



Review

Immuno-spin trapping from biochemistry to medicine: Advances, challenges, and pitfalls. Focus on protein-centered radicals[☆]

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ABSTRACT

Background: Immuno-spin trapping (IST) is based on the reaction of a spin trap with a free radical to form a stable nitron adduct, followed by the use of antibodies, rather than traditional electron paramagnetic resonance spectroscopy, to detect the nitron adduct. IST has been successfully applied to mechanistic *in vitro* studies, and recently, macromolecule-centered radicals have been detected in models of drug-induced agranulocytosis, hepatotoxicity, cardiotoxicity, and ischemia/reperfusion, as well as in models of neurological, metabolic and immunological diseases.

Scope of the review: To critically evaluate advances, challenges, and pitfalls as well as the scientific opportunities of IST as applied to the study of protein-centered free radicals generated in stressed organelles, cells, tissues and animal models of disease and exposure.

Major conclusions: Because the spin trap has to be present at high enough concentrations in the microenvironment where the radical is formed, the possible effects of the spin trap on gene expression, metabolism and cell physiology have to be considered in the use of IST and in the interpretation of results. These factors have not yet been thoroughly dealt with in the literature.

General significance: The identification of radicalized proteins during cell/tissue response to stressors will help define their role in the complex cellular response to stressors and pathogenesis; however, the fidelity of spin trapping/immuno-detection and the effects of the spin trap on the biological system should be considered.

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Abbreviations: AG, aminoglutethimide; BSA, bovine serum albumin; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; ESR, electron spin resonance; HPLC, high performance liquid chromatography; hoMb, horse myoglobin; huHb/Mb, human hemoglobin/myoglobin; I/R, ischemia/reperfusion; IST, immuno-spin trapping; LC, liquid chromatography; LPS, lipopolysaccharide; MRI, magnetic resonance imaging; MS, mass spectrometry; OA, octanoic acid; SOD, superoxide dismutase; TPO, thyroid peroxidase

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1. Introduction

The “gold-standard” technique that allows the unambiguous detection of free radicals is electron spin resonance spectroscopy [ESR, also called electron paramagnetic resonance (EPR)] because this technique is based on fundamental physics and makes no assumptions [1–4]. There is no doubt that ESR has a number of undisputed advantages over other methods of studying free radicals [5]. However, the greatest limitation of ESR in the study of free radicals in cells and tissues is its poor sensitivity in relation to the steady-state concentrations of free radicals under physiological conditions, or even in the response to severe stress. Typically, the steady-state concentration of free radicals under normal conditions is less than 1 nM or even 1 pM, which is far below the best sensitivity of ESR of 3 nM. These limitations of ESR

have led to an intensive search for alternatives for the investigation of free radicals in biological systems.

The limitations of direct ESR in the study of short-lived free radicals led, in the 1960s, to the development of spin trapping [6], in which a free radical adds to the carbon end of the nitrene function of a spin trap (Fig. 1). This reaction produces a much more stable free radical, a nitroxide radical adduct or radical adduct that can be seen by ESR spectroscopy [6]. The greatest advantage of using spin trapping in the study of free radicals is the increased stability leading to increased radical adduct concentrations, which are often above the 3 nM limit of ESR detection [2]. This has led to a renaissance of ESR-spin trapping in the study of oxidative processes in biological systems [7,8]. Indeed, a number of free radical metabolites, proteins and nucleic acids have been detected by ESR-spin trapping both *in vitro* and *in vivo* [2].

Many spin traps, such as nitroso and nitrene compounds, have been used to study free radicals in biological systems [7,9]. Because of their physico-chemical properties, membrane permeability [10,11], effectiveness at trapping free radicals [9], and low toxicity [12], nitrene spin traps have been employed both as reagents to detect radicals using ESR spectroscopy [7,9] and as pharmacological agents against oxidative stress-mediated injury [13,14]. The most popular of these spin traps is 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) [15], which has been cited in Medline over 1300 times. In a radicalized macromolecule, for example a radicalized protein, the spin trap adds to an atom in a solvent-exposed site with high electron spin density [6]. The greater the stability of the radical adduct, the higher the concentration of the radical adduct for a given rate of free radical generation [7]. The lifetime of the radical adduct is usually the most important factor in deciding the success of an ESR-spin-trapping experiment [2]. In addition, it is important to note that unlike direct ESR, the spin-trapping methodology depends on the absolute fidelity of the spin-trapping reaction [16]. Importantly, nitrene spin traps are known to react with free radicals and non-radicals via electrophilic and nucleophilic addition reactions [17]. Two alternate mechanisms of radical adduct artifacts with DMPO have been recently investigated and discussed [16,18]: inverted spin trapping (one-electron oxidation of the spin trap) and the Forrester–Hepburn (nucleophilic addition of the spin trap) mechanisms. In biological systems the Forrester–Hepburn mechanism, which is initiated by a nucleophilic addition of a nucleophile to the spin trap, would be the major mechanism of

generation of potential artifactual DMPO-molecule adducts. See [16,18] for a comprehensive chemical discussion of these two sources of artifacts in spin trapping.

The specificity of the reactions of nitrene spin traps with free radicals has already made spin trapping with ESR detection the most universal and specific tool for the detection of free radicals in biochemical systems as well as in cells, tissues and animals [19]. Unfortunately, ESR-spin trapping of protein radicals *in vivo* has severe limitations. Some of them are: 1) the cost of acquiring the instrument; 2) the instability of radical adducts in tissues, which may compromise reproducibility; 3) the lossiness of the dielectric sample, and 4) the low radical adduct concentration due to the presence of antioxidants that can compete with DMPO.

2. Principle of immuno-spin trapping

To an ESR spectroscopist, the conservation of the unpaired electron is the most important aspect of the reaction of a protein-centered radical with a spin trap to form a radical adduct. To an organic chemist, the most unique feature of the reaction is the formation of a new covalent bond between the DMPO and the free radical in a reaction that is specific for free radicals. To an immunologist, the reaction of a free radical with a spin trap marks the creation of a novel epitope; and to a biochemist this is a novel way to identify a protein target of oxidation in states of stress that may lead to an understanding of the chemical basis of a free radical process in biologically-relevant scenarios.

DMPO is very stable and nearly redox inert, being reduced to the hydroxylamine only at the very low potential of -1.68 V and oxidized only at the very high potential of 1.87 V versus normal hydrogen electrode. Once formed DMPO–protein adducts can exist in three redox forms: (1) the nitroxide radical adduct, (2) the corresponding hydroxylamine formed by a one-electron reduction of the radical adduct, and (3) the corresponding nitrene formed by a one-electron oxidation of the radical adduct [20] (see Fig. 1). The nitroxide radical adducts and their corresponding hydroxylamine adducts are all unstable and decay over time. The nitrene adduct is the most thermodynamically stable product of the reaction of a free radical with a nitrene spin trap that can be studied in tissues excised from an experimental animal treated with the spin trap.

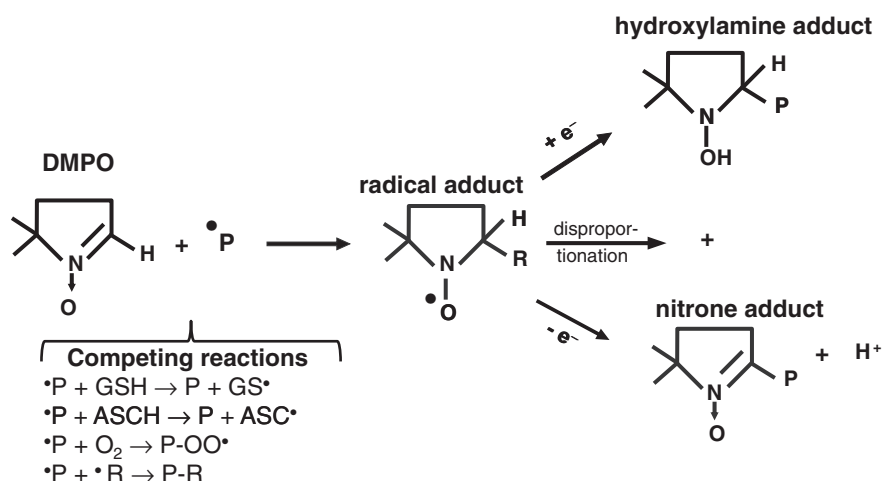


Fig. 1. Spin trapping and fate of protein–DMPO adducts. A protein radical (a radical site in a protein) reacts with DMPO to form a radical adduct. Depending on microenvironment conditions and structural characteristics of the target protein, the radical adduct can be reduced to hydroxylamine or oxidized to a stable nitrene adduct. It can also disproportionate to generate both the hydroxylamine and nitrene adducts. In cells and *in vivo* there are a number of competing reactions that can affect the yield of DMPO–protein adducts. Reduced glutathione (GSH) and L-ascorbate (ASCH) [28] can react with protein radicals faster than the rate of reaction with DMPO, resulting in reduced yield of protein–DMPO nitrene adducts, a repaired protein and a less reactive radical (*i.e.*, GS• and ASC•). Oxygen, the best spin trap in nature, can also react with protein radicals to form protein–peroxyl radicals, which very slowly react with DMPO. Protein radicals can react with other radical sites in the same or different proteins to form cross links (*e.g.*, Tyr–Tyr, His–His or Trp–Trp). Protein radicals can also react with lipid radicals, •NO₂/•NO or drug/toxicant radicals, thus resulting in protein–lipid or protein–drug/toxicant adducts (P–R).

These basic concepts brought our attention to the development of an immunoassay for the DMPO motif in protein–DMPO nitron adducts (Fig. 2), thus bringing the power of immunological techniques to the growing field of free radical biochemistry in medicine [21]. We called this technique immuno-spin trapping (IST) [22]. The basic idea of IST arose from the observation in Mason's laboratory that when the DMPO•hoMb (horse heart myoglobin) radical adduct decayed, the unique chemical bond formed by the reaction of the hoMb [Tyr103] radical and DMPO persisted [23]. MS analysis showed that this ESR-silent species was the one-electron oxidation product of the radical adduct, the nitron adduct. Once the nitron adduct is formed, it is a specific marker for the radical and for any analytical method that can be used to detect it [e.g., immunochemistry, high-performance liquid chromatography (HPLC), mass spectrometry (MS), MS/MS (tandem-MS), NMR, and magnetic resonance imaging (MRI)] [21,24]. The impressive sensitivity, power and relative ease of immunological techniques led to the production of antibodies to DMPO as a specific radical probe.

3. Anti-DMPO antibodies detect the DMPO motif of protein–DMPO nitron adducts

Polyclonal and monoclonal antibodies have now been produced against the nitron spin trap DMPO [23]. Because of the hapten characteristics of DMPO (i.e., its low molecular weight), to raise an antiserum that specifically recognizes DMPO, it was necessary to synthesize a DMPO–protein immunogen [23]. The production of the immunogen required the synthesis of a DMPO-linked compound, in this case octanoic acid (OA), followed by its reaction with a carrier protein. The nitron group

does not occur in nature and has proven to be highly antigenic, as is the related nitro group [20]. The compound 5,5-dimethyl-2-(8-octanoic acid)-1-pyrroline *N*-oxide (DMPO–OA) was chosen as a hapten because it mimics a nitron–protein adduct and provides more access for antigen presentation during the immune response. Originally, the DMPO–OA hapten was conjugated to chicken egg albumin to produce the immunogen, which was further used to immunize rabbits and produce the anti-DMPO polyclonal antiserum. More recently, a monoclonal anti-DMPO (hybridoma clone N1664A) has been developed from the original DMPO conjugated to chicken egg albumin [25]. Both polyclonal and monoclonal anti-DMPO immunoreagents are commercially available.

In the original experiments, anti-DMPO bound to Mb–DMPO adducts was 50% displaced by 30 nM DMPO–OA, which showed the high affinity of the DMPO motif–antibody interaction. The sensitivity advantage of IST over traditional ESR–spin trapping is that a stable product, not a transient adduct, is being measured [20]. Recently, Summers et al. produced a chicken IgY antiserum against a DMPO–OA–BSA immunogen and used it to detect DNA–DMPO adducts [26]. Importantly, anti-DMPO IgY binds nonspecifically to agarose beads commonly used for immunoprecipitation experiments; therefore, the same authors [26] suggested the need for a precleaning step to eliminate this nonspecific binding by incubating the IgY with 1% agarose in a blocking buffer for 2 h at room temperature. Moreover, rabbit monoclonal IgG and goat anti-DMPO antibodies against DMPO–OA–BSA and DMPO–OA–keyhole limpet *hemocyanin* have been produced and are awaiting validation (Ramirez Laboratory, unpublished data).

4. Considerations in the use of DMPO to study protein radicals in cells, tissues and whole animals

If a free radical does not react with a spin trap, it will rapidly decay to more stable, non-radical products and become undetectable by spin trapping. For this reason DMPO must be present while the free radical still exists [20]. Therefore, stored samples of cells or tissues are of no use for spin trapping or IST assays.

In most cases in cells or whole animals, the higher the concentration of DMPO the better for IST assays because both reduced glutathione (GSH), and L-ascorbate will react faster [27,28] with free radicals than DMPO will, or because they exist in a higher concentration than the spin trap (e.g. GSH) (see competing reactions in Fig. 1). Therefore, DMPO in cells or tissues will trap a relatively small fraction of the free radicals formed unless DMPO concentrations are high enough to outcompete other reactions of the free radicals, including, for example, peroxy radical and dityrosine formation [21]. *In vitro*, DMPO concentrations as high as 100 mM are routinely used in incubation studies for 24 h with no changes in cell viability [29].

In this situation, the antioxidant nature of DMPO becomes apparent, and secondary and tertiary events including tissue damage and toxicity can be prevented [29]. For this reason a thorough study may require a range of non-cytotoxic DMPO incubation conditions (i.e., concentrations and incubation times). For exploratory work 2–100 mM DMPO is adequate. The final concentration of DMPO should be as high as possible, but not so high that it affects the biochemistry of the system under study [29].

In view of the necessary high concentrations of DMPO, it is fortunate that DMPO has low toxicity. At 50 mM, DMPO does not affect trypan blue exclusion or the clonogenicity of Chinese hamster ovary cells, but it does prevent colony formation by 9 L tumor cells [30]. DMPO administered to Sprague Dawley rats at 2.32 g/kg body weight (average concentration of 20.5 mM) is nontoxic, so necessarily high concentrations of DMPO can be used with little side effect [12].

In addition to all the usual controls, a typical IST assay in cells or whole animals should have an additional control, the no-DMPO control [31,32]. As with all polyclonal antibodies, non-specific binding can occur with the anti-DMPO antiserum. For instance, with the polyclonal rabbit anti-DMPO, non-specific binding to catalase in hepatocytes has

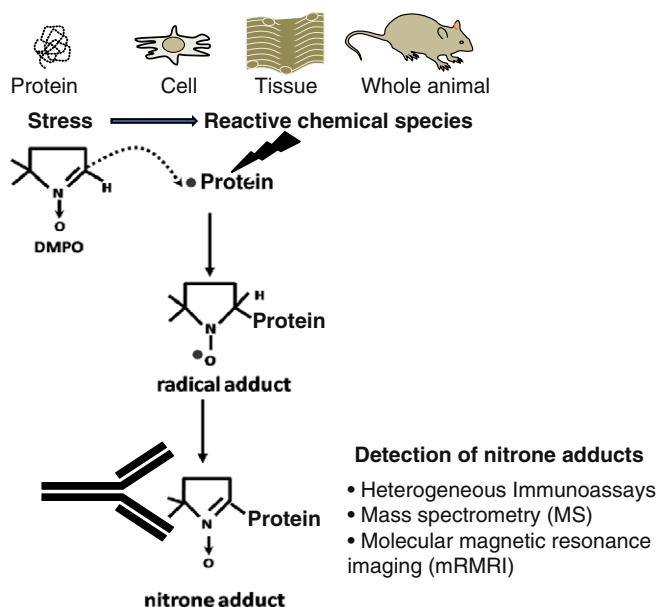


Fig. 2. Principle of immuno-spin trapping of protein radicals. Immuno-spin trapping can be applied to investigate protein radicals in purified systems, cells, tissues and in the whole animal. The production of protein radicals is caused by one-electron-mediated oxidation of specific residues in a protein. These residues can be primary or secondary targets. Protein radicals are trapped *in situ* by DMPO to form protein–DMPO radical adducts. With time, a radical adduct decays to form a stable DMPO–protein nitron adduct. The DMPO motif of a protein–DMPO nitron adduct can be detected with an anti-DMPO antibody using immunoassays. Protein–DMPO nitron adducts can be pulled down by immunoprecipitation from complex mixtures such as homogenates of organelles, whole cells or tissues and then characterized using MS. To preserve tissue architecture, protein–nitron adducts can be observed using the anti-DMPO antibody and fluorescent or immunogold techniques. Protein–DMPO nitron adducts can also be detected using non-invasive techniques such as molecular magnetic resonance imaging (mMRI).

been noticed [33]. This non-specific binding is independent of DMPO and therefore was obvious in the no-DMPO control. The greatest strength of IST, like spin trapping with ESR itself, is that false positives, although possible, are very rare. This absence of false positives is in contrast to other methods of detecting free radical-mediated oxidative modification of macromolecules. For example, the carbonyl assay, the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine, and many other assays are characterized by high background signals of unknown origin. However, one should also consider artifactual sources of nitron adducts produced by the chemistry of spin trapping [16,18].

Any *in vivo* microenvironment that has the capability of forming a free radical certainly will have the ability to oxidize the radical adduct to its stable nitron [20]. Although the oxidation of the radical adduct to the nitron is a necessary step for detection with anti-DMPO, in principle the oxidation could occur *ex vivo* without compromising the assay. Unless either the radical or hydroxylamine adduct chemically fragments, as is known to occur for the superoxide and thiyl radical adducts, the nitron adduct, as the only stable product, must accumulate until all of the radical and hydroxylamine adducts decompose one way or another [17].

As with any method, attention must be given to certain apparently small details if failure is to be averted. First, a horseradish peroxidase secondary antibody will be positive everywhere a protein with peroxidase activity is present, which usually occurs in tissues. This is true for all immunochemical detection using a horseradish peroxidase-labeled secondary antibody. When hemoproteins are known to be present, our laboratory uses an alkaline phosphatase-conjugated secondary antibody [31,32]. Moreover, DMPO and nearly all organic compounds are contaminated with trace quantities of H_2O_2 and reducing substances. All chemicals should be as pure as possible, because the assay with met-hoMb/ H_2O_2 is as sensitive for H_2O_2 as Amplex Red. In the hoMb/ H_2O_2 system, the DMPO-hoMb nitron could easily be detected by ELISA with only 1 μM H_2O_2 [23]. Whether the assay detects a hoMb radical or H_2O_2 is only a matter of perspective.

5. IST study of protein radicals in purified proteins

Since the production of the anti-DMPO antibody [23], a number of protein radicals have been detected by DMPO-based IST [21,32], including myoglobin [23,34–36], hemoglobin [22,35,37,38], cytochrome c [39], neuroglobin [40], Cu, Zn-superoxide dismutase (SOD1) [41–43] and Mn-SOD [44], catalase [33], myeloperoxidase [45,46], eosinophil peroxidase [47], lactoperoxidase [48,49], microglobulin [50], albumin [43,51], thyroid peroxidase (TPO) [25], α -lactalbumin [52] and Ras GTPase [53]. Oxidants used to generate these radicals included H_2O_2 , peroxyxynitrite, $\text{Cu}^{2+}/\text{H}_2\text{O}_2$, hypochlorous acid, *tert*-butylhydroperoxide, peroxyxynitrate, and reactive species generated from ketoprofen plus UV-A, riboflavin plus UV-A irradiation and nano- TiO_2 plus UV-A irradiation. Below, we briefly discuss a few examples of these studies of protein-centered radicals formed in models that use purified proteins treated with a bolus or slow generation of oxidants in the presence of DMPO, and highlight the medical significance of the biochemical findings, challenges, pitfalls and further considerations of the IST assay.

Myoglobin radical formation was observed in rat heart homogenates exposed to H_2O_2 by ESR-spin trapping [54] and IST [23]. The site of DMPO trapping on hoMb was determined to be Tyr103 by MS/MS and site-specific mutagenicity experiments [34,55]. The LC-ELISA-MS-based strategy [36] is a powerful approach to identifying the sites of DMPO reaction with specific radicalized amino acid residues in the model hemoproteins, such as sperm whale Mb, horse heart Mb and human oxyHb. Importantly, this study by Lardinois et al. [36] showed that peptides containing Tyr-DMPO adducts are better preserved under neutral or basic conditions. In fact, if a DMPO-protein nitron adduct is not found, it does not mean that the protein was not radicalized, but that the reaction of DMPO with the radical site may have been slower than

the reaction of the radical by other pathways (see competing reaction in Fig. 1).

Although a number of studies have identified sites of DMPO binding to proteins, these sites may or may not be the major sites of free radical damage or the cause of the observed effect on the change in function of the protein. Indeed, one of the major problems that potentially affect both traditional spin trapping and IST data is the rapid electron transfer between amino acid residues. Radical chemistry studies indicate that electrons can delocalize between Tyr, Trp, Cys, Met, and probably His residues, in proteins *via* electron or hydrogen atom transfer reactions. Then the spin trap will react with them at different rates, causing disequilibrium in the populations of radical sites in the protein. Therefore, a minor radical site in a protein can appear to be a major site of damage because of its rapid reaction with the spin trap or its greater accessibility. Thus the major radical site detected by DMPO-based IST in a protein may be determined more by the rate of addition of DMPO (and subsequent rate of oxidation of the radical adduct than by the original concentration/abundance of the radical. Bonini et al. [33] found that *in vitro* and in mouse hepatocytes, the reaction of catalase with HOCl produced a protein radical, which is a primary event leading to catalase aggregation and loss of function. Catalase was immunoprecipitated with an anti-catalase antibody from hepatocytes treated with HOCl and DMPO, and then probed with an anti-DMPO antibody [33]. However, protein radicalization in cells exposed to a stressor and DMPO can be obtained when the anti-DMPO is first used to immunoprecipitate total adducted proteins, which can then be separated and identified [29]. A protein's sensitivity to radicalization in a stressed cell depends on many factors, including its proximity to the source of oxidants, local concentration of oxidants, protein conformation, antioxidants in its microenvironment, etc. [56].

Ehrenschaft et al. [25] used IST to investigate protein radical formation in the reaction of TPO with H_2O_2 , its normal substrate. IST has reduced the amount of protein needed for detection of radicals by 1000-fold, from milligrams to micrograms [25]. We expect that the use of IST in TPO studies will provide a valuable tool for revealing the catalytic processes of this essential peroxidase. In order to translate these findings to the field of therapeutic interventions, it would be necessary to adjust these experiments to a cell or *in vivo* model of thyroid deregulation by iodine deficiency or exposure to thyroid toxicants.

6. Protein radicals in models of diseases

Detection of protein radicals *in vivo* remains a major challenge in free radical research. Protein-DMPO radical adducts have been studied using IST in models of amyotrophic lateral sclerosis or Lou Gerig's disease [57], idiopathic agranulocytosis [45,46,58–60], host-parasite interaction [61,62], sepsis [63–65], LPS-induced lung injury [63], ischemia/reperfusion (I/R) [39,66–70], asthma [43,47], diabetes [71,72] and obesity [73,74]. These studies may provide a molecular mechanism of tissue dysfunction in toxicology and pathology. Some considerations in the trapping of radicals and detecting protein-DMPO *in vivo* have been discussed in Section 4.

Before briefly discussing a few advances, challenges and pitfalls of IST, two key points should be considered in the study of protein radicals *in vivo*. Firstly, the fact that a particular protein can be detected as a DMPO nitron adduct does not mean that it is important in pathology or damage. It may be exactly the opposite. The radicalized residue in a protein may have been detected because it did not undergo other reactions rapidly, and thus may be essentially an irrelevant species in pathology. It may be that radicals detected by IST are those that do not react rapidly with oxygen (e.g., Tyr and Trp residues) or do so reversibly (e.g., Cys). Thus, in general, only radicals that do not undergo very rapid alternative reactions (see Fig. 1) appear to react competitively with DMPO and, hence, give species that can be detected by spin trapping and IST. Secondly, the reaction of DMPO with reactive chemical species can affect the effective concentration of the spin trap to react with protein radicals. Recently, we found that DMPO can prevent the activation of macrophages by

interfering with ROS-mediated signaling pathways that lead to the activation of the master regulator of the inflammatory response nuclear factor (NF)- κ B [75]. Most recently, Villamena's team [17] has investigated the thermodynamics of the reactions of DMPO and other nitrones with a number of reactive nitrogen species, including \bullet NO, \bullet NO₂, ONOOCO₂[−] and ONOO[−], which are known to be generated in LPS-activated macrophages. This work indicated that these reactions of spin traps with reactive species should be carefully considered because they can diminish the local concentrations of the spin trap at sites of inflammation where protein radicalization more likely occurs.

Protein radicals have been determined in host–parasite interaction models. One of these studies from Radi's team has shown peroxynitrite-mediated oxidative killing of *Trypanosoma cruzi* trypomastigotes with formation of DMPO nitron adducts when phagocytized by J774.1 macrophages activated with interferon- γ and lipopolysaccharide (LPS) [62]. This study showed the trapping of a protein radical in a unique targeted compartment by loading a parasite with the spin trap, but did not consider protein radical formation in other compartments of the activated macrophage. See Section 7 and [29] for a discussion on the study of protein radicals in whole macrophages activated with LPS.

Recently Chatterjee and collaborators [64] reported the radicalization and trapping of carboxypeptidase B1 in the spleens of mice treated with a single bolus dose of LPS – a model of sepsis and DMPO. However, a careful assignment of a protein target of oxidation in cell or tissue homogenates should include an anti-DMPO Western blot of the total homogenate instead of immunoprecipitated anti-DMPO adducts with or without further detection of the specific protein [29,76].

Dogan et al. [70] observed that DMPO prevented hepatic damage caused by reperfusion as assessed by reduction in the markers of hepatic damage, most likely by trapping protein and DNA radicals. Interestingly, their data showed that most nitron adducts were localized in the cytosol as well as the mitochondria and the nucleus. The marked reduction in I/R induced thiobarbituric acid-reactive substances and 4-hydroxynonenal adducted proteins may indicate that DMPO can also trap lipid-centered radicals or, alternatively, interfere with the chain of lipid peroxidation as previously suggested [77]. However, the development of a high-throughput immunoassay to determine DMPO-lipid nitron adducts remains a challenge.

A recent study from Towner's team [78] used a novel anti-DMPO probe (Fig. 3A) to study *in vivo* protein/lipid radicals in tissues of diabetic mice using a non-invasive, molecular magnetic resonance imaging (MRI) technique (Fig. 3B). The location of the anti-DMPO probe in excised tissues (lungs, liver and kidneys) was confirmed by confocal imaging *via* conjugation of streptavidin-Cy3, which targeted the probe biotin moiety. To our knowledge this is the first report showing protein radicals in a mild *in vivo* model of chronic low-grade inflammation. Appropriate controls included non-diabetic mice administered the anti-DMPO probe and diabetic mice administered a non-specific IgG contrast agent of a similar molecular weight to the anti-DMPO probe [78]. However, because of the molecular size of the anti-DMPO MRI molecular probe developed by Towner's group, the permeability throughout the intercellular space may limit its utility to detect protein/lipid radicals in hypoxic environments where blood perfusion is usually poor, but where inflammation and ROS can cause free radical-mediated damage. This group is now considering generating molecular MRI probes using nanoparticles and Fab antibody fragments, which may decrease the molecular size of the molecular imaging probes.

7. The future of immuno-spin trapping of protein radicals

Protein carbonyl formation is one acceptable marker of protein oxidation [79]. See also [80] in this special issue. Previously we found that trapping of protein radicals with DMPO prevented carbonyl formation [51]. A similar effect was observed in RAW264.7 macrophages activated with LPS. Ramirez's team has developed a high-throughput, cell-based, anti-DMPO immunoassay that was used to quantify protein–DMPO

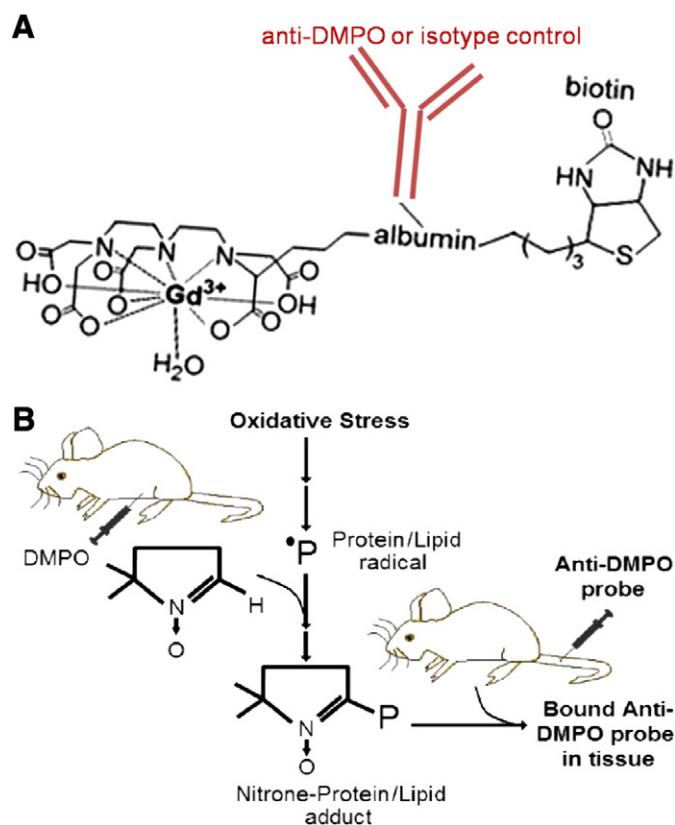


Fig. 3. *In vivo* molecular MR imaging (mMRI) of DMPO nitron adducts. A) Schematic representation of the anti-DMPO probe used to detect free radicals forming membrane-associated-DMPO nitron adducts; B) scheme of the experimental design to form membrane-associated radicals and detection of these radicals using the probe shown in A with mMRI. See Ref. [78] for further details.

nitron adducts in LPS-activated RAW264.7 cells and helped to define the best conditions for further characterizing, identifying and localizing protein radicals formed in LPS-primed cells [29,81]. This development may help advance the study of free radicals in high-throughput formats.

Radicalomics, the study of all protein radicals formed in time and space at a defined time or condition, is a very challenging area not only because free radicals can be formed in proteins [9], DNA [82], lipids [77] and even polysaccharides [83], but because several different free radicals can be formed within a single macromolecule and its constituents (amino acids, nucleotides, fatty acids and monosaccharides). More challenging yet is the fact that in a single constituent, the unpaired electron can delocalize between atoms and thus would determine the site where the DMPO will react and whether the radical adduct will be oxidized to the corresponding nitron. These facts explain why a standard for nitron adducts has not been developed yet.

A broad search for identification and localization of specific radicalized proteins in stressed cells and the mechanism behind these findings is a challenge that Ramirez's laboratory has taken up. DMPO was found to trap radicalized proteins to form protein–DMPO nitron adducts, to decrease protein carbonyl formation, and to block LPS-induced cell death [29]. Microarray and isobaric tags for relative and absolute quantitation (iTRAQ) analyses showed that DMPO affects the transcriptome and proteome, respectively, in RAW 264.7 macrophages exposed to LPS (Ramirez laboratory, unpublished data). Moreover, confocal microscopy and cell fractionation data showed the subcellular localization of proteins labeled with DMPO (Fig. 4A). Cell fractionation data from macrophages treated with LPS and DMPO for 24 h clearly showed that DMPO nitron adducts are mainly formed in the mitochondrial and microsomal fractions [32].

To identify radicalized and DMPO-tagged proteins, total homogenates of macrophages treated with LPS and DMPO were analyzed using an

anti-DMPO Western blot and LC/MS-MS (Fig. 4B). Importantly, proteins identified in the anti-DMPO-positive bands might or might not be proteins labeled with DMPO [29]. For instance, the authors rationalized that some proteins not labeled with DMPO can be physically associated with proteins that are covalently bound to the spin trap and wrongly assigned as DMPO nitron adducts. To simplify the analysis, we prepared an anti-DMPO molecular catcher (Fig. 4C) to pull down proteins or complexes of proteins in which at least one protein was adducted with DMPO [29]. This was the first report on radicalomics that warrants further corroboration and study of specific proteins labeled with DMPO during macrophage activation and their role in the fate of macrophages at sites of inflammation. In addition, the identified proteins need to be corroborated by knocking down specific proteins and mutating specific residues where DMPO adduction should be lost.

The identification of radicalized proteins during cell stress may allow them to be included in interactomes that will help define their role in the complex cell response to stressors. One of the major challenges in the characterization of free radical oxidation of specific proteins in cells or tissues from animals is the difficulty of isolating these DMPO–protein adducts and further identification of the specific residues where DMPO is bound. Cell and tissue fractionation experiments can help, but it is important to consider that DMPO adduction to some proteins and peptide

fragments can be unstable under certain circumstances such as acid or basic pH [36]. In the damage and pathology discussed in Sections 4 and 6, the significance of the identification of radical sites in a specific protein and the identity of the protein labeled with DMPO also applies to macrophage proteins labeled with DMPO.

8. Concluding remarks

In this review article we have critically discussed recent advances, challenges and pitfalls of the investigation of protein-centered radicals using DMPO-based IST in models of purified proteins as well as in cells, tissues and whole animals. Indeed, IST has been successfully applied in mechanistic *in vitro* studies, and recently, macromolecule-centered radicals have been detected in models of drug-induced agranulocytosis, hepatotoxicity, cardiotoxicity, and I/R, as well as in animal models of neurological, immunological, and metabolic diseases. As in spin trapping, the fidelity of IST is determined by the reaction of a free radical with a spin trap, as well as the immune-specificity of the antibody, the technique used to visualize the antigen-antibody complex, and the techniques used to separate, localize and identify the site-specific reaction of the spin trap within the primary sequence of the protein. In addition, unlike other techniques used to determine protein oxidation, such as carbonyl

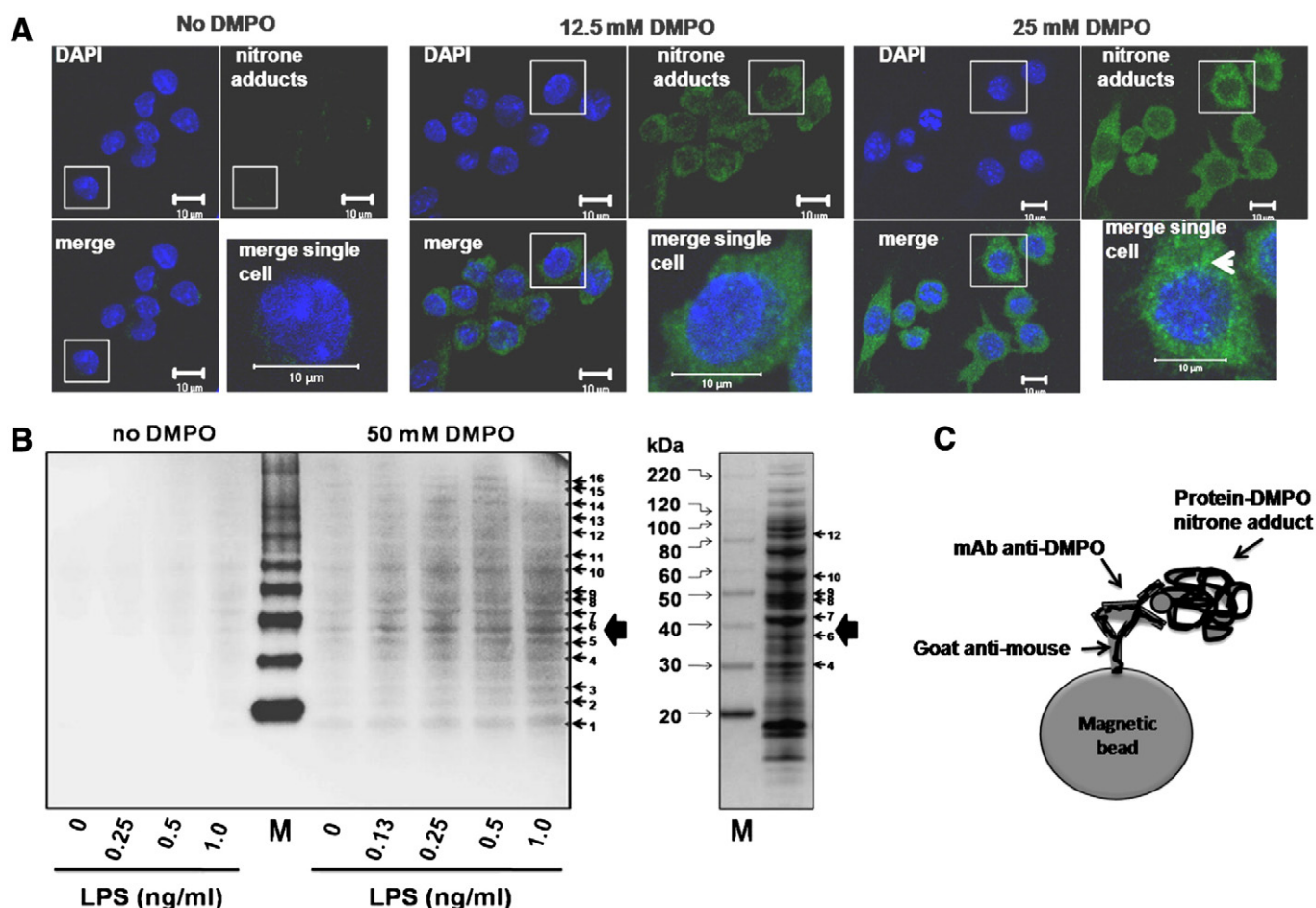


Fig. 4. Localization and identification of protein–DMPO nitron adducts in RAW 264.7 macrophages treated with LPS and DMPO. A) Single-plane confocal images of nitron adducts formed in cells treated with 1 ng/ml LPS and 12.5 or 50 mM DMPO for 24 h. Green indicates nitron adducts and blue indicates nuclei. Insert is a high-power magnification of the image of a single and representative cell. The white arrowhead indicates the perinuclear localization of most nitron adducts. B) Western blot analysis of protein–DMPO nitron adducts in homogenates of cells treated with LPS and/or DMPO for 24 h. Right panel, coomassie blue staining of the homogenate of cells treated with 1 ng/ml LPS and 50 mM DMPO for 24 h, separated in a reducing gel and showing 7 representative bands that correspond to anti-DMPO-positive bands in the Western blot. C) Schematic representation of an anti-DMPO molecular “catcher”–protein–DMPO nitron adduct complex used to pull-down proteins labeled with DMPO. M indicates Magic Mark Western XP molecular weight marker (Invitrogen). Modified from [29].

and nitration assays, and because of the promiscuity of the free radical reactions, we have been unable to produce a standard to provide quantitative data. However, DMPO nitron adducts can be presented as a signal relative to the amount of proteins or DNA in the sample or the relative intensity of images. Note that DMPO could trap a variety of macromolecular (and small) free radicals even though there are distinct reaction rate constants for each type of free radical. This relatively weak selectivity or specificity in trapping radicals should also be considered in assays of other types of macromolecular radicals.

Because the spin trap has to be present at high enough concentrations in the microenvironment where the radical is formed, the possible effects of the spin trap on gene expression, metabolism and cell physiology have to be considered. In trying to understand the role of protein radicalization in stressed cells or tissues, most published studies have been limited to immunoprecipitation of specific proteins from cell homogenates and further confirmation of DMPO adducted to a protein. However, because of the promiscuity of free radical reactions, mostly controlled by thermodynamic, kinetic and structural features, it is expected that more than one protein or amino acid residue can be radicalized. Consequently, to define the overall impact of protein radicalization on physiology, it is necessary to immunoprecipitate proteins labeled with DMPO and then confirm their identity, the site of reaction with DMPO, the location, and any change in function. Now the next goal should be to identify the specific proteins undergoing oxidation and to study the role of these modifications in pathogenesis and cell response to stress.

Finally, herein we have mentioned a number of examples of the use of IST to identify radicalized proteins in purified proteins, organelles, cells and tissues. We have also highlighted the important challenges regarding IST data interpretation: 1) the impact of the spin trap in physiology and gene expression; 2) the fidelity of spin trapping; 3) electron transfer and disequilibrium in the concentration of radical sites in the protein; 4) radical decay mechanisms and the significance of radicalized proteins in the cellular response to stress; 5) non-adducted proteins pulled down with DMPO–protein nitron adducts; and 6) the need for appropriate controls. Only after meeting these challenges will we be able to assign a role to free radical modification of proteins in medicine.

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