and peroxidant in the perfused liver is readily ascertained.

Accumulation of Neutrophils

The fact that neutrophils may contribute considerably to the ROS in the liver in vivo, where complement activation of neutrophils is expected, is shown in Figure 5 using the perfused liver with luminol as an enhancing agent (4). The liver, previously stressed with a hypoxic/ischemic insult, shows no change of baseline of chemiluminescence over time. The addition of myristic acid as an activator of neutrophil function causes a roughly 12-fold increase of chemiluminescence signal over a significant interval (5).
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In Figure 4, the effect of increasing rates of t-butyl hydroperoxide infusion into perfused rat liver (top) on chemiluminescence is shown. The graph indicates enhanced chemiluminescence following administration.

![Figure 4](image)

**Figure 4.** Effect of increasing rates of t-butyl hydroperoxide infusion into perfused rat liver (top) in chemiluminescence.

In Figure 5, the chemiluminescence of normal isolated perfused rat liver post ischemic insult reflow begins at time zero. The traces show the effect of PMA administration: ■ Control; ● PMA administration.

![Figure 5](image)

**Figure 5.** Luminol-enhanced chemiluminescence of normal isolated perfused rat liver post ischemic insult; reflow begins at time zero. The two traces show the effect of PMA administration: ■ Control; ● PMA administration.

To explore the neutrophil response in more detail, the assay of chemiluminescence by myristic acid-activated chemiluminescence was timed with respect to the low-flow ischemic stress and reperfusion stress that lasted uniformly for 30 min (4). The animal was sacrificed at various times following recovery from ischemic stress as indicated in the diagram and the largest response of the perfused liver was found to be shown 6 hr after the stress (Figure 6).

![Figure 6](image)

**Figure 6.** Free radical chemiluminescence (luminol enhanced) at indicated times after 30 min ischemia in perfused rat liver: ■ Control; ● PMA administration; ▲ 6 hr; ○ 24 hr.

A more detailed time study of the same phenomenon is shown in Figures 7 and 8, which plot the integrated photon output at a number of times after the oxidative stress. This shows a clear and sharp maximum at 6 hr. If we take note of Karnovsky (6), this interval most likely represents the combination of the accumulation of neutrophils and the time window within which their activation may be observed. In fact, this model affords a most convenient assay of radical stress in vivo using a sensitive perfused organ assay and is used in the following evaluation of the therapeutic effect of liver enzyme transfection.

**Oxidative Stress in Ischemia and Reflow**

Figure 9 illustrates the key role of creatine kinase in facilitating energy flow from cytoplasmic work to mitochondrial ATP synthesis. Phosphocreatine affords an extra

![Figure 9](image)

**Figure 9.** The creatine kinase shuttle from cytosol to mitochondrial matrix redox state.
energy reserve that tides the system over periods of energy deficit. Koretsky (7) has afforded a mouse with transfection of creatine kinase into the liver so that feeding the animal with creatine affords 20 to 30 mM phosphocreatine in the cytosol from the accumulated creatine pool and the activity of creatine kinase. The effects of this extra energy pool upon the ability of the mouse to withstand ischemia and reflow are indicated in Figure 10, which gives a series of NMR spectra of the perfused liver from two kinds of mice, wild and transfected. The bottom trace is a control, and on the right side is the wild type where three peaks of ATP and negligible peaks of inorganic phosphate indicate the resting metabolic state of the healthy liver. To the left is the corresponding resting state for the liver of the transfected creatine-fed mouse (creatine kinase). The phosphocreatine to phosphate ratio approximates that of the resting skeletal muscle, i.e., approximately 10. Both specimens are subjected to low-flow ischemia for an interval of 30 min, so that ATP has disappeared from both livers and only inorganic phosphate remains, as indicated by the single peak at approximately 4 ppm from PCr.

In the first 15-min interval following reflow, the wild type shows a double peak of inorganic phosphate and a trace of ATP resynthesis, while the mutant shows a recovery of ATP and PCr almost identical to that of the preischemic state. In the second interval of reflow, the wild type has lost the trace of ATP observed previously with no change of inorganic phosphate, a situation that deteriorates further with time; the phosphate balance in the wild type is seriously deranged. After reflow, the phosphate pool present in ischemia has been released to the perfusate, evidence of leakiness of the cells. On the other hand, the transfected liver continues to maintain PCr and ATP over the interval of the study with only a slight loss of PCr at 60 min. Thus, this simple transfection has made the difference between failure and recovery after ROS stress in mouse liver model.

**Discussion and Summary**

The results presented here suggest that the recovery from ischemia and reperfusion, or indeed the ability to maintain cell integrity, is, as a matter of first principle, a bioenergetic problem. Free radical damage to bioenergetic function of the cells is shown in the hepatic model to be critical to its survival following the reflow phenomenon.

The creatine kinase transfection and creatine feeding affords a significant protection from ROS stress due to ischemia and reperfusion. The beneficial effects are in consonance with preliminary data obtained by creatine feeding of athletes, particularly at Oxford University where improved endurance in athletes has been noted.

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