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In vivo detection of free radical metabolites

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Abstract - During the past two decades, the ESR spin-trapping technique has been used extensively to detect free radicals generated by metabolism of xenobiotic compounds *in vitro*. Although this ESR technique has proven useful *in vitro*, it has only recently been applied to drug metabolism *in vivo*. Spin trapping has successfully been employed to study the metabolism of halocarbons, hydrazines and hydroperoxides in perfused organs and in whole animals. The results of these current studies are reviewed herein.

INTRODUCTION

The production of free radical metabolites has been thoroughly studied *in vitro* using ESR techniques; detection of these reactive species *in vivo* has only recently been undertaken. There are several reasons for the late development of this area. Although many drugs and industrial chemicals are metabolized through free radical intermediates, most biochemicals are metabolized via two electron processes. When free radical metabolites are produced, their detection is difficult due to the transitory nature of these species. The problem of detection is confounded by the slow rate of production of free radicals in animals relative to that observed in chemical systems; therefore, it is very important to obtain the highest possible sensitivity. Unfortunately, the molar sensitivity of biological samples is decreased because they contain a large fraction of water so only small samples can be studied. Water is the worst solvent for ESR due to its high dielectric constant. Although the detection of free radical metabolites *in vivo* is a challenging task, their existence must be demonstrated in a whole animal or there will always be some question as to their actual existence in biology.

Several techniques have been used to overcome the problem of working with aqueous samples. The dielectric constant of water can be lowered by freezing so that larger samples can be analyzed. The freeze quench method is useful for enzymatic systems where solutions can be frozen in milliseconds (ref. 1). This method is not as applicable to tissues because frozen tissues must be ground to fit into ESR sample tubes, and this leads to mechanically induced radicals or artifacts (ref. 2). In addition, the resulting powder spectra are poorly resolved, and their interpretation in complex biological systems is very difficult. The same problems of artifacts and poor resolution are observed when tissue is lyophilized (refs. 3,4). Larger samples can be analyzed using low-frequency ESR; this method could theoretically be used to study radical production directly in small animals (i.e., *in vivo* spectroscopy). Unfortunately, sensitivity is directly dependent on frequency; thus, low-frequency instruments are unlikely to achieve the molar sensitivity needed to directly detect the low concentrations of free radical metabolites generated *in vivo*. Spin trapping appears to be the most convenient approach to the detection of free radicals *in vivo* because it facilitates a higher steady-state concentration of free radicals (as free radical adducts) and therefore overcomes the sensitivity problems inherent to detection of radicals in biological systems. Since the concentration of endogenous radicals in biological tissues is generally near the sensitivity limit of ESR spectroscopy, the spin-trapping technique is not limited by background signals.

SPIN TRAPPING *IN VIVO*

The technique of spin trapping involves the addition of a primary free radical across the double bond of a diamagnetic compound (the spin trap) to form a radical adduct more stable than the primary free radical. This technique involves the indirect detection of primary free radicals that cannot be directly observed by conventional ESR due to low steady-state concentrations or to very short relaxation times, which lead to very broad lines (ref. 5).

All of the reported *in vivo* spin trapping investigations have used the nitrone spin traps, phenyl-*tert*-butylnitrone (PBN), α-2,4,6-trimethoxy-PBN ($(CH_3O)_3$PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Radical adducts of nitrone spin traps typically exhibit six-

line ESR spectra. Hyperfine splittings in the radical adduct ESR spectrum do not usually arise from atoms of the primary radical; instead, they are due solely to the nitrogen and β-hydrogen of the spin trap. Identification of the primary free radical trapped in vivo depends on a comparison of hyperfine splitting values of the radical adduct with those of bona fide nitroxides prepared either chemically or enzymatically and analyzed under exactly the same experimental conditions. If the radical is trapped at a nucleus with nonzero spin such as ^{14}N, then additional hyperfine splittings occur which greatly facilitate the identification of the radical adduct. Thus, the identification of C- and O-centered free radicals is simplified by isotopic substitution with ^{13}C or ^{17}O, respectively.

Although the major difficulty of the spin-trapping technique is production of a radical adduct stable enough to be detected in biological samples, other factors must be considered when spin traps are administered in vivo. For example, spin traps may affect the experimental system by inhibiting or stimulating enzymes, or by producing toxicity. The latter possibility has not seemed to affect in vivo work to date, although this issue has not been addressed in detail. There are several good reviews which address in vitro applications of the spin-trapping technique (refs. 5-14). These reviews also discuss the effects of spin traps on enzymes and more general problems of spin trapping such as artifacts.

DETECTION OF RADICAL ADDUCTS IN FOLCH EXTRACTS OF TISSUE

Results of the first in vivo spin-trapping study were reported in 1979; in these experiments, the spin trap PBN and carbon tetrachloride were given to a rat through a stomach tube (ref. 15). After 2 hrs, the liver was extracted with a solution of 2:1 chloroform/methanol (the Folch extraction for lipids), and the chloroform layer was analyzed by ESR. The spectrum was assigned to PBN/$^{\cdot}CCl_3$ by comparing the experimental hyperfine coupling constants with those of the bona fide radical adduct generated both in a microsomal system and by photolysis of carbon tetrachloride. When ^{13}C carbon tetrachloride was used, an additional splitting was observed in the radical adduct spectrum; this result provided a more definitive identification of the trapped radical (refs. 16,17).

In more recent studies, carbon-centered adducts have been detected in the organic extracts of liver from animals treated with other halogenated hydrocarbons. Studies with the volatile anesthetic halothane are of clinical interest since this compound produces hepatitis in humans. When phenobarbital-pretreated rats were given halothane under hypoxic conditions, both liver damage and free radical production were observed; the radicals were trapped by PBN and extracted from the liver (refs. 18-20). Although the trapped radical species remains to be identified unambiguously, reductive debromination is probably responsible for the reported carbon-centered free radical formation. Chloroform, iodoform, bromoform, and bromodichloromethane are other halogenated hydrocarbons that are metabolized to free radicals in vivo (ref. 21). After treating gerbils with carbon tetrachloride and PBN, free radical adducts were detected in Folch extracts of liver, kidney, heart, lung, testis, brain, and blood, with radical adduct concentrations decreasing in the order given. The radical adduct was identified as PBN/$^{\cdot}CCl_3$ in Folch extracts from liver; the radical adducts observed in other tissue extracts could not be positively identified (ref. 22).

Free radical formation from halocarbon metabolism results in lipid peroxidation. Thus, spin adducts detected after treatment with carbon tetrachloride that do not exhibit ^{13}C hyperfine coupling from $^{13}CCl_4$ are probably derived from carbon- or oxygen-centered lipid radicals. Lipid-derived radical adducts have been detected in several in vitro studies (refs. 16,23-25). A carbon tetrachloride-dependent, non-carbon tetrachloride-derived species assigned to a lipid-derived radical was observed in organic extracts of livers from rats treated with $(CH_3O)_3PBN$ and carbon tetrachloride (ref. 25). However, O-demethylation of the spin trap precludes a simple explanation of these latter studies. Definitive identification of lipid radicals is inherently difficult, since ^{13}C-labelled fatty acids are unavailable and chromatography or mass spectroscopy of these heterogeneous radical adducts is a formidable task.

Lipid peroxidation is an important toxicological process because it results in damage to biological membranes, which can lead to loss of cellular function. Lipid radical adducts may be the only evidence of radical species that initiate lipid peroxidation but do not form stable adducts themselves. For example, 3-methylindole is metabolized in vitro to form a nitrogen-centered free radical that can be trapped with PBN. In contrast, Folch extracts of lungs from goats treated with 3-methylindole and PBN yielded only a carbon-centered radical adduct. Experiments with the GSH precursor cysteine and the GSH-depleting agent diethylmaleate suggested that the concentration of carbon-centered radical adduct varied inversely with in vivo levels of GSH (ref. 26). When rats were dosed with PBN and then exposed to a non-lethal burst of gamma-irradiation, carbon-centered lipid-derived radical adducts were detected in Folch extracts of brain, spleen, liver, and heart

(ref. 27). An ethanol and high fat diet has also been used to produce the $(CH_3O)_3PBN$/lipid radical adduct in Folch extracts of hearts and livers from animals treated in vivo (ref. 28).

The Folch extraction was used in all of the in vivo investigations of carbon-centered free radicals derived from halocarbons and lipids. The greatest advantage of this extraction method is that the radical adduct is transferred from a sample with a high dielectric constant, the biological tissue, to a solvent with a lower dielectric constant, the chloroform solution; this enables the use of larger sample volumes. In addition, the sample can be easily concentrated by evaporation of the chloroform layer. The greatest limitation of organic extraction is that only non-polar radical adducts such as those of $^{\cdot}CCl_3$ and lipid-derived radicals can be detected.

DETECTION OF RADICAL ADDUCTS IN BIOLOGICAL FLUIDS

Our approach to in vivo spin trapping has been to examine biological fluids such as urine, bile and blood directly for spin adducts. For these studies, the TM_{110} ESR cavity and a 17 mm flat cell have been used to achieve the largest possible aqueous sample size in the active region of the cavity (approximately 100 μl). This approach yields both high molar sensitivity and high resolution. Degassing of samples is not usually necessary due to the low solubility of oxygen in water (approximately 250 μM), although it will occasionally narrow the sharpest lines. The only background signals that have been detected are the ascorbate semidione doublet and Mn^{2+} (only in bile). The biological fluids are not extracted, so chemical reactions causing the appearance or disappearance of radicals that may occur during the extraction process itself are avoided. The detection of free radical metabolites in biological fluids is, in principle, similar to the detection of the products of drug metabolism by HPLC as practiced in pharmacology departments or by the pharmaceutical industry.

Detection of a radical adduct in a biological fluid was first reported in 1986. In this study, rats were given carbon tetrachloride and PBN; urine was collected from the living rats and analyzed by ESR. A novel radical adduct, PBN/$^{\cdot}CO_2^-$, was detected in the urine samples (ref. 29). When ^{13}C carbon tetrachloride was used, an additional splitting was observed in the ESR spectrum, indicating that this radical adduct, like PBN/$^{\cdot}CCl_3$, was carbon tetrachloride-derived. PBN/$^{\cdot}CO_2^-$ was also detected in the effluent perfusate of livers which were perfused with carbon tetrachloride and PBN, and in the urine of rats after administration of bromotrichloromethane and PBN (ref. 30). Bromotrichloromethane and carbon tetrachloride are metabolized by a common pathway and yield the same metabolites, although the former is more readily dehalogenated due to the relative weakness of the C-Br bond.

The perfusion of liver with either carbon tetrachloride or bromotrichloromethane results in hepatocellular necrosis as measured by release of lactate dehydrogenase (LDH) from cells; appearance of PBN/$^{\cdot}CO_2^-$ precedes LDH release (ref. 30). Perfusion of the liver with nitrogen-saturated instead of oxygen-saturated buffer accelerates LDH release. The concentration of PBN/$^{\cdot}CO_2^-$ in perfusate at the beginning of lysis is inversely proportional to the length of time required until LDH is detected (ref. 30). Although PBN/$^{\cdot}CO_2^-$ concentration is statistically correlated with the time period prior to LDH detection, this relationship does not imply causation; therefore, the stable trapped product PBN/$^{\cdot}CO_2^-$ can only be considered a marker for the species actually responsible for membrane damage. The correlation of radical adduct production with an index of toxicity (LDH release) suggests that free radicals are involved in the onset of hepatocellular necrosis. The fact that PBN (5 mM) did not inhibit the hepatotoxicity induced by carbon tetrachloride and bromotrichloromethane can be explained by the very low rates of radical trapping characteristic of PBN (ref. 31); that is, PBN only scavenges a small fraction of the radicals produced in this system.

When rats were administered PBN intraperitoneally and carbon tetrachloride intragastrically, both PBN/$^{\cdot}CCl_3$ and PBN/$^{\cdot}CO_2^-$ were detected in bile samples collected at multiple timepoints after treatment (ref. 32). The toxicity of carbon tetrachloride is known to be increased either by hypoxic conditions or by phenobarbital pretreatment. Both of these in vivo manipulations increased the biliary concentration of PBN/$^{\cdot}CCl_3$. Either hypoxia or phenobarbital pretreatment was required for the detection of PBN/$^{\cdot}CO_2^-$. Bile facilitates the detection of ionic, polar, and non-polar radical adducts because it consists of both an aqueous phase and hydrophobic environment (ref. 32).

The mechanism of toxicity of phenylhydrazine and a series of hydrazine-based drugs has received a great deal of attention due to their ability to induce hemolytic anemia. This condition results from cell destruction that is initiated by the reaction of a hydrazine derivative and oxyhemoglobin within red cells. In vitro ESR investigations have provided considerable evidence that implicates free radicals in the processes leading to red blood cell hemolysis.

An immobilized radical adduct (a^N_{zz} = 31.8 G and a^H_{zz} = 9.5 G) was detected in the blood of rats which received an intraperitoneal injection of DMPO followed by an intragastric dose of phenylhydrazine greater than or equal to 1 mg/kg (ref. 33). Hydrazine also induced the production of this adduct, although the concentration was less than that detected with phenylhydrazine. The adduct co-chromatographed with oxyhemoglobin and was detected *in vitro* using purified rat hemoglobin, phenylhydrazine and DMPO, indicating that the immobilized radical adduct was hemoglobin-derived. When whole rat blood was pretreated *in vitro* with a sulfhydryl-depleting reagent (either iodoacetamide, maleimide, or N-ethylmaleimide) prior to the addition of phenylhydrazine, inhibition of immobilized radical adduct formation was observed, indicating that radical production is dependent on the presence of reduced sulfhydryl groups. On the basis of these results, the immobilized radical adduct has been assigned to a DMPO/hemoglobin thiyl radical adduct. This is the first report of a biological macromolecule-derived free radical formed as a consequence of free radical metabolism. Another immobilized radical adduct, the PBN/hemoglobin thiyl radical adduct (a^N_{zz} = 30.8 G), was detected when DMPO was replaced by PBN *in vivo* (ref. 34). The DMPO/phenyl radical adduct was also detected in chloroform extracts of whole blood in agreement with *in vitro* results (ref. 33). In subsequent work, the DMPO/hemoglobin thiyl radical adduct was detected when rats were dosed with DMPO and a hydrazine-based drug (either hydralazine, iproniazid, or phenelzine). Of these drugs, only phenelzine induced DMPO/hemoglobin thiyl radical adduct formation both *in vivo* and *in vitro*; iproniazid formed this adduct only *in vivo*, whereas hydralazine formed this adduct only *in vitro*. Isoniazid, another hydrazine-based drug, did not induce radical adduct formation in either system. Pretreatment of rats with bis-*para*-nitrophenylphosphate, an arylamidase inhibitor, decreased radical adduct formation induced by iproniazid *in vivo*. This result suggests that iproniazid is hydrolyzed in the liver to a more reactive metabolite (e.g, isopropyl hydrazine) which is subsequently released into the blood stream. In contrast, phenylhydrazine and phenelzine react directly with red blood cells and do not require metabolism to yield the DMPO/hemoglobin thiyl radical adduct. As hydralazine did not yield this adduct *in vivo*, we proposed that hydralazine is metabolized *in vivo*, possibly via acetylation, to a less reactive compound. The DMPO/hemoglobin thiyl radical adduct has also been detected in the blood of rats following administration of either *tert*-butyl hydroperoxide, cumene hydroperoxide, ethyl hydrogen peroxide, 2-butanone hydroperoxide, 15(S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid, or hydrogen peroxide (ref. 35). This adduct was not detected in previous *in vitro* ESR spin-trapping studies dealing with the interaction of organic hydroperoxides and oxyhemoglobin because the hemoglobin concentration was too low (refs. 36,37). Under these conditions, only radicals resulting from the decomposition of the hydroperoxide (i.e., alkyl and alkoxyl radicals) were observed. In summary, a DMPO/hemoglobin thiyl radical adduct was formed *in vivo* by the reaction of phenylhydrazine (refs. 33,34), some hydrazine-based drugs (ref. 34) and organic hydroperoxides (ref. 35) with oxyhemoglobin and detected via the ESR spin-trapping technique.

CONCLUSION

Radicals formed *in vivo* can be spin trapped and detected by ESR in Folch extracts of tissue or in biological fluids such as bile, blood and urine. Only non-polar radical adducts such as those of $^{\cdot}CCl_3$ and lipid-derived radicals can be detected with organic extraction, whereas ionic radical adducts of $^{\cdot}CO_2^-$ and hemoglobin thiyl can only be detected in predominately aqueous biological fluids such as urine and blood, respectively. In principle, ionic, polar and non-polar radical adducts can be detected in bile.

Identification of urinary metabolites derived from drugs and pesticides is required by both the U.S. Food and Drug Administration and Environmental Protection Agency. The ESR studies described above suggest that these requirements may be extended to include the spin-trapped products of free radical metabolism. Unlike the stable products of detoxification, which are detected by conventional analytical techniques, the detection of radical adducts proves the existence of highly reactive intermediates which are most likely formed via toxification reactions. Although the scope of most *in vivo* spin-trapping investigations has been limited to the metabolism of halogenated hydrocarbons, enough additional examples of metabolic free radical formation are known to demonstrate that this technique may be widely applicable. If this is the case, then the detection and identification of free radical metabolites *in vivo* should be more useful to the understanding and even to the prediction of toxicities than are traditional analytical techniques, which are inherently limited to the detection of stable metabolites. Due to their reactivity, free radicals are capable of interacting with a number of cellular constituents *in vivo*; these reactions may lead to damage that ultimately results in a free radical-mediated pathology. Thus, any free radical metabolites detected *in vivo* would be of great toxicological significance.

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