Chapter 3

Free Radicals and Medicine

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Abstract: EPR has been employed in attempts to understand the basis of specific pathophysiologies in which free radicals have a postulated role. Examples described include pulmonary free radical damage, free radicals and sickle cell disease, free radicals in amyotrophic lateral sclerosis, melanin and free radicals and the potential role of oxidative stress in the induction of cancer. The final section of the chapter describes the use of NMR as the spectroscopic measure of spin-trapped radicals, after they have reacted further to form diamagnetic species.

1. INTRODUCTION

This chapter is a joint effort that aims to provide an illustrative view of the current understanding of the relationship between free radicals and disease. This is a vast subject and while EPR has an important role in its study, the role of EPR is far from dominant. Therefore, instead of trying to provide a comprehensive review of the role of free radicals in medicine, we have chosen to provide an overview through some general remarks, followed by more detailed considerations of several illustrative examples in which EPR has been employed in attempts to understand the basis of specific pathophysiologies in which free radicals have a postulated role. The first author has drafted the overview section and the primary author(s) for each specific section are indicated in those sections. The terms EPR and ESR are used in accordance with the preference of the authors of each section.

1.1 Theoretical Considerations for Expecting Links Between Free Radicals and Medicine

The theoretical basis for expecting free radicals to be related to disease has evolved over the years. Initially the hypothesized links between free radicals and disease were based on loosely reasoned considerations that because free radicals are so reactive, and cells are so well organized, that the occurrence of free radicals *in vivo* would likely lead to disease. This type of reasoning became somewhat more focused on the role of free radicals in cancer (Swartz, 1979). The current status of concepts of the role of free radicals in cancer is illustrated in the section drafted by Gutierrez.

The next phase of theories potentially linking free radicals and disease was based on the specific potential of free radicals to initiate strong oxidizing reactions. The fundamental rationale comes from the fact that living organisms include many highly oxidizable components, yet usually require the presence of oxygen in order to carry out basic metabolic functions. Interest in these aspects became greatly increased with the discovery of the natural occurrence of large amounts of superoxide dismutases in both the blood and cells. Subsequently a role of free radicals in almost every conceivable disease has been proposed on various theoretical grounds related to the potential for uncontrolled oxidations. Such theories have general support by observations that the extent of many pathologies can be modified by changing the availability of superoxide (especially by modifying the activity of superoxide dismutases) or the strongly oxidizing functions of macrophages. But the observations did not provide rigorous proof of causality, and the chemical reactions through which the superoxide caused the observed effects has remained obscure.

Until very recently, it has been assumed that the damage caused by free radicals occurred because of direct reactions with key molecules, damaging sufficient numbers of the key molecules to alter function. This has been puzzling when looked at critically, because when considering the specific free radicals that are expected to be involved, it has been difficult to construct plausible reaction schemes that would account for the observed effects. The hydroxyl radical and closely related species (e.g. the perferyl radical), are so reactive that they must be generated on or immediately adjacent to the target molecules, because their diffusion distance is usually the distance to the next molecule. Therefore it would take very special circumstances for such radicals to be important in a disease. On the other hand, the superoxide radical is so unreactive that it is difficult to understand how it can lead to the very profound effects that clearly are associated with excess or inappropriate production of superoxide.

Because of recent advances in molecular biology, however, it now seems reasonable to expect that within the next few years we will have, finally, an adequate theoretical basis for understanding the roles of free radicals in biology. Then it will be much more feasible to develop methods to control or ameliorate the undesirable processes that they can cause.

The keys to the potential of much more effective progress have been the discoveries that oxidizing species and the amount of oxygen can have profound effects on gene activation and cell signaling. This has, finally, provided a potentially satisfactory mechanism for understanding the association of free radicals (and other oxidizing species) with pathophysiology. It has become clear that there are a large number of activators and modulators of genes and cytokines that respond to the presence of oxidizing species. Currently the understanding of the role of free radicals and other oxidizing species in cell signaling is still at an early stage, but rapid progress is likely as the cascades of effectors that are activated or inactivated by the amount of oxidizing species and/or oxygen are delineated. It seems likely that in many cases the origin of the free radicals will be from inappropriate responses of the naturally occurring defense systems, mediated through cell signaling. The old paradigm of deleterious actions of free radicals occurring through direct damage from the free radicals is likely to be applicable only in exceptional circumstances (e.g. ionizing radiation). Thus the experimental approaches in which roles of free radicals are likely to be important aspects for understanding probably will be in the arena of molecular biology, and the role of EPR in these studies may be critical. This is perhaps even more exciting than the vague postulates of free radicals being "bad" and leading to disease, because the responses of cells to the presence of oxidizing species are probably very fundamental properties of cellular systems. These mechanisms have enabled life to evolve in an environment with an abundant potential for deleterious oxidations, due to an atmosphere that is 20% oxygen. With this understanding, the value of EPR and related techniques to follow free radical reactions is likely to increase, as the techniques are applied in experiments that are more appropriately framed.

It is not surprising then, that recently theories suggesting links between free radicals and pathophysiological, physiological, and therapeutic processes have become more specific, linked to specific intermediates and/or specific cell signaling pathways. These are areas where it seems more likely that important and useful links will be found. EPR is likely to have an important role in elucidating what already has been shown to be a very complex set of processes.

1.2 Conceptual Approaches for EPR Experiments on Links Between Free Radicals and Medicine

Initially investigators sought evidence for the role of free radicals in disease by direct observations with EPR, reasoning that there should be increased amounts of free radicals in the related diseases. In general increases were not found, although in a few cases in studies of cancer some unexpected EPR signals were seen (Swartz, 1986). As noted in the review, more thorough consideration of this approach leads to the conclusion that even if free radicals are involved, there are seldom specific reasons for there to be increased amounts of free radicals that can be detected by EPR. The role of free radicals could be critical but transient. And even if there was a continuing role of free radicals, the more reactive that they are, the less likely it would be that they could be detected directly by conventional EPR spectroscopy. Hence it is not surprising that there has been little concrete evidence for a general link between the amount of free radicals and pathological or physiological processes.

Subsequently EPR has been used more specifically to detect free radical intermediates associated with a wide range of physiological, pathophysiological, and therapeutic processes that are potentially related to medicine. These studies have ranged from serially observing tissues in which tumors are expected to development in models of carcinogenesis, to looking for free radical intermediates in drugs. The more specific the hypothesis and the rationale for anticipating the occurrence of free radicals, the more likely it is that such approaches will be productive. In virtually all situations, it is unlikely that measurements of free radicals will be sufficient to test a hypothesis or reach a firm conclusion pertinent to a disease or its treatment. Appropriate additional studies, including tests of factors that may affect the amount or type of free radicals usually will be needed. The later sections of this chapter provide some illustrative examples of studies in which the role of free radicals in disease have been studied under appropriately rigorous conditions.

1.2.1 Experimental approaches for studying links between free radicals and medicine in experimental animals

A wide and effective range of approaches have been used for studies in animal models relevant to clinical medicine. The available approaches are quite wide because unlike the situation in human subjects, there is much less concern about the long term effects of materials that are administered to carry out the studies and/or the invasiveness of the approach. Recently, as described in much more detail in ch. 9 on *in vivo* studies it has become

possible to carry out studies in intact animals as well as in model systems and cells.

Sometimes it is possible to have direct detection of free radicals, if they occur in high enough concentrations and have sufficient stability. This approach is especially applicable when substantial amounts of a substance are administered, such as with therapeutic drugs or toxins. The occurrence of free radical intermediates of drugs is among the most likely situations where direct observations with EPR are likely to be productive. Sometimes it may be possible to have sufficient amounts of radicals to observe them directly in aqueous systems under physiological conditions, including normal temperatures for the organism (Fujii et al., 1994; Mader et al., 1995). Often this will not provide sufficient sensitivity and rapid freezing will be required. While the use of frozen samples often results in the loss of resolvable structure in the EPR spectrum, the ability to have a much larger sample (because of the much lower dielectric loss in ice, the sample volume can be much larger) and the enhanced sensitivity of lower temperatures usually result in very significant increases in sensitivity versus aqueous samples.

For most studies, however, it is likely that there will be a need to employ spin trapping or other techniques to observe the free radicals. Several different experimental approaches using spin traps have been successfully employed with cells and experimental animals including:

a) *use of model systems (in vitro).* This is the most widely used approach. The concentration of spin traps can be made as high as needed when live organisms are not used. When the studies are carried out in cell systems careful attention has to be paid to effects of the spin traps on the cells (toxicity) and effects of the cells on the spin adducts (stability of the adduct) (Khan et al., 2003).
b) *ex vivo approaches in which the trapping occurs in vivo and samples are obtained for study in vitro.* This approach has been used productively, especially by Mason and colleagues (ch. 5). It involves the administration of the spin trap to the animal and then obtaining samples through biopsies or removal of biological fluids. It can provide excellent evidence for the occurrence of free radical reactions *in vivo,* but considerable care is needed to avoid artifacts or misleading results. The potential problems include loss of spin adducts during the extraction steps and generation of spin adducts during the processing of the samples.
c) *direct spin trapping in vivo with measurements made directly in the organism under physiological conditions.* In principle this is a very desirable approach, providing direct evidence without intervening processing steps and, also, the potential to provide kinetic data under physiological conditions. This approach, however, has lower sensitivity than *ex vivo* methods, because the latter can study the samples at X-band

or even higher frequencies, while direct *in vivo* studies require the use of L-band or even lower frequencies, with the consequent power sensitivity of such frequencies. Both *ex vivo* and direct *in vivo* approaches share the problem of potential instability of the spin adducts, which often are converted to non-paramagnetic products in the presence of functioning cells (Khan and Swartz, 2002; Timmins et al., 1999).

d) *spin trapping using combined NMR and EPR.* This is a new approach whose utility has not been fully evaluated. In principle it provides a means to use the widespread availability of *in vivo* NMR, and that is very attractive. The section by Berliner in this chapter provides an excellent summary of this approach.

e) *indirect assays based on reactions of free radicals with nitroxides or other paramagnetic labels.* This has been especially advocated by Utsumi's group, using the rate of disappearance of nitroxides. While in principle this could be a very productive approach, there are some potential fundamental limitations because of the non-specificity of the observed parameter.

1.3 Experimental Approaches for Studying Links Between Free Radicals and Medicine in Human Subjects

In the near future it may be possible to develop techniques for studies in human subjects. The potential limiting factors for such studies include the technical problems of carrying out EPR measurements in human subjects and, for techniques that involve the administration of spin traps or other substances, the complex and difficult process for obtaining permission to administer substances to human subjects (Swartz, 2003). These also are discussed in some detail in ch. 9. Here we will briefly summarize those aspects that are especially pertinent to measurements of free radicals in human subjects.

The technical problems for using EPR in human subjects revolve around accommodating the human body in the system and obtaining sufficient sensitivity. The geometric constraints can readily be overcome and at least one specifically designed whole body clinical EPR spectrometer already is in operation. This is at Dartmouth where a conventional but large-sized permanent magnet has been specifically built by Sumitomo Special Metals for the facility. It provides a 400 gauss field with a gap that can accommodate human subjects on a cart, or sitting or standing within the gap of the magnet.

For studies in which the direct observable is a free radical form of a drug, etc., the only potential limitations are the sensitivity that can be achieved and

the pertinence of the radical to the processes under investigation. Unfortunately, there appears to be a very limited number of drugs or toxins that generate sufficient amounts of free radicals to be observed directly.

All other approaches, e.g. spin traps, interactions with nitroxides, require the administration of a compound. This adds the requirement that the administered compound has been demonstrated to be safe for use in humans. This is potentially a very limiting aspect, because of the time and expense involved in obtaining the data for achieving such clearance.

There are a few drugs that may be of use that already have been cleared for use in human subjects. In particular, one of the usual EPR responsive trapping agents for nitric oxide, the dithiocarbamates, have been approved for use in humans as chelators of potentially toxic metals and as the alcohol avoidance agent ("antabuse").

There are some possibilities for the approval of some spin trapping agents and nitroxides as the result of initiatives to use them as therapeutic agents. The nitrone spin traps are being evaluated for use in humans because they appear to have potential therapeutic benefits, probably through their effects on the induction of nitric oxide synthesizing enzymes. The nitroxides have been put forth as potential therapeutic agents for the dismutation of superoxide and/or as protective gents against damage by ionizing radiation. If these initiatives result in the approval of nitrones or nitroxides for use in humans, then it would be much more feasible to obtaining permission to use them as spin trapping agents in humans.

2. PULMONARY FREE RADICAL DAMAGE (R. MASON)

2.1 Chemical Toxicants

2.1.1 Paraquat

The herbicide paraquat serves as a model for investigating free radical-mediated pulmonary toxicity because it has no known metabolism other than the free radical metabolism. In microsomal systems, the enzymatic reduction of paraquat to its cation radical is catalyzed by the flavoenzyme NADPH-cytochrome P-450 reductase. The paraquat radical is stable in the absence of oxygen. In the presence of oxygen, paraquat is re-formed, and superoxide is generated in a catalytic fashion with no net change occurring to the paraquat molecule. This process has been termed futile metabolism (Mason 1979, 1982). The mechanism of paraquat poisoning in man is a superoxide-mediated toxicity that is completely analogous to the herbicide mode of

action. The lung is the site of injury by paraquat because it accumulates there (Rose and Smith, 1977). The energy-dependent uptake of paraquat and the subsequent free radical formation are cell-specific. Paraquat free radical formation occurs with clara cells and alveolar type 2 cells, but not with alveolar macrophages (Horton et al., 1986). Diquat, morfamquat, and other bipyridylium compounds do not affect the lung as seriously, but these compounds do cause liver damage and are reduced by rat hepatocytes to their respective radical cations (DeGray et al., 1991).

2.1.2 Nitrofurantoin

In early investigations of the mechanism of rat hepatic mitochondria! and microsomal nitroreductase (Mason and Holtzman, 1975 a), ESR and kinetic evidence demonstrated that the first step in these nitroreductase reactions is the transfer of a single electron to nitro compounds to give the corresponding nitro anion free radical. For instance, in the case of nitrofurantoin, the interaction of the free electron with the nitrogens and protons gives a complex hyperfine pattern that has been analyzed to demonstrate that the free radical is simply nitrofurantoin plus an extra electron (Rao et al., 1987, 1988a). Nitrofurantoin increased the NADPH-supported oxygen consumption by pulmonary microsomes sevenfold over the basal rate, and this stimulation was partially reversed by superoxide dismutase. The presence of superoxide anion radical strongly suggested reductase under aerobic conditions (Mason and Holtzman, 1975b). As expected, the disproportionation of hydrogen peroxide by catalase also decreased the nitrofurantoin-stimulated oxygen uptake.

The effect of superoxide dismutase and catalase on the nitrofurantoin-stimulated oxygen consumption by microsomes is consistent with the formation of nitroaromatic anion radicals under aerobic conditions and with the rapid air oxidation of these radical intermediates, which results in the catalytic generation of superoxide and the well-known oxygen inhibition of nitroreductases. The nitrofurantoin-catalyzed reduction of oxygen to superoxide and the hydrogen peroxide that forms from this superoxide may be responsible for some of the toxic manifestations that occur during nitrofurantoin therapy (Mason and Holtzman, 1975b). For instance, the occasional cases of pulmonary edema and fibrosis caused by nitrofurantoin therapy are similar to the effects of paraquat poisoning. Subsequent work with animal models supported this proposal (Peterson et al., 1982; Boyd et al., 1979). Boyd and coworkers (1979) have shown that the acute toxicity of nitrofurantoin is markedly increased by vitamin E deficiency, an oxygen-enriched atmosphere, or a diet high in highly polyunsaturated fats. Further studies by Peterson and colleagues (1982) showed that decreasing the

activity of selenium-dependent glutathinone peroxidase in 8-day-old chicks enhanced the acute toxicity of nitrofurantoin.

2.1.3 Smoke-Mediated Free Radicals

Both cigarette smoke and the smoke from burning buildings form free radicals even after combustion stops. Both the *in vitro* effects of this smoke and the use of ESR in these studies have been extensively reviewed (Pryor 1992). Smoke inhalation by rabbits formed unidentified PBN radical adducts in plasma (Yamaguchi et al., 1992; Murphy et al., 1991). Cotton smoke formed radical adduct concentrations similar to those caused by 2.5 atm of oxygen (Yamaguchi et al., 1992). Formation of a burst of PBN radical adducts by a brief exposure to cigarette smoke required pretreatment by bacterial endotoxin (Murphy et al., 1991).

2.1.4 Ozone

The spin trapping technique detected free radicals produced *in vivo* by ozone exposure (Kennedy et al., 1992). When rats were exposed for 2 hr to either 0, 0.5, 1.0, 1.5, or 2.0 ppm ozone with 8% CO_2 to increase their respiratory rate, a six-line 4-POBN/radical spin adduct signal was detected by ESR in lipid extracts from lungs of rats treated with 4-POBN. Only a weak signal was observed from rats exposed to 0 ppm ozone (air with CO_2 only). A correlation was observed between the radical adduct concentration and the lung weight/body weight ratio, an index of lung damage. These results demonstrate that ozone induces the production of free radicals in rat lungs during inhalation exposure and that radical production may be involved in the induction of pulmonary toxicity by ozone.

2.1.5 Asbestos

It has been postulated that the *in vivo* toxicity of asbestos results from its catalysis of free radical generation. We examined *in vivo* radical production using ESR and the spin trap 4-POBN; 180 day-old rats were intratracheally instilled with either 500 μg crocidolite asbestos or saline. Twenty-four hours later, histologic examination revealed a neutrophilic inflammatory response. ESR spectroscopy of the chloroform extract from lungs exposed to asbestos gave a spectrum consistent with a carbon-centered radical adduct, while those spectra from lungs instilled with saline revealed a much weaker signal. This same radical formation persisted and, even one month after instillation, could be detected in the lungs of rats exposed to asbestos.

2.1.6 Oil Fly Ash Particles

Exposure to air pollution particles can be associated with increased human morbidity and mortality. Lung exposure to oil fly ash (an emission source air pollution particle) causes *in vivo* free radical production as evidenced by ESR analysis of chloroform extract from lungs of animals exposed to oil fly ash, which gave a spectrum consistent with a carbon-centered radical adduct (Kadiiska et al., 1997). This signal was also reproduced by instilling animals with the soluble fraction of the oil fly ash, which contains soluble metal compounds. The same signal was observed after instillation of either a mixture of vanadium, nickel, and iron sulfates or $VOSO_4$ alone, metals which are prevalent in oil fly ash. Therefore, this generation of free radicals appears to be associated with the soluble metals in the oil fly ash.

2.2 Biological Toxins

2.2.1 Lipopolysaccharide

Intratracheal instillation of lipopolysaccharides (LPS) activates alveolar macrophages and infiltration of neutrophils, causing lung injury/acute respiratory distress syndrome. Free radicals are a special focus as the final causative molecules in the pathogenesis of lung injury caused by LPS. *In vivo* free radical production by rats was detected after intratracheal instillation of LPS. ESR spectroscopy of lipid extract from lungs exposed to LPS for 6 h gave a spectrum consistent with that of a POBN/carbon-centered radical adduct tentatively assigned as a product of lipid peroxidation. To further investigate the mechanism of LPS-initiated free radical generation, rats were pretreated with the phagocytic toxicant $GdCl_3$, which significantly decreased the production of radical adducts with a corresponding decrease in neutrophil infiltration. NADPH oxidase knockout mice completely blocked phagocyte-mediated, ESR-detectable radical production in this model of acute lung injury, demonstrating that superoxide formation by NADPH oxidase is the root cause of this free radical formation.

3. FREE RADICALS AND SICKLE CELL DISEASE (N. HOGG)

There has been a long association between sickle-cell disease and the formation of oxidants from the partial reduction of molecular oxygen. In general this association has focused on either the deposition of redox active

iron as a mediator of biomolecule oxidation, or on accelerated autoxidation of HbS as opposed to HbA, leading to enhanced superoxide formation. In both cases increased oxidative stress in sickle cell disease results in the oxidation of biological macromolecules which contributes to the pathogenesis of the disease (Hebbel et al., 1982; Klings et al., 2001; Natta et al., 1992; Repka and Hebbel, 1991; Schacter, 1986; Sheng et al., 1998) of free radicals and oxidants in the control of vascular function (as opposed to oxidative damage).

Endothelial dysfunction, which is most often indicated by loss of endothelial-dependent relaxation, can be regarded as a disease of altered free radical balance. For example the scavenging of nitric oxide by superoxide has long been thought to be contributing factor to hypertension and it is becoming apparent that this same mechanism may be responsible for altered endothelial signaling in atherosclerosis, diabetes, and other cardiovascular pathologies, and may also be a control mechanism of normal cardiovascular responses such as the modulating effects of shear stress on vessel relaxation. Recently two nitric oxide scavengers have been identified in the lumen of patients with sickle cell disease.

The first of these, xanthine oxidase, has long been understood to be a physiological source of superoxide, however, the recently discovered elevation of vessel wall xanthine oxdase may be an important component of endothelial dysfunction in sickle cell disease. Aslan et al. (2001) have demonstrated that liver injury in both sickle-cell patients and a mouse sickle cell model can result in the release of xanthine oxidase into the blood stream. Accumulation and/or uptake of this enzyme by the vascular endothelium can lead to enhanced superoxide production and impairment of endothelium-dependent relaxation.

Another molecule that has only recently been implicated as a nitric oxide scavenger in sickle cell disease is plasma hemoglobin. A major paradox of nitric oxide function has been the presence of high (10 mM) concentrations of hemoglobin in the vascular lumen. Calculations have clearly shown that this level of hemoglobin should preclude nitric oxide radical from having any appreciable steady-state concentration in the vessel wall. This paradox has been recently addressed by the discovery that compartmentalized hemoglobin reacts with nitric oxide at significantly slower rates (up to 1000 times) than cell-free hemoglobin. The effects of increased levels of cell-free hemoglobin on vascular function have only recently been realized. As recently demonstrated by Reiter et al. (2002), the levels of plasma cell free hemoglobin are substantially elevated in patients with sickle cell disease, averaging 4 μM and as high as 20 μM. The hemoglobin appears to be in the ferrous form as the plasma from sickle cell patients exhibits a greater ability to scavenged nitric oxide. Measurements of blood flow in the forearm

indicate that plasma hemoglobin can severely limit nitric oxide-dependent vasodilation. An important corollary of these studies is that pharmacologically administered nitric oxide (for example by inhalation) can act to 'scavenge the scavenger' by oxidizing plasma hemoglobin to its met or ferric (Fe^{III}) derivative. Ferric hemoglobin then has limited ability to scavenge peripheral nitric oxide, thus potentially restoring NO bioavailablility. The level of methemoglobin in plasma (as demonstrated by electron paramagnetic resonance spectroscopy) is increased when sickle-cell patients inhale nitric oxide, together with the level of nitrosylhemoglobin (Reiter et al., 2002). Cell-free hemoglobin circulating through the pulmonary circulation is a much more amenable target for nitric oxide therapy than guanylyl cyclase and it may be the case that the effects of nitric oxide inhalation in other clinical conditions are at least in part mediated through plasma hemoglobin oxidation. It is interesting to speculate that the ability of hydroxyurea, recently identified as an NO congener, to relieve sickle cell disease may also be at least in part related to its ability to oxidize hemoglobin in the extracellular compartment (Glover et al., 1999; Huang et al., 2002; Jiang et al., 1997).

4. FREE RADICALS IN MOTOR NEURON DISEASE OR AMYOTROPHIC LATERAL SCLEROSIS (ALS) (B. KALYANARAMAN)

4.1 Motor Neuron Disease

Motor neuron disease or ALS (also known as Lou Gehrig's disease) is a fatal illness that progressively causes the degeneration of the nerve cells that control the muscles, paralyzing the body but sparing the mind (Price et al., 1994). Approximately 10% of ALS cases are familial with the rest being sporadic. The genetic defect in familial ALS (FALS) has now been linked to *Sod 1,* the gene encoding the cytosolic Cu, Zn, superoxide dismutase (SOD1) (Brown, 1995, 1998). At present, approximately 70 or so missense FALS mutations in *Sod 1* at 26 different amino acid positions have been identified. The mechanisms by which FALS-linked *Sod 1* mutants cause selective degeneration of motor neurons of the spinal cord, brain stem, and cerebral cortex remain unclear (Brown, 1995, 1998). Of the various mechanisms proposed, the hypothesis suggesting that the FALS-linked mutants acquired a cytotoxic "gain-in-function" has found increased acceptance (Brown, 1998). The "gain-in-function" of *Sod 1* mutants had been postulated to arise from an increased peroxidase or hydroxyl radical activity. The EPR technique has played a key role in identifying the

structure of the oxidant formed from the peroxidase activity of SOD 1 (Singh et al., 1998).

4.2 Bicarbonate-dependent Increased Peroxidase Activity -- Role of Carbonate Anion Radical

Nearly 30 years ago, Hodgson and Fridovich demonstrated that the copper-bound hydroxyl radical ($SOD\text{-}Cu_2^{+}\text{-}^{\bullet}OH$), a putative oxidant generated in the reaction between SOD1 and H_2O_2, could oxidize several anionic ligands (e. g., formate, azide, and nitrite anion) (Hodgson and Fridovich, 1975). In addition to these anions, several other molecules (which are too bulky to find access to the active site of Cu, Zn and SOD) were also oxidized. These experiments were performed in a bicarbonate buffer.

The x-ray crystal structure of SOD1 indicates that access to the active site is via a narrow channel that restricts the entry of large molecules. However, a relatively small anion like HCO_3^- could reach the active site of SOD1 and undergo oxidation to the carbonate anion radical ($CO_3^{\bullet-}$) by $SOD\text{-}Cu^{2+}\text{-}^{\bullet}OH$. $CO_3^{\bullet-}$ is a diffusible oxidant that could leave the active site and cause oxidation of various substrates in free solution. Bicarbonate thus effectively exports oxidation from the sterically hindered active site to large molecules in bulk solution (Liochev and Fridovich, 1999). This model which gives a new perspective on the peroxidative mechanism of SOD1, is pivotal for explaining the published discrepancies in spin trapping reports using SOD1 and FALS SOD1 mutants (Goss et al., 1999; Zhang et al., 2002).

4.3 Reaction between Nitrones and Carbonate Anion Radical Causes Hydroxylation

Evidence for increased peroxidase activity from FALS SOD1 mutants (e. g., A4V with alanine substituted by valine at amino acid 4, G93A with glycine replaced by alanine at amino acid 93) was first obtained by monitoring the oxidation of nitrone spin trap 5, 5'-dimethyl-1-pyrroline N-oxide (DMPO) to its hydroxylated adduct (DMPO-OH) (Wideau-Pazos et al., 1996). The EPR spectrum of DMPO-OH is characterized by an equivalent hyperfine interaction (=15 G) from the nitroxide nitrogen and the β-hydrogen atom. Investigators attributed the formation of DMPO-OH to trapping of hydroxyl radical (see Zhang et al., 2000 for references). Significant differences in DMPO-OH signal intensity were observed between the wild type SOD1 and FALS mutant SODs. However, the fact

that bicarbonate was needed for DMPO-OH formation in this system has been previously ignored.

We reinvestigated the mechanism of formation of DMPO-OH using oxygen-17 labeled water and hydrogen peroxide. Results from these experiments unambiguously demonstrated that nearly all of the oxygen in DMPO-OH originated from water. We proposed that either a nucleophilic addition of ^{17}O-labeled H_2O to the DMPO-carbonate radical intermediate or an addition of $[^{17}O]$-H_2O to the DMPO cation radical intermediate.

Independent evidence for the intermediacy of $CO_3^{\cdot}$ was obtained from photolysis studies using the pentammine carbonate complex of Co (III). The UV photolysis of this cobalt complex has been shown to generate the authentic $CO_3^{\cdot}$ radical. This system yielded the same type of radical adducts in the presence of substrates (e. g., azide, ethanol, and formate) as detected in the $SOD1/H_2O_2/HCO_3^-$ system (Zhang et al., 2000, 2002).

Bicarbonate-dependent peroxidase activity of SOD1 was found to be responsible for hydroxylation and oxidation of azulenyl nitrone to an aldehyde. Aldehyde formation was attributed to trapping of hydroxyl radicals by the azulenyl nitrone (Gurney et al., 1998). Recently, we showed that in the absence of bicarbonate, oxidation of azulenyl nitrone to azulenyl aldehyde was negligible in phosphate buffers containing SOD1, H_2O_2, and the chelator DTPA (Zhang et al., 2002). Photolysis of solutions containing the cobalt complex that generates the carbonate radical anion and azulenyl nitrone yielded azulenyl aldehyde. Based on these results, we proposed that $CO_3^{\cdot}$ is responsible for $SOD1/H_2O_2/HCO_3^-$ mediated oxidation of azulenyl nitrone. Bicarbonate-dependent SOD1 peroxidase activity is, thus, inhibitable by nitrones. Bicarbonate-dependent SOD1 peroxidase activity was shown to be higher in FALS mutants (Brown, 1995, 1998).

4.4 Nitrones as Therapeutic Drugs in ALS Animal Models

The first *in vivo* spin trapping evidence for increased free radical formation was provided using the SOD1-G93A transgenic mouse model for FALS (Gurney et al., 1998). The investigators found that when azulenyl nitrone was administered to the nontransgenic, wild-type transgenic, and mutant transgenic mice of different ages (30, 60, and 90 days), increased levels of azulenyl aldehyde were detected in spinal cord extracts of mutant mice but not in transgenic wild-type and nontransgenic mice. Concomitantly, azulenyl nitrone treatment prolonged the survival of FALS overexpressing mice.

More recently, it was shown that treatment of mutant G93A-SOD1 transgenic mice with nitrone trap DMPO significantly delayed paralysis and

prolonged survival (Li et al., 2002). However, DMPO-derived products were not analyzed. In conclusion, *in vitro* and *in vivo* experiments suggest that nitrone spin traps can potentially mitigate oxidative stress in FALS mutant overexpressing cells and mice and protect against progressive motor neuron death.

5. MELANIN, FREE RADICALS, AND PATHOPHYSIOLOGY (T. SARNA, P. PLONKA AND M. ZAREB)

Melanins are virtually the only naturally occurring stable free radicals in mammals. EPR spectroscopy can be used as a unique non-destructive tool for studying melanin in *in vitro* in cultivated cells (Pilas and Sarna, 1985; Cieszka et al., 1995; Hill et al., 1997), in isolated tissues and organs *ex vivo* (Enochs et al., 1993a; Slominski et al., 1994; Plonka et al., 2002), and even under *in vivo* conditions (Lukiewicz and Sarna, 1972; Katsuda et al., 1990). The putative function of the pigment is protection against light-induced damage, perhaps mediated by free-radical damage (Woods et al., 1999).

The interactions of melanin probably involve both radical and non-radical aspects of the quinone-hydroquinone equilibria that underlie many of its properties (Sarna and Swartz, 1993). Because of their redox activity and ability to bind charged materials, including metal ions and drugs, melanins potentially can affect many physiological, pathophysiological, and therapeutic processes (Enochs et al., 1994; Larsson, 1993; Mars and Larsson, 1999). In addition, due to the high reactivity of some of the intermediates, melanin synthesis has also been linked to cytotoxicity (Halaban and Lerner, 1977; Pawelek and Lerner, 1978).

Because of these complex and diverse properties, the study of melanins with EPR has been a large and productive field that has pertinence to the subject of this chapter: free radicals and medicine. As an example of such studies, we consider here two aspects in which the ESR Center at the Medical College of Wisconsin has been especially involved, ocular melanins and neuromelanins.

Specialized cells that synthesize melanin in humans are found not only in the skin epidermis and in the hair follicles, they are also present in the eye, ear and in restricted regions of the brain (Boissy, 1998). Although populations of these extracutaneous melanin-synthesizing cells have some morphological and functional similarities to the various cutaneous melanocytes, it is expected that significant differences between extracutaneous and cutaneous melanocytes should exist (Boissy, 1998). Melanin synthesis usually occurs in specific organelles, the melanosomes,

and is controlled by specialized enzymes (Orlow, 1998; Boissy, 1998; Pawelek and Chakraborty, 1998), but the formation of neuromelanin (NM) in dopaminergic and noradrenergic neurons of the substantia nigra (SN) and locus coeruleus (LC), appears to be enzyme-independent and the neuromelanin granules show significant similarities to inactivated lysosomes (Barden and Brizzee, 1987).

5.1 Ocular Melanin

In primates, two different cell types are involved in ocular melanogenesis – melanocytes of the uveal tract and neuroepithelial cells of the retinal pigment epithelium (RPE). All true melanocytes, including ocular melanocytes, originate from the neural crest; in contrast, the RPE cells are derived from neuroepithelial cells of the developing forebrain of the embryo (Noden, 1991; Mann, 1964). Both types of the melanin-synthesizing cells of the eye express melanocyte-specific proteins (Boissy, 1998).

Allthough biological functions of ocular melanin have not been unambiguously determined, a growing body of experimental evidence and epidemiological data suggest that both the iridial (uveal) and RPE melanin may have important photoprotective and antioxidant properties (Sarna, 1992; Sarna and Różanowska, 1994). Clearly, ocular pigmentation, particularly in the uveal tract, protects the retina from overexposure by absorbing and scattering the impinging light. Melanin in the choroid and RPE contributes to visual acuity by preventing light reflection from the fundus. It has long been recognized that the severity of the visual abnormalities in albinism correlates with the degree of hypopigmentation (Kinnear et al., 1985). However, even with corrective tinted lenses that help photophobia and, in a minor way, vision by increasing contrast, the results in albinos are rather disappointing (Taylor, 1978). It has been postulated that the albino visual system may represent a case of arrested development (Wilson et al., 1988). Thus, melanin synthesis in the RPE appears to be of crucial importance for normal development of the visual system of an organism, and the presence of RPE is not only responsible for the maintenance of the neural retina but also for its successful embronic development and organization (Boissy et al., 1993; Raymond and Jackson, 1995).

Age-related macular degeneration (AMD) is the major cause of late onset blindness in developed countries (Evans and Wormald, 1996; Klein et al., 1992). There is currently no established etiology that could serve as a basis for preventive medicine and no cure for this disease. Light can damage photoreceptor cells in the mammalian retina (Organisciak and Sarna, 2001; Rapp, 1995; Ham et al., 1980) and population-based studies indicate an association between advanced AMD and a patients' exposure to blue or

visible light over the preceding 20 years (Cruickshanks et al., 1993; McCarty and Taylor, 1999; Taylor et al., 1992). The primary lesion in AMD may occur in the RPE (Zarbin, 1998) and result from oxidative damage (Beatty et al., 2000; Cai et al., 2000).

Due to the close structural and functional association between the RPE and photoreceptors, it is generally agreed that any oxidative damage to the RPE, which causes RPE dysfunction, could contribute to the photoreceptor degeneration that characterizes AMD. Thus, the antioxidant status of the RPE may be a key factor in determining the tissue's health, and the health of the photoreceptors that the RPE supports. An RPE antioxidant that may be particularly relevant when photic stress is implicated in tissue damage is the light-absorbing pigment melanin (Sarna, 1992). It has been postulated that RPE melanin can protect the RPE from oxidative stress by sequestering redox active metal ions, quenching electronically excited states of certain photosensitizing dye molecules and by scavenging reactive free radicals (Sarna et al., 1998; Rozanowska et al., 1999). Significantly, melanin content of the human eye seems to be inversely correlated with the incidence of AMD (Weiter et al., 1985; Young, 1988), and the risk of suffering from AMD is about 40 times higher in whites than in blacks (Pauleikhoff and Holz, 1996), even though the inter-racial differences in the amount of ocular melanin mainly concern the uveal pigmentation. However, it is important to realize that antioxidant abilities of melanin may change with age. Indeed, a recent EPR study has shown that the amount of RPE melanin decreases by a factor of 2.5 between the first and ninth decade of life (Sarna et al., 2002). In an independent study, the melanin content of RPE melanosomes from human donors of different age was analyzed by EPR after drying the pigment granules (Bilinska et al., 2002). Surprisingly, the content of melanin, when normalized to a single pigment granule, was found independent of age. On the other hand, RPE melanosomes from younger individuals could contain significantly higher percentage of strongly bound water, that those from older individuals (Bilinska et al., 2002). RPE melanosomes from older human donors exhibit increased photoreactivity: they induce faster oxygen photouptake and accumulation of superoxide anion spin adducts (Rozanowska et al., 2002). Thus with age, RPE melanin may contribute to oxidative stress in the outer retina both as a result of a decreased antioxidant efficiency and an increased aerobic photoreativity. Interestingly, W-band EPR spectroscopy may provide a sensitive measure for differentiating RPE melanin from human donors of different age and monitoring possible chemical changes of the melanin that are likely to be aggravated with aging (Rozanowska et al., 1993). In this preliminary study a striking pattern in the changes of magnetic parameters of melanin radicals with age was observed. Although the molecular nature of these changes is

not yet understood, they may result from life-long oxidative modifications of the RPE melanin.

Neuromelanin (NM) is an insoluble brown-black or greyish in appearance pigment that accumulates with age in the midbrain of primates, most notably of humans (Van Woert et al., 1966). Based on standard histochemical tests and various physicochemical analysis, including EPR spectroscopy, NM has been classified as a true melanin (Barden and Brizzee, 1987; Enochs et al., 1993b). Characteristic degradation products of NM indicate that this melanin has chemical properties similar to both eumelanins and pheomelanins (Odh et al., 1994). It appears that the synthesis of NM occurs via oxidative polymerization of dopamine and its sulfur containing derivatives. As a synthetic model of NM, a polymer obtained by autooxidation of dopamine and cysteine is often used (Shima et al., 1997). Biological functions of NM remain unknown. Unlike cutaneous and ocular melanins, neuromelanin is never exposed to environmental light. Therefore, no photoprotective role of NM is likely.

It has been postulated that under normal physiological conditions NM may have a cytoprotective function by the sequestration of redox-active metal ions (Swartz et al., 1992; Zareba et al., 1995; Korytowski et al., 1995). On the other hand, the presence of NM may also be responsible for the vulnerability of pigmented neurons observed in Parkinson's disease (PD). This neurological disorder is characterized clinically by akinesia, rigidity, and tremor (Stern, 1990). Histological analysis of Parkinsonian brains shows extensive degeneration of the pigmented dopaminergic neurons of the substantia nigra. In PD, the neuromelanin containing cells of the SN appear to be more vulnerable to degeneration than the non-pigmented neurons. However, there seems to be no strict correlation between the vulnerability of the neurons and their melanin content (Kastner et al., 1992).

The formation of NM in the human brain, probably is a by-product of oxidative metabolism of dopamine, known to occur at high rate in catecholaminergic neurons of the SN and LC. While both autooxidative reactions and enzymatic oxidation of dopamine are accompanied by the generation of hydrogen peroxide, the former process can also lead to the formation of potentially cytotoxic semiquinones and quinones (Cohen, 1983; 1989; Graham 1984). Therefore, the dopaminergic neurons of the SN and LC are intrinsically at risk of oxidative stress. Despite the presence of antioxidative mechanisms in catecholaminergic neurons for maintaining their neurotransmitters in a reduced state, the system is not perfect and some semiquinones and quinones may escape from reduction and randomly form products that cannot be catabolized and gradually accumulate in lysosomes, eventually becoming neuromelanin granules (Enochs et al., 1994). It can be argued that when formed, NM can exert its antioxidant action by binding of

advantitious multivalent metal ions, such as iron and copper (Swartz et al., 1992). There is evidence that SN neurons in patients with PD exhibit an elevated level of oxidative stress (Jenner, 1991). Thus the ratio of reduced to oxidized glutathione is decreased in Parkinsonian brains, compared to normal brains (Riederer et al., 1989; Sofic et al., 1992).

In addition, a decreased level of polyunsaturated fatty acids and increased levels of malondialdehyde, a product of lipid peroxidation, were found in PD brains (Dexter et al., 1989). Whether these changes are the cause of the disease or they result from an increased dopamine turnover in the remaining neurons of a PD patient, remains unclear.

One of the most important properties of NM that may determine its biological functions is the ability of NM to bind metal ions (Enochs et al., 1993b). High levels of metal ions, particularly iron; were found in the human SN (Zecca and Swartz, 1993; Gerlach et al., 1995; Shima et al., 1997). Although ferritin is known to be responsible for accumulation of iron in many tissues, it is believed that in SN iron is bound to NM (Jellinger et al., 1992; Zecca and Swartz, 1993; Gerlach et al., 1995). Indeed, recent measurements suggest that ferritin is not present in SN neurons (Moos, 2000), which implies that neuromelanin may be a key buffering system for iron in pigmented neurons (Zecca et al., 2002).

The antioxidant efficiency of neuromelanin was studied in model systems. The study has clearly shown that the yield of hydroxyl radicals generated via iron ion-catalyzed free radical decomposition of hydrogen peroxide dramatically decreased in the presence of synthetic NM (Zareba et al., 1995). Both synthetic and natural NMs were able to inhibit peroxidation of lipids induced by iron/ascorbate or thermolabile azo-compounds (Korytowski et al., 1995). A hypothesis, about the role of NM in the multifactioral etiology of PD was proposed by Enochs et al. (1994): oxidative degradation of NM that can occur with aging or as a result of an intense or chronic oxidative stress in the pigmented neurons of SN could, via positive feed-back mechanism, further increase the level of the oxidative stress. Oxidatively modified NM is expected to have a decreased binding affinity for iron and other metal ions. It would, therefore, not only bind fewer metal ions compared to “native” NM, but even raise the cytoplasmic level of potentially cytotoxic species by release of the accumulated ones.

6. FREE RADICALS AND CANCER- POTENTIAL ROLES OF OXIDATIVE STRESS IN THE INDUCTION OF CANCER (P. L. GUTIERREZ)

The genetic basis of human cancer has been well documented. Widespread genomic instability is a hallmark of tumor cells (Jeong, 2003; Nowell, 1976). An average of about 1/3 of all tumor types appear to exhibit a genomic instability manifested by subtle nucleotide sequence alternations that activate proto-oncogenes or inactivate tumor suppressor genes. Chromosomal rearrangements are likely to be a result of aberrant recombination repair that occurs during normal cellular mitotic growth (Przybytkowski, 2003; Friedberg, 1985; Friedberg et al., 1995; Kucherlapati and Smith, 1988). A very large number of genes has been identified as tumor promoter genes or tumor suppressor genes (Cheng, 2003; Stanbridge, 1990; Weiberg, 1989). As far back as 1993, genetic analysis of tumor cells has suggested that between 6 to 12 of these genes are altered during the development of a tumor (Renan, 1993). The number of altered genes far exceeds the number of mutations that would be predicted by the spontaneous mutation rate in human cells. Of the several theories to account for this discrepancy, the "mutator hypothesis" for tumorigenesis has gained much attention (Loeb, 1991, 1994). In this hypothesis, an early spontaneous mutation in one of many genes that maintain the genetic information in chromosomes becomes altered. This leads to genetic instability, or a mutator phenotype, either by accumulation of replicative errors or by gross chromosome rearrangement. A higher-than-normal mutation rate then leads to the combination of mutations in genes that regulate growth, invasion, or metastasis. Post-replication mismatch repair systems enhance the fidelity of DNA replication by correction of replicative errors. It has recently been shown that mutations in mismatch repair genes are associated with cancers (e.g. Jeong, 2003; Worrillow, 2003).

Oxidative stress caused by reactive oxygen species (ROS) can lead to genomic instability. Oxygen is metabolized inside the cell by a series of one electron reductions with the generation of reactive and potentially damaging ROS which include superoxide radical anion, H_2O_2, and the hydroxyl radical (Halliwell and Gutteridge, 1989). A pro-oxidant status in the cell can lead to the accumulation of ROS. Humans are exposed to substantial amounts of oxidative stress from a variety of sources. Halliwell has estimated levels of up to $100\ \mu M\ H_2O_2$ in the urine of individuals ingesting such foods as instant coffee (Long et al., 1999a,b).

Oxygen and its metabolites are known to function as intracellular signals, playing a role in apoptosis (Butts et al, 2003; Liu et al., 2003; Barr and Tomei, 1994; Buttke and Sandstrom, 1994,1995; Corcoran et al., 1994;

Davies, 1995; Forrest et al., 1994; Greenspan and Aruma, 1994; Hockenbery, 1995; Sandstrom et al., 1994) as well as in the stimulation of cell growth via the induction of a number of transcriptional activation factors such as *c-fos, c-jun, egr*-1, NF-κB and metabolizing enzymes (Anderson et al., 1994; Burdon, 1995; Crawford et al., 1988; Curran and Morgan, 1995; Janknecht et al., 1995; Khan and Wilson, 1995; Koong et al., 1994 a,b; Chen and Giaccia, 1994; Nose et al., 1991; Piechaczyk et al., 1994; Remade et al., 1995; Schreck et al., 1991; Xanthoudakis and Curran, 1994; Xanthoudakis et al., 1994; Yao et al., 1994; Lambert et al., 1994; Vile et al., 1994; Firth et al., 1994). ROS are believed to play a causative role in the degenerative diseases of aging including cancer (Ames and Shigenaga, 1993; Ames et al., 1993; Peterszegi et al., 2003; Cantuti-Castelvetri et al., 2003). Endogenously, activated neutrophils and macrophages generate large quantities of NO, $O_2^{\cdot}$, H_2O_2, OCl^- as part of the inflammatory response (Chanock et al., 1994; Densen et al., 1995, Ginsburg and Kohen, 1995; Laskin and Pendino, 1995), and a variety of other normal cells can also be stimulated to produce low levels of superoxide and hydrogen peroxide (Burdon, 1995). Ames has estimated that one third of the cancers in developing countries can be attributed to chronic infections (Ames et al., 1993). When oxidative stress due to these reactive species from whatever source exceeds the capacity of the defense systems of the cells to intercept and neutralize them (i.e. pro-oxidant status), toxicity (Mytar et al., 1999), genomic instability and mutations in cells result. For example, tumor promoters stimulate the production of reactive oxygen species which may play a critical role in the progression to malignancy (Beckman et al., 1994; Gopalakrishna et al., 1994; Hu et al., 1995; Marnett and Ji, 1994; Lin and Shih, 1994; Panandiker et al., 1994). Also, an African green monkey kidney cell line (CV-1) transfected with the H_2O_2-generating enzyme peroxisomal fatty acyl-CoA oxidase. When exposed to the enzyme substrate linoleic acid for 2-6 weeks, 4% of the cells became tumorigenic (Chu et al., 1995).

ROS targets are many, including membranes and proteins. The most critical potentially carcinogenic targets of ROS are probably deoxynucleotides and DNA, which generate in DNA 7,8-dihydro-8-hydroxy-2'-deoxyguanine (8-OHdG), 7,8-dihydro-8-hydroxy-2'-deoxyadenine (8-OHdA), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G), and 4,6-diamino-5-formamidopyrimidine (Fapy-A) (Bertoncini and Meneghini, 1995; Breen and Murphy, 1995; Dizdaroglu, 1993; Feig et al., 1994; Giese et al., 1995; Halliwell and Aruoma, 1991; Steenken, 1989). Guanine is the most easily oxidized of the nucleic acid bases (an electron-loss center created in a system containing the four nucleic acid bases will eventually migrate to guanine) (Steenken, 1989). The keto and enol forms of this damaged guanine are found in equilibrium. Thus, the interchangeable

use of 8-oxodG and 8-OHdG found in the literature. We will use the 8-oxoG terminology in this discussion. Since the last step in formation of 8-oxoG and 8-oxoA is an oxidation while the last step in the formation of Fapy-G and Fapy-A is a reduction (Breen and Murphy, 1995; Steenken, 1989), it has been suggested that 8-oxoG formation is favored under the more oxidizing (pro-oxidant) conditions found in cancer cells (Malins et al., 2003; Malins, 1993; Malins et al., 1993), or alternatively, that a pro-oxidant condition exists in cells destined to become malignant favoring the accumulation of 8-oxoG. For instance, the DNA of hepatitis B virus infected mouse liver cells destined to become tumorigenic had elevated levels of 8-oxoG (Hagen et al., 1994). Whatever the reason, 8-oxoG is considered to be a major stable product generated by ROS attack on DNA and it is excreted in the urine of humans where it is used as a biological marker of *in vivo* oxidative DNA damage (Halliwell, 1993; Shigenaga et al., 1994).

The formation of 8-oxoG in DNA, if not repaired, leads to misincorporation of dA opposite to the 8-oxoG lesion. The misincorporated dA leads to an A/8-oxoG mispair resulting in G·C → T·A transversions Moriya, 1993; Cheng et al., 1992; Moriya et al., 1991). The A/8-oxo G mispair also interferes with DNA-processing enzymes such as methylases (Weitzman et al., 1994) and restriction enzymes (Turk and Weitzman, 1995). It is interesting to note that G·C → T·A transversions occur frequently as mutations of the p53 tumor suppressor gene in human lung, breast, and liver cancers (e.g. Hollstein et al., 1991). More importantly, in p53, H_2O_2 induces a G to T transversion at both G-residues of codon 249 (AGG) and a C to A transversion at codon 250 (CCC) (Hussain et al., 1994).

In order to minimize the deleterious effects of reactive oxygen species, all aerobic organisms possess extensive defense systems. The primary defense is achieved by decomposing superoxide and hydrogen peroxide, which thus minimizes the formation of the ensuing hydroxyl radical (·OH). This primary antioxidant defense system includes various enzymatic and nonenzymatic mechanisms such as superoxide dismutase, catalase, glutathione peroxidase, and ascorbic acid (e.g. Halliwell and Gutteridge, 1989). In addition, although controversial, data indicate that BCL-2, the antiapoptotic protein also has antioxidant powers (Amstad et al., 2001). The reactive oxygen species that escape from the primary defense system have a significant chance of damaging DNA. Several repair enzymes in both prokaryotes and eukaryotes have been shown to remove 8-oxoG residues from DNA providing a secondary line of defense. The mammalian repair enzyme hOGG1, the human homolog of the yeast OGG1 is able to repair C/8-oxoG DNA damage back to CG in cells due to its 8-oxoG glycosylase /AP lyase activity (Gu et al., 2001; Aburatani et al., 1987; Arai et al., 1997; Friedberg et al., 1995; Hazra et al., 1998; Nash et al., 1996; Radicella et al.,

1997; Ramotar and Demple, 1993; Roldan-Arjona et al., 1997; Shibutani et al., 1991; Van der Kemp et al., 1996). The mammalian MTH1 enzyme, like the E. Coli MutT homolog eliminates 8-oxodGTP damage from the deoxynucleotide pool (Maki and Sekiguchi, 1992). Based on sequence homology with yeast OGG1, hOOG1 has been cloned (Aburatani et al., 1997; Arai et al., 1997; Lu et al., 1997; Radicella et al., 1997; Roland-Arjona et al., 1997; Rosenquist et al., 1997; Shibutani et al., 1991). If the 8-oxoG lesion is not removed by the hOGG1 protein before DNA replication, then misincorporation of dA opposite to the 8-oxoG lesion occurs (Hazra et al., 1998; Moriya et al., 1991; Nghiem et al., 1988; Shibutani et al., 1991). The mammalian hMYH enzyme, like E. Coli MutY, removes this misincorporated adenine from an A/8-oxoG mispair, with the formation of C/8-oxoG, which is then a substrate for hOGG1. This is considered the third level of defense (Au et al., 1989; McGoldrick et al., 1995; Slupska et al., 1996; Tsai-Wu et al., 1991; Yeh et al., 1991). The MutY gene has been cloned and sequenced (Boiteux et al., 1987; Michaels et al., 1990; Michaels et al., 1991; Slupska et al., 1996). In addition, it has been shown that expression of the *E. coli* MutM gene which expresses Fpg, an enzyme with the same activity as hOGG1 in mammalian cells, reduces the mutagenicity of X-rays (Laval, 1994). Furthermore, germ line mutations in human genes *h*MSH2, or *h*MLH1, which express proteins with the ability to correct DNA mismatches and misalignments lead to genetic instability in microsatellite repeat sequences in hereditary nonpolyposis colon cancer (Jeong et al., 2003; Bronner et al., 1994; Papadopoulos et al., 1994), It thus seems likely that the hOGG1/hMYH repair pathways are linked to protection against certain cancers.

An elevation of oxidative modifications in DNA has recently been reported in human cancers of the breast, prostate (Malins and Haimanot, 1991; Malins et al., 1993, Malins et al., 2003) and other tissues (LePage et al., 2000; Olinski et al., 1992). The 8-oxoG lesion has been suggested to be a putative link to cancer development (Malins et al., 2003; Malins, 1993) and a predictor for breast cancer (Malins et al., 1995). This modification occurs by the attack of hydroxyl radicals that arise from H_2O_2 mediated by trace metal ions such as Fe^{2+} and Cu^+ (Luo et al., 1994). Since H_2O_2 diffuses readily across the nuclear membrane, hydroxyl radicals are formed near DNA and modify it by attacking the purine and pyrimidine bases (Meneghini and Martins, 1993). Breast cancer is not commonly associated with pre-existing chronic inflammatory conditions, unlike bowel cancer where a causal relationship between ulcerative colitis and bowel cancer has been suggested (Weitzman and Gordon, 1990). Breast lesions (benign or malignant) are, however, commonly infiltrated with macrophages, which lead to oxidative stress. This in turn could lead to the progression of some

cells from benign to malignant and/or facilitate the transformation to a more aggressive metastatic tumor. There are a number of tumorigenic cell lines including one from breast that chronically elaborate large quantities of H_2O_2 (Szatrowski and Nathan, 1991) that can lead to chromosome mutations and cancer (Emerit, 1994). In addition, the most lethal form of locally advanced breast cancer is termed inflammatory breast cancer due to the acute inflammatory changes observed in patients with this type of malignancy (Jaiyesimi et al., 1992). As mentioned above, humans are exposed to considerable oxidative stress as judged by measurements of up to 100 μM H_2O_2 in urine (Long et al., 1999a,b). These levels vary with a normal diet. Halliwell concluded that urine contains "autooxidizable proteins" which upon exposure to oxygen result in the generation of O_2^- to generate H_2O_2 (Long et al., 1999a). Exposing cells to H_2O_2 can shift the cell to a pro-oxidant state, which facilitates ROS damage to a variety of targets. We have shown this to be true in the benign human breast epithelial cell line MCF-10A (Gu et al., 2001). MCF-10A is a spontaneously immortalized human breast cell line, which has characteristics of human breast epithelium such as: lack of tumorigenicity in nude mice and lack of anchorage independent growth (Soule et al., 1990). We treated these cells with 450 μM H_2O_2 5 separate time after allowing for growth (P5 line) or gradually at 50 μM H_2O_2 increments until reaching μM H_2O_2 (GP line). The peroxide decay and the concomitant production of OH radicals was measured by EPR using DMPO as mentioned earlier in this chapter (Figure 1).

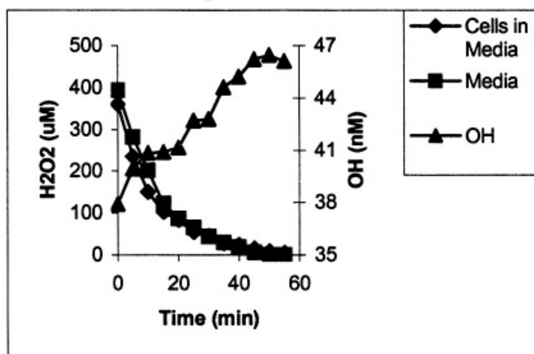


Figure 1 Hydrogen peroxide decay with concomitant formation of hydroxyl radicals. A colorimetric assay was used in the detection of hydrogen peroxide and DMPO/EPR in the detection of hydroxyl radicals.

After exposure to hydrogen peroxide, the cells became resistant to this oxidant and went on to exhibit different degrees of some of them being able to grow in immunodeficient mice (GP line). This transformation towards malignancy can be related to the cell's ability to bypass apoptosis to ensure survival, cell cycle anomalies modulated by oxidative stress-induceable

genes and the lack of repair of 8-oxoG in DNA (Gu et al., 2001). With respect to the latter damage, the role of hOGG1 and hMYH repair enzymes in mammalian tumor progression is to maintain genetic stability by avoiding G·C → T·A transversions. This is based on the work on *E. coli* MutY and MutM gene mutants. Cells with a single mutation in these genes are moderate mutators; however, cells with a double mutation in MutY and MutM genes have mutation rates three orders of magnitude higher than the wild-type cells (Nghiem et al., 1988; Cabrera et al., 1988). Overexpression of these genes will reduce the transversion mutations in tumor promoter genes or tumor suppressor genes and thus reduce the risk of tumorigenesis. Thus, if indeed the role of 8-oxoG in breast cancer tumorigenesis is established, one application to the treatment of cancer would be elevating the enzymes that repair such lesion over the endogenous levels present in healthy tissue.

With respect to cell responses to H_2O_2, we found that H_2O_2 induced p53 in MCF-10A but not in the H_2O_2-transformed line P5. In addition, all transformed lines overexpress BCL-2 when compared to MCF-10A (data not shown). It has been recently shown that the transfection of MCF-10A cells with the BCL-2 gene resulted in a 5 fold increase in BCL-2 protein expression over the parental MCF-10A line (Upadhyay et al., 1995) and that apoptosis did not occur in the transfectants at concentrations of 500 μM H_2O_2. In contrast, this concentration of H_2O_2 induced apoptosis in the parental non-transfected line (Upadhyay et al., 1995). This H_2O_2-induced apoptosis in MCF-10A cells is p53 dependent (Upadhyay et al., 1995).

7. USING NMR AND EPR WITH SPIN TRAPS (L. J. BERLINER)

The other contributors to this book have presented excellent approaches and examples to the use of EPR for observing radical species in biological systems. However, there are many further potential applications that currently cannot be done because of limited radical concentration or stability. This section describes alternate approaches to overcoming some of these obstacles, combining the two magnetic modalities that Jim Hyde has used so productively, often in combination with the use of spin traps. Spin traps have the potential advantages of being able to accumulate ("trap") highly reactive radicals that could not be otherwise observed directly, due to sensitivity limits and kinetic considerations. This is clearly the case for the hydroxyl radical, $\bullet OH^{\cdot}$, which may be directly observed only by fast freeze quenching at liquid nitrogen temperatures and quickly undergo secondary radical reactions and interconversions. Obviously, quickly immersing a

mouse into liquid nitrogen negates an *in vivo* experiment! Many of the applications, successes and pitfalls of *in vivo* spin trapping are reviewed in this volume by Mason (ch. 5). A major problem that is difficult to overcome is the tendency of the living cell to reduce radicals; consequently, the nitroxyl product of a nitrone radical adduct is normally bioreduced to the diamagnetic hydroxylamine. This section discusses potential alternatives utilizing NMR as the ultimate spectroscopic probing method. It also considers the use of more conventional NMR techniques to further characterize the spin traps and their metabolism.

7.1 NMR Spin Trapping

Many years ago Selinsky et al. (1989) suggested the use of ^{19}F NMR to study free radical reactions with fluorinated spin traps. They monitored organic free radical reactions in the test tube by both EPR and NMR. In later work outlined below, this technique was coined as "NMR spin trapping." The success of this approach depends on the following:

1. Do the diamagnetic (decomposition or transformed) products of a spin trap adduct accumulate to sufficient levels for detection by NMR?
2. Does the resultant NMR spectrum allow extrapolation back to a specific radical adduct?

The definition encompasses not only the spin trap chemistry described below, but also "MRI spin trapping" described in the last section below, in which the T_1 weighted NMR images of water protons are measured in the presence of stable, paramagnetic, biologically generated radical adducts or complexes. The enhanced relaxation of the water protons at the site of the radicals effectively 'localizes' the site of radical generation.

7.1.1 Phosphorus containing nitrone spin traps DEPMPO

Tordo and coworkers (Frejaville et al., 1995) described a series of nitrone spin traps, based initially on DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide), a phosphorus containing analog of DMPO (2,2-dimethyl-pyrroline-N-oxide). The advantages were that oxygen radical adducts of this trap showed a somewhat longer half life compared with DMPO. Despite these improvements, the in-vivo "stability" of paramagnetic spin trap adducts is sharply reduced relative to stable nitroxyl radicals, probably due to biological reduction of the resulting nitroxide to the hydroxylamine, but perhaps also by other destructive chemical processes.

It seems prudent to take advantage of the phosphorus moiety of DEPMPO by utilizing ^{31}P-NMR to probe its chemistry during and after radical reactions to understand the pathways of its radical adduct

degradation. As described below, a series of biologically relevant DEPMPO adduct reaction products for •OH and $•O_2^-$, as well as methyl radical ($•CH_3$) and hydroxymethyl radical ($•CH_2OH$) were characterized by ^{31}P-NMR.

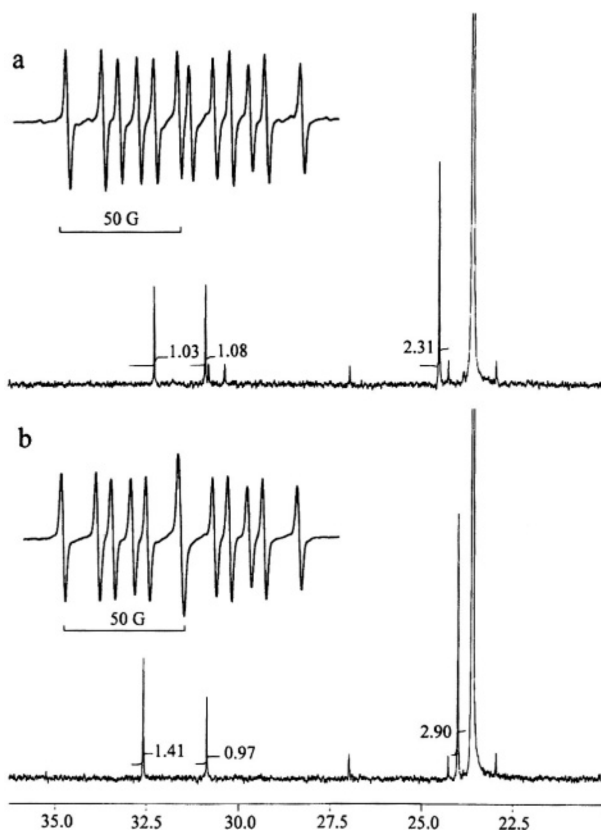


Figure 2 ^{31}P-NMR and ESR spectra of DEPMPO/$\cdot CH_3$ and DEPMPO/$\cdot CH_2OH$ reaction products. a) ^{31}P-NMR spectra obtained from 0.1 M DEPMPO in 0.1 M cacodylate buffer, 20 mM DTPA, pH 7.0, 10% DMSO, detected 2 h after addition of 5 mM $FeSO_4$ and 5 mM H_2O_2 at T=300K. *Inset:* corresponding ESR spectra observed at 10 min; b) the same reactions but in the presence of 10% methanol instead of DMSO. *Inset:* corresponding ESR spectra observed at 10 min.. Experimental spectra were in a good agreement with simulations using the calculated parameters a_N = 14.56 G, a_H = 21.8 G, a_P = 46.95 G for DEPMPO/$\cdot CH_3$ and a_N = 14.5 G, a_H = 20.7 G, a_P = 49.95 for DEPMPO/$\cdot CH_2OH$ adducts, which are in agreement with literature data. From Khramtsov et al. (1999) with permission.

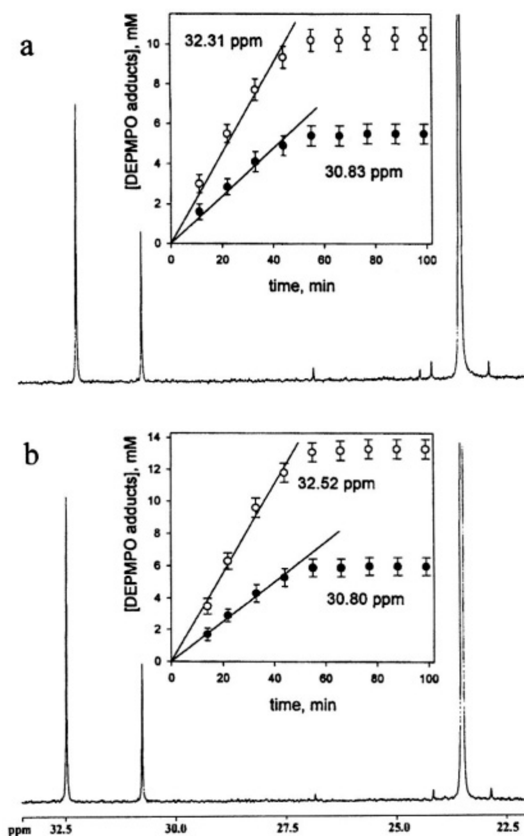
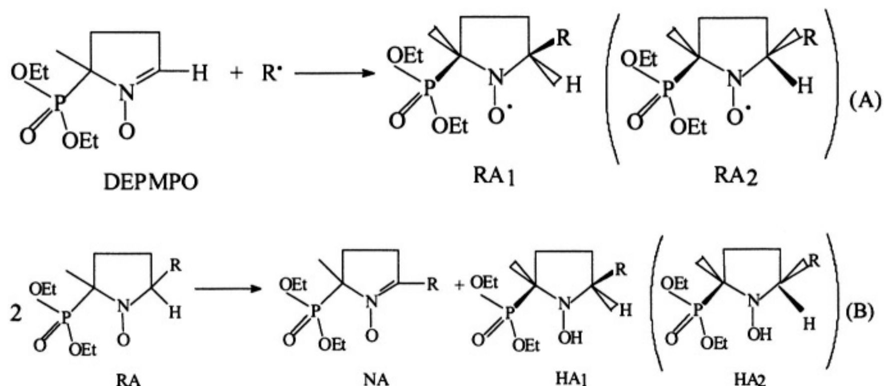


Figure 3. Quantitative determination of DEPMPO/·OH using ESR and NMR. Filled squares: concentration of DEPMPO adduct from the double integrated ESR spectrum; open circles: concentration calculated from the integral intensity of the ^{31}P-NMR peak at 27.05 ppm; filled circles: the same integral intensities of the ^{31}P-NMR peak multiplied by 2.0 to reflect the mechanism in Scheme B. The bent curve was calculated proposing bimolecular decay of DEPMPO-OH from initial concentration equaling the concentration of Fenton reagents ($[FeSO_4]$ = $[H_2O_2]$) with the rate constant k_d=15.8 $M^{-1}s^{-1}$ (namely [DEPMPO-OH]= $[H_2O_2]/([H_2O_2]\cdot k_d \cdot t + 1)$, where t=10 min. was the time at which the EPR spectra were obtained). The straight line was inserted as a guide to evaluate how closely the experimental data reflected a 1:1 stoichiometry between hydroxyl radical produced and product formed. Note that both the ESR data (at concentrations lower than 50 mM) and the adjusted NMR data show a linear concentration dependence on the Fenton concentration reagents. From Khramtsov et al. (1999) with permission.

7.1.1.1 DEPMPO

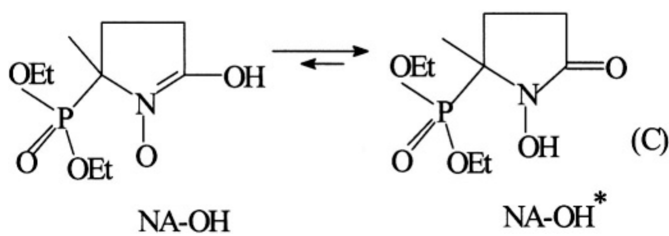
The reaction of DEPMPO with radical, R• as shown in Scheme A, emphasizes the fact that two stereoisomers are obtained since the spin trap contains an asymmetric center (yielding, in effect, two pairs of EPR spectra). However, the radical signals decay within two hours, yielding the ^{31}P NMR spectra depicted in Figure 2. For example, in Figure 2a (DEPMPO/·CH_3), there is evidence of several new, unique ^{31}P peaks at 24.54 ppm with additional peaks at 30.83 and 32.52 ppm. (A small 'DEPMPO/•OH' resonance at 27.05 ppm also occurs since the reaction conditions involve Fenton •OH chemistry (Khramtsov et al., 1999).



These peaks originate from bimolecular disproportionation chemistry as outlined in Scheme B, where *both* reaction products were diamagnetic: a new nitrone (NA) and the hydroxylamine of the spin adducts, HA_1 and HA_2. This accounts for the three ^{31}P NMR peaks, one of which is 50% total intensity, two of which are 25% each (the ratio depends slightly on the stereospecificity of the initial radical addition). Since the concentrations of spin trap adduct are significantly lower *in vivo,* disproportionation would not be appreciable; however, *direct reduction* of the DEPMPO adduct to the hydroxylamines HA_1 and HA_2 is highly facile. Hence, when ascorbic acid was added to the radical reaction system, direct conversion to HA_1 and HA_2 occurred. Consequently the two new ^{31}P lines at 32.31 and 30.83 ppm reflected the direct "history" of spin adduct formation of the methyl radical adduct of DEPMPO and its subsequent disproportionation or *in vivo* bioreduction.

At high concentrations the bimolecular decay of the DEPMPO/•OH EPR adduct spectrum reaction is relatively rapid. The adduct remains for only a few minutes unless balanced by extremely high steady state levels of new radical production (Liu et al., 1999; Timmins et al., 1999). As shown in Figure 3, the maximum intensity of the new ^{31}P line at 27.05 ppm never

exceeded 50% of the theoretical yield of the diamagnetic product from an •OH adduct. Scheme C suggests that the nitrone product of disproportionation (depicted in Scheme B) is in tautomeric equilibrium with the more stable N-hydroxypyrrolidone, NA-OH, to which the ^{31}P NMR line at 27.05 ppm was attributed. In addition, as shown in the scheme, the hydroxylamine can spontaneously eliminate H_2O, regenerating DEPMPO. Similar results were found with superoxide radical where, in fact, the ^{31}P NMR peaks were identical to those observed after reaction with •OH, suggesting a conversion of DEPMPO/•O_2^- to DEPMPO/•OH (or transformation of their diamagnetic products). *In vivo,* however, this is unlikely to be important because the local concentrations of spin adduct are unlikely to high.



7.1.2 Fluorine containing nitrone spin traps – FDMPO

From a sensitivity viewpoint, nitrones that contain trifluoromethyl groups might be more promising than those containing other stable isotopes. This was borne out with new spin trap, FDMPO (4-hydroxy-5,5-dimethyl–2-trifluoro methylpyrroline-1-oxide). The parent nitrone yields a single NMR resonance at –66.0 ppm, which yields a net ten-fold higher sensitivity than with ^{31}P NMR of DEPMPO.

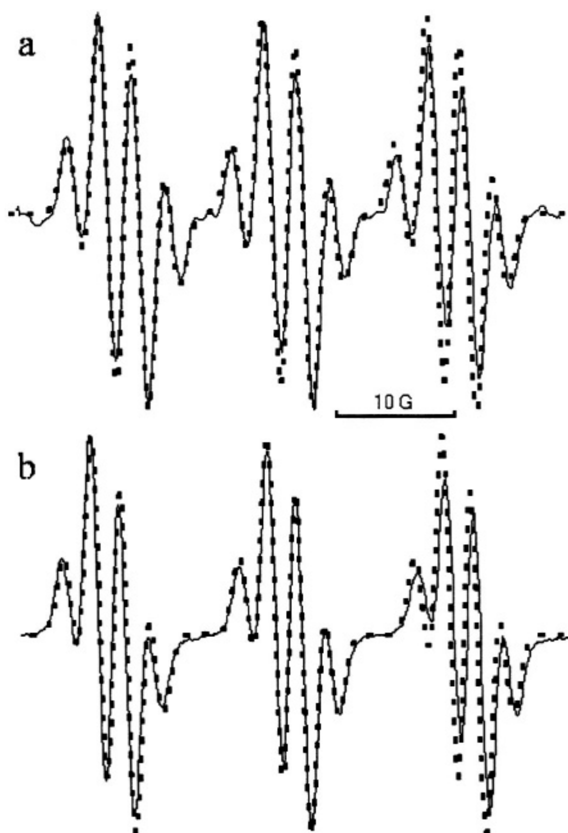


Figure 4. X-band ESR spectra of FDMPO spin adducts with ·OH (a) and $\cdot CH_2OH$ (b). Spectra were obtained in 0.1 M K-phosphate buffer, pH 7.0, 2 mM DTPA, 0.1 mM H_2O_2, 50 mM FDMPO, after addition of 0.1 mM $FeSO_4$ solution, and in 10% (v/v) methanol (b). Spectrometer settings were: microwave power 20 mW; modulation amplitude, 0.63 G; sweep time, 1 min. The dotted lines are calculated spectra. From Khramtsov et al. (2001) with permission.

Figure 4 shows simulated and observed EPR spectra for the •OH and $\bullet CH_2OH$ adducts, respectively (Khramtsov et al., 2001). The EPR signals were stable for over a day since bimolecular decay (as noted in Scheme B) is much less probable with the absence of an alpha proton. In order to observe the products by ^{19}F NMR, it was necessary to reduce the paramagnetic adduct(s) with ascorbic acid. However, despite the significantly increased *in vitro* stability of the paramagnetic adducts, it is likely that bioreduction would occur fairly rapidly. Figure 5 depicts ^{19}F results for the $\bullet CH_3$ adduct of FDMPO in a system containing ascorbate. The inset shows the precursor EPR spectrum.

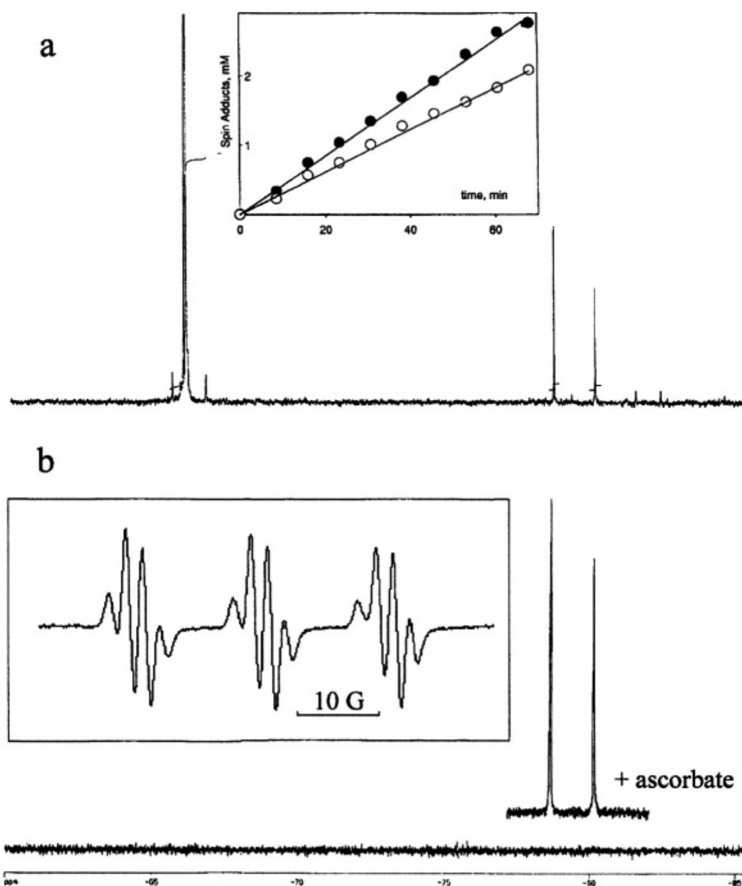


Figure 5. ^{19}F-NMR spectra of FDMPO/CH_3 adduct products: (a) in a Fenton "iron recycling" system in 0.1 M K-phosphate buffer, pH 7.0, 2 mM DTPA, 20 mM H_2O_2, 50 mM FDPMPO, 10% v/v of DMSO, 20 mM of ascorbate, 30 min after addition of 0.5 mM FeSO4 solution; number of scans, 208; *inset:* kinetics of product accumulation. The symbols (•) and (o) denote integral intensities of the 19P-NMR peaks of reduced FPMPO/·CH_3 products at –78.6 ppm and–80.0 ppm, respectively; (b) 19F-NMR spectra of 3 mM of FDMPO/CH_3, synthesized in Scheme B, in 0.1 M phosphate buffer, pH 7.0, before and after addition of 10 mM ascorbate; number of scans, 120; inset: EPR spectrum of 0.1 mM FDMPO/CH_3 adduct; spectrometer settings were microwave power 20 mW; modulation amplitude, 0.63 G; sweep time, 1 min. From Khramtsov et al. (2001) with permission.

The •O_2^- adduct gave identical NMR spectra to that with •OH. However, overall, the fluorine containing spin traps are less likely to undergo destructive metabolism assuming toxicity levels will not be a major problem. Overall the reactivity of FDMPO was comparable to that of DMPO as

measured by competitive kinetics. Consequently, the trifluoromethyl NMR spin traps have greater potential than traps containing other stable isotopes.

7.1.3 Carbon-13 containing nitrone spin traps [Methyl-^{13}C]-

The remaining biological isotope, ^{13}C, is at *ca* 1% natural abundance. In order to distinguish a particular molecule of interest, one must synthesize it enhanced with ^{13}C. In a novel application by Bose-Bsau et al. (2001). [Methyl-$^{13}C_3$]-2-methyl-2-nitrosopropane (MNP) was employed, in the presence of H_2O_2, as an NMR spin trap for tyrosyl radical in met-myoglobin. Relatively stable paramagnetic adducts were detectable by EPR; however, the direct identification and characterization of the final adduct on the protein and the specific chemistry involved was possible only after reduction of the paramagnetic adduct by ascorbate and the use of 2D high resolution NMR to assign the relevant Tyr residue and the chemistry of the modification. Previous spin trapping studies on myoglobin had shown the presence of tyrosyl radicals that could be trapped with MNP (Barr et al., 1996), but the EPR results were ambiguous as to which residue was preferentially labeled. These recent results were definitive in identifying Tyr 103 in equine myoglobin and that the adduct formation occurs at the C-3 carbon of the amino acid. Figure 6 depicts the reaction scheme that most likely occurs. In addition, the ^{13}C-^{1}H HMQC-NOESY studies identified the nearby protons of Phe 106.

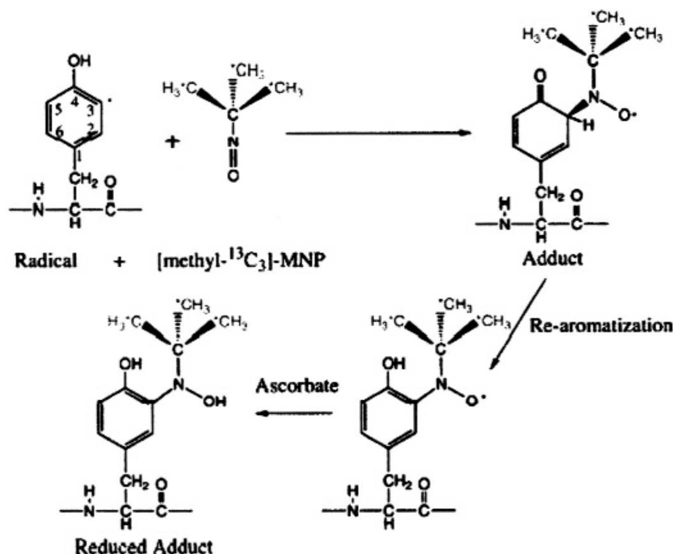


Figure 6. Scheme 1. Adduct formation at Tyrosine C-3, rearomatization, and reduction by ascorbate to the hydroxyl amine. From Bose-Bsau et al. (2001 with permission).

7.1.4 MRI Spin Trapping

The direct localization and imaging of free radical distributions is much more challenging with EPR due both to aspects of stability discussed above as well as linewidth limitations. Despite almost three orders of magnitude higher sensitivity (eg, a free electron vs a proton) the much broader linewidths usually encountered in EPR places limitations on both resolution and overall sensitivity. There are additionally other limits to distinguishing tissue boundaries with radical distributions; these are simply more difficult to visualize by EPR. On the other hand, MRI, which is a mature, commercialized technology, offers exquisite anatomical resolution based on the protons of water (i.e. 100 M). Several physical aspects, such as nuclear relaxation times, diffusion rates, oxygen magnetic susceptibility effects, etc., allow multifaceted approaches to EPR and MRI image resolution. On the other hand, paramagnetic compounds serve as excellent contrast agents in MRI by inducing proton (nuclear) relaxation enhancement from rapidly exchanging water molecules. Consequently, MRI spin trapping potentially allows visualization of free radical biology at the site of its generation, by using the spin adducts as "contrast agents".

7.1.5 Nitric Oxide (NO)

NO may be 'spin trapped' with dithiocarbamate compounds and Fe(II), which form a stoichiometric 2:1 complex for axial coordination of NO. The water soluble trap, MGD, forms the $(MGD)_2Fe(II)$ NO complex where a diagnostic EPR spectrum is found with septic shock mice (which has been almost the sole example with sufficient sensitivity *in vivo).* Recently Kaneko et al. (2002) demonstrated *in vivo* observations of NO in the brain of epileptic mice models. The relaxivity of this complex is relatively frequency independent for both T_1 and T_2 at 20 and 85 MHz, respectively. Uncomplexed MGD, Fe(II) or $(MGD)_2$-Fe(II) show essentially negligible relaxivity while the $(MGD)_2Fe(II)$ NO complex yields appreciable relaxation enhancement (Fujii et al., 1999). Consequently, in MRI measurements, only the NO complex will yield image contrast enhancement, frequently at the site of NO generation. As shown in Figure 7, 1.5T T_1-weighted MRI images of septic-shock rats showed increased enhancement in the liver with time. This was suppressed by injecting the animals with a specific iNOS inhibitor, L-NMMA. Consequently, both the localization and "biochemistry" of NO generation can be visualized at high-resolution *in vivo.*

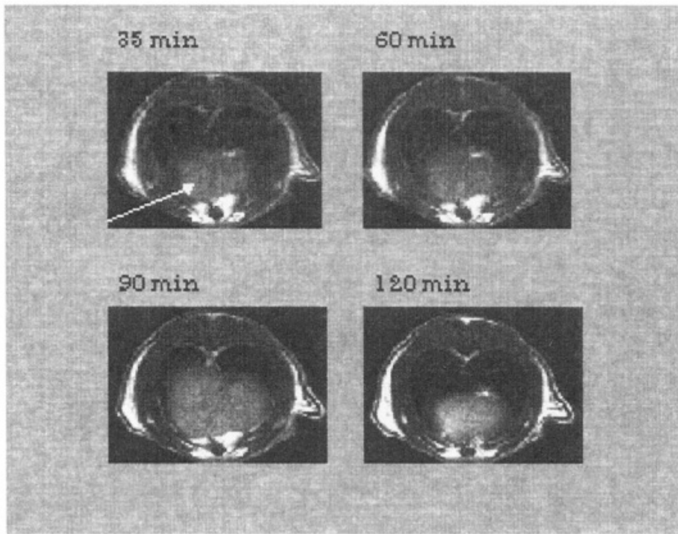


Figure 7. Transverse T_1-weighted MR images of the liver in LPS-doped rats. MR images were measured at the times indicated for two different slices of the liver. Six hours after LPS injection, the NO spin-trap (3 ml of $(MGD)_2$-Fe(II), MGD: 100 mM, Fe: 20 mM) was administered i.p. The slice thickness was 2 mm, and each slice was separated by 1 mm. The image enhancement was drastically reduced upon treatment with the NOS inhibitor, L-NMMA. Adapted from Fujii et al. (1999) with permission.

7.2 Prospects for the Future

Most of the new approaches outlined here are still in their infancy. The future, however, looks promising Feasibility studies have been quite successful. Most of the stable isotopes incorporated in the spin traps described above are easily resolvable in the presence of other biological milieu. Sensitivity is particularly promising with ^{19}F NMR, a technique that has never been exploited to its full capacity *in vivo.*

Of particular note is the use of paramagnetic relaxation enhanced MRI. The power of paramagnetic contrast agents to MRI has been proven time and again. Several sophisticated pulse sequences exist which take advantage of small susceptibility/relaxation effects. In addition, the development of new, targeted paramagnetic spin traps and spin probes promise to provide the requisite localization. The ability to observe biological radical production at the site of generation offers the ultimate advantage in diagnosis.

8. SUMMARY AND CONCLUSIONS

There clearly are many areas in which free radicals are intrinsically involved in both physiology and pathophysiology. There also are many situations where the involvement of free radicals is minimal or not significant. EPR spectroscopy, combined with thoughtful experimental approaches can be a powerful method for resolving many of the questions that arise regarding the role of free radicals in disease. The National Biomedical ESR Center at MCW has had, and will continue to have an important role in providing critical EPR spectroscopy tools and original experimental results that will provide an understanding of the role of free radicals in disease. There appear to be sufficient important problems to make this a productive field for many years.

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