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Immuno-spin trapping of protein and DNA radicals: "Tagging" free radicals to locate and understand the redox process☆

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ABSTRACT

Biomolecule-centered radicals are intermediate species produced during both reversible (redox modulation) and irreversible (oxidative stress) oxidative modification of biomolecules. These oxidative processes must be studied *in situ* and in real time to understand the molecular mechanism of cell adaptation or death in response to changes in the extracellular environment. In this regard, we have developed and validated immuno-spin trapping to tag the redox process, tracing the oxidatively generated modification of biomolecules, *in situ* and in real time, by detecting protein- and DNA-centered radicals. The purpose of this methods article is to introduce and update the basic methods and applications of immuno-spin trapping for the study of redox biochemistry in oxidative stress and redox regulation. We describe in detail the production, detection, and location of protein and DNA radicals in biochemical systems, cells, and tissues, and in the whole animal as well, by using immuno-spin trapping with the nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide.

The oxidative modification of biomolecules has been systematically observed under normal and pathological conditions [1–3]. However, to understand the role that redox biochemistry has in health and disease requires the identification of the biomolecule(s) that is the target of such modification, the specific residue(s) at which the radical was first generated, the nature of such modifications, the cellular and subcellular locations of biomolecule-centered free radicals in metabolically active systems, and their localization in the whole animal.

Oxidatively generated damage to biomolecules, e.g., proteins and DNA, produces protein- and DNA-centered radicals that generally occur through initial abstraction of an electron or hydrogen atom [4,5] (Scheme 1). These biomolecule-centered radicals often decay quickly; they can react either with oxygen, resulting in fragmentation or posttranslational modifications [6,7], or with neighboring biomolecules or antioxidants, resulting in aggregation or scavenging, respectively [8]. A free radical is an atom, ion, or molecule that is usually very reactive and unstable because it has one and only one unpaired electron in an outer orbital, which explains its paramagnetic properties. Exceptions include paramagnetic transition metals like copper. The unpaired electron gives these species paramagnetic properties that make them suitable for detection by electron spin resonance (ESR) spectroscopy, the "gold standard" technique used to detect free radicals [9]. However, because of the high reactivity of protein- and DNA-centered radicals, they are generally stable for only microseconds to seconds before they decay to produce diamagnetic (ESR-silent) species, although stable protein radicals such as the tyrosyl radical of ribonucleotide reductase do exist [10].

In the spin trapping technique, a reactive radical ($R^{\cdot}$) adds across the double bond of a diamagnetic compound, known as a spin trap, to form a much more stable free radical, a nitroxide radical adduct or radical adduct, which can then be examined by ESR [9,11] (Scheme 1). This technique is called ESR-spin trapping. Spin trapping was a critical technical advance in the detection of free radicals in biology because the radical adducts, for example, lipid–radical adducts, have lifetimes of minutes and, in a few cases, even hours, which means that biological free radicals can be detected in many biological systems *in vitro* and, in some cases, even in biological fluids (bile, blood, and urine) from living animals [9,12,13]. The analyses of protein and DNA radicals by ESR or ESR-spin trapping are usually performed in

☆ This paper is dedicated to the memory of Dr. Earl R. Stadtman and Dr. Colin F. Chignell for their seminal discoveries on the biochemistry of protein oxidation and its role in cell biology.

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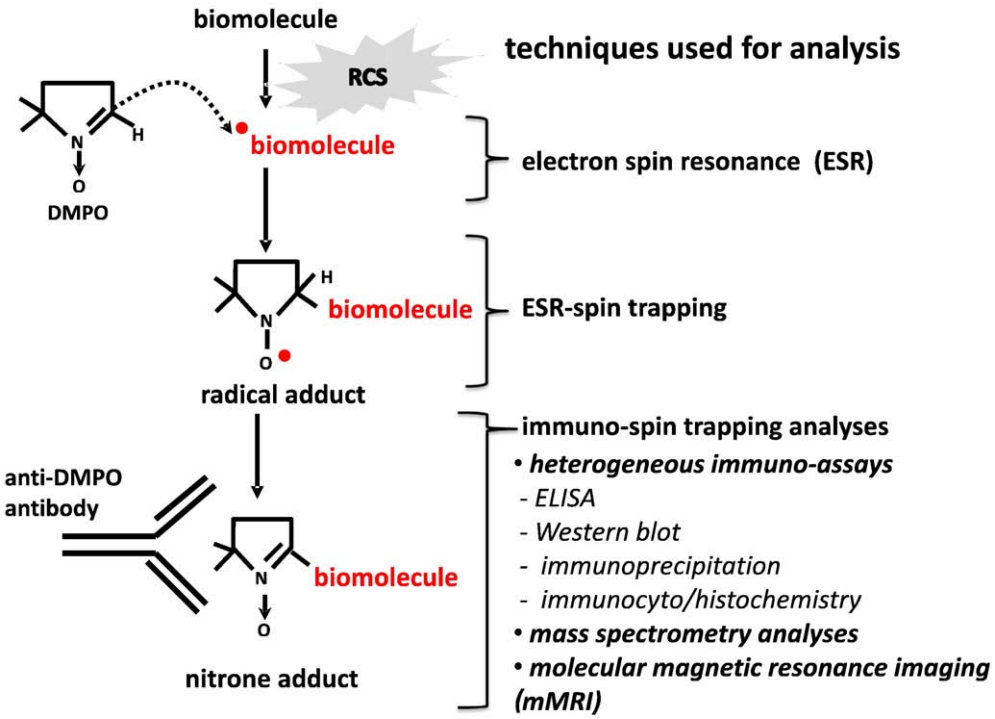


Scheme 1. Study of biomolecule-centered radicals. Reactive chemical species (RCS) (e.g., reactive oxygen and nitrogen species) react with biomacromolecules (proteins, lipids, carbohydrates, and nucleic acids), leading to the formation of biomolecule-centered radicals. Once generated, these free radicals can be detected using electron spin resonance (ESR), also called electron paramagnetic resonance. However, these species decay to diamagnetic (ESR-silent) species by a variety of mechanisms. The trapping of the radical with the nitrone 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) produces another radical species known as a nitroxide radical adduct or radical adduct, which is longer lived than the parent radical and can be studied by ESR. This technique is called ESR-spin trapping. After the paramagnetic radical adduct decays, the spin trap usually remains stably bound to the molecule and the nitrone adduct can be detected using immunoanalyses with the anti-DMPO antiserum, mass spectrometry, and in vivo molecular magnetic resonance imaging.

chemical systems by exposing the isolated cellular biomolecules [14,15] or their components (amino acids, fatty acids, bases, nucleosides, nucleotides, and sugars) [16,17] to oxidizing conditions (i.e., peroxidases/peroxides, hypohalous acids, Fenton systems, ozone, and irradiation) in the absence or presence of a spin trap followed by analysis by ESR [18] (for an example, see Fig. 1A). However, as a practical matter, the ESR or ESR-spin trapping analysis of protein and DNA radicals and their radical adducts produced in functioning cells is complex because the time required to prepare homogenates or to isolate the DNA from the biological matrix is typically much longer than the decay of the parent radical(s) or radical adduct(s) [19].

Previously, we have published step-by-step protocols for the immuno-spin trapping analysis of protein- [20] and DNA-centered [19] radicals. Those protocols have been used as a basis for expanding the field of biomolecule-centered free radical detection in cell, tissue, and whole animal models (Table 1). In this update, the production and detection of protein and DNA radicals in biochemical, cell, tissue, and whole-animal systems using immuno-spin trapping with the nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) are described. Immunoanalyses, mass spectrometry (MS), and molecular magnetic resonance imaging (mMRI) are used to investigate the resulting nitrone adducts.

Principles

Among the spin trap compounds, the nitrone spin trap DMPO is the least toxic and has convenient pharmacokinetics (uptake, distribution, metabolism, and excretion) in biological systems, i.e., cells, parasites, and animals [21]. DMPO, which is soluble in water and organic solvents, can access any cellular compartment and thus can trap, *in situ* and in real time, protein- and DNA-centered radicals whenever and wherever they are produced. The adducts thus formed (DMPO–biomolecule adducts) remain stably bound in most cases, thereby facilitating their extraction and immunoanalysis as nitrone adducts, which are usually as stable as DMPO itself [19,20,22] (Figure 1C). Accordingly, we have developed a new technology to detect protein [22–24] and DNA radicals [19,25], which we have named immuno-spin trapping (Scheme 1). See Table 1 for a complete list of references on immuno-spin trapping.

Immuno-spin trapping combines the specificity of spin trapping with the specificity and sensitivity of antigen–antibody interactions by detecting the nitrone moiety in DMPO–protein or DNA–radical-derived nitrone adducts (hereafter referred to as nitrone adducts) with a rabbit antiserum against DMPO (Scheme 1). Protein and DNA radicals are trapped *in situ* and in real time by DMPO and form radical adducts. The radical adduct decays by oxidation, to form a nitrone adduct that is recognized by the anti-DMPO antiserum. The anti-DMPO antiserum recognizes DMPO, but not the molecule to which DMPO is bound. The nitrone moiety in DMPO makes it highly antigenic.

The anti-DMPO antibody was produced as described in Detweiler et al. [23]. Briefly, DMPO was conjugated to an octanoic acid (OA) chain in the C2 position of the pyrroline *N*-oxide ring. The resulting hapten (DMPO–OA) simulates the trapping of a radical by DMPO. The DMPO–OA complex was then conjugated to ovalbumin (OVA) to produce an immunogen (DMPO–OA–OVA) that was used to immunize rabbits following an immunization plan. This antiserum was used to develop and validate immuno-spin trapping [24] and recognizes the hapten (DMPO–OA), free DMPO, and protein– or DNA–DMPO nitrone adducts. Basic step-by-step protocols for immuno-spin trapping of protein- [20] and DNA- [19] centered radicals have been published elsewhere and are updated in this paper.

Because the anti-DMPO antiserum recognizes DMPO, the immunoanalyses of DMPO–biomolecule nitrone adducts are heterogeneous, i.e., they require separation of biomolecule-centered radicals from free DMPO and DMPO–small molecule-centered radicals

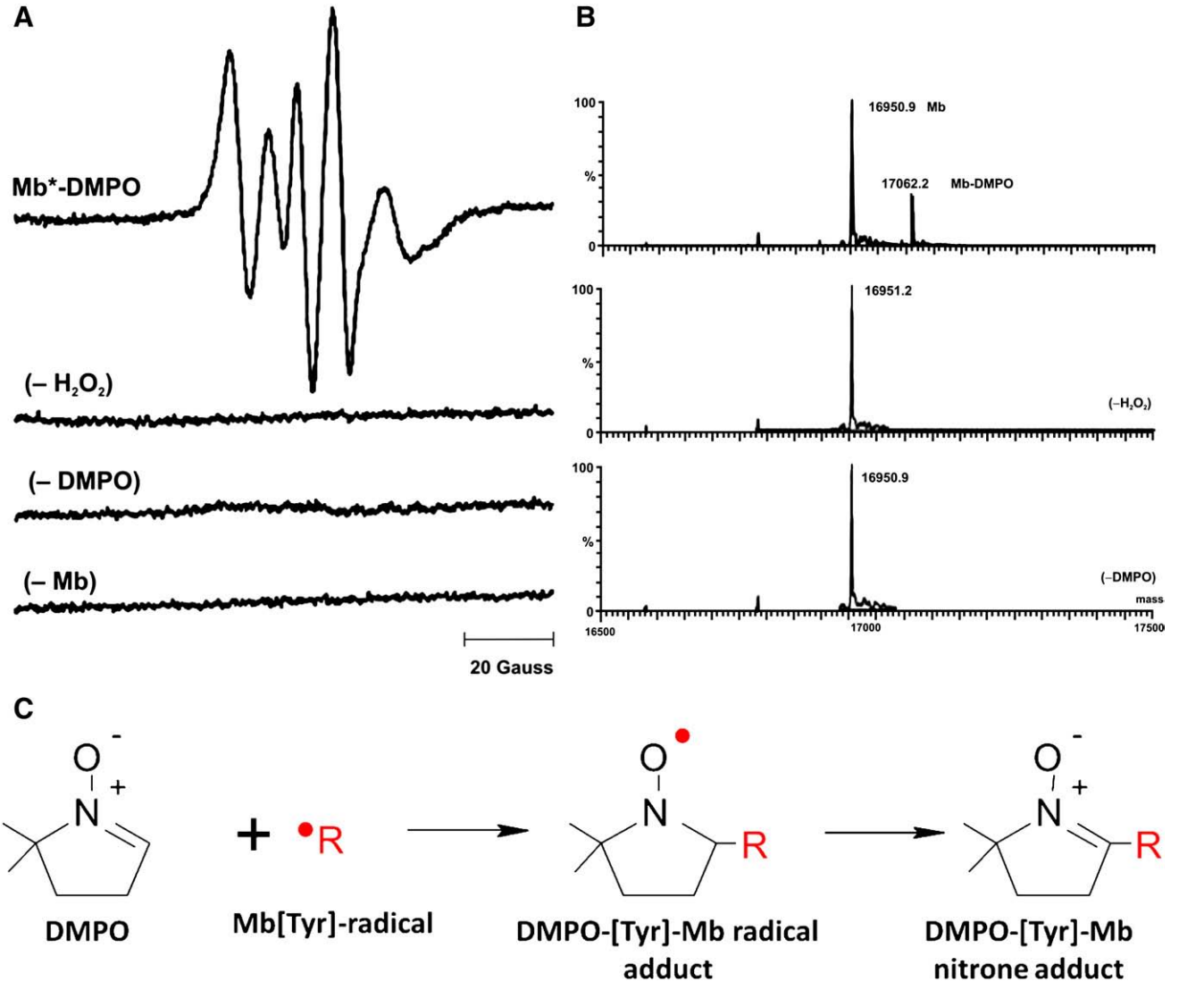


Fig. 1. DMPO traps biomolecule-centered radicals by forming a new covalent bond with the biomolecule. Incubation of horse heart myoglobin (Mb), which has a tyrosine residue at position 103, with hydrogen peroxide in the presence of the nitrone spin trap DMPO produces a radical adduct. (A) The ESR spectrum that is characteristic of DMPO bound to a Tyr residue in Mb forming a radical adduct. The omission of one or more components in the reaction prevents the formation of the radical adduct, as shown. (B) The deconvoluted electrospray ionization mass spectra of the intact protein showing the change in molecular mass of myoglobin by 111 Da, corresponding to the addition of DMPO and loss of two protons. This change is not observed if DMPO or H_2O_2 is omitted. (C) Schematic representation of the trapping of the myoglobin–tyrosyl radical with DMPO forming the nitroxide radical adduct and the decay to the nitrone adduct that is recognized by the anti-DMPO antiserum. (A) and (B) were modified with permission from [23].

(Note: DMPO-dithiothreitol nitrone adducts bind to solid supports) [26]. It is a major advantage, however, that the nitrone group does not exist in nature, thus eliminating the possibility of a false positive from endogenous cellular components. Thus immuno-spin trapping involves three main steps [19,20]: (i) trapping macromolecule-centered radicals to form stable nitrone adducts *in situ* and in real time, (ii) separation and/or extraction of the nitrone adducts, and (iii) immunodetection/localization of the nitrone adducts.

Materials

Note: Not all the reagents and equipment that follow are needed for each variant of immuno-spin trapping assay. Before starting, read the entire protocol to determine which reagents and equipment will be needed for a particular option of the immuno-spin trapping assay.

We recommend the preparation of myoglobin (Mb) or hemoglobin (Hb) nitrone adducts for initial training in immuno-spin trapping and as positive controls [20,23].

For production of nitrone adducts in biochemical systems

- Chelex 100 ion-exchange resin, analytical grade, 100–200 mesh (Bio-Rad Laboratories, Hercules, CA, USA; Cat. No. 142-2832).
- 100 mM chelexed sodium phosphate buffer (PB). This is prepared by treating 100 mM sodium phosphate buffer with Chelex following the published method [27].
- Calcium- and magnesium-free Hanks' balanced salt solution ($HBSS^-$) without phenol red (Invitrogen; Cat. No. 14175-103).
- Diethylenetriaminepentaacetate (DTPA; Sigma; Cat. No. D1133). Prepare a 100 mM solution in 1 M NaOH and adjust the pH to 7.2 ± 0.2 with concentrated HCl before adding to the reaction mixture or to $HBSS^-$.
- DMPO, ultrapure (Alexis; Cat. No. ALX-430-090-6001). Its molar concentration is about 10 M; extinction coefficient at 228 nm (ε_{228})= 7800 M^{-1} cm^{-1}.
- DNA sodium salt, from calf thymus (Sigma; Cat. No. D3664). To prepare a calf thymus DNA stock solution (1 mg/ml), dissolve 2 mg of lyophilized calf thymus DNA in 2 ml of 100 mM chelexed PB, pH

Table 1
Immuno-spin trapping analyses of biomolecule-centered free radicals

System	Immuno-spin trapping technique	Model	Reference
Whole animal	mMRI	STZ-induced diabetes and SOD1^{G93A}Tg rats	[48]
Tissue	Immunohistochemistry and fluorescence and confocal microscopy	SOD1^{G93A}Tg rats, STZ-induced diabetes, and acetone-induced protein radicals in liver	[37,43]
Cell	Immunocytochemistry, flow cytometry, cell-based ELISA[a]	Hemoglobin in red blood cells, parasites, myeloperoxidase in HL-60 cells, catalase radical in hepatocytes, DNA-centered radicals in macrophages	[19,20,24,25,34–36,38,39,42,53–56]
Homogenate	ELISA, Western blot, protein staining-LC-MS/MS	Heart and brain homogenate, mitochondria from spinal cords of SOD1^{G93A}Tg rats, SOD1-driven protein radicals, DNA-centered radicals	[20,23,25,34,35,37,41,42]
Isolated protein	ELISA, Western blot, MALDI-MS, LC-ESI-MS/MS	Myoglobin, hemoglobin, cytochrome c, human serum albumin, SOD1, thyroid peroxidase, lactoperoxidase, myeloperoxidase, NDH, neuroglobin, Mtb KatG	[20,23,24,26,30–35,40–42,50,57–63]
Isolated DNA	ELISA, immuno-slot blot	Calf thymus and macrophage DNA-centered radicals	[19,25]

mMRI, molecular magnetic resonance imaging; MS, mass spectrometry, LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; ELISA, enzyme-linked immunosorbent assay; Mtb KatG, *Mycobacterium tuberculosis* catalase-peroxidase; NDH, NADH dehydrogenase; STZ, streptozotocin; SOD1, Cu,Zn-superoxide dismutase; HL-60, human leukemia-60 cells.
[a] Cell-based ELISA of protein radicals is under development (Ramirez's laboratory).

7.4. Dialyze the DNA solution against 100 mM PB in a 10 kDa cut-off Dialyzer cassette (Pierce) overnight with three changes of buffer. Collect the DNA solution and determine the DNA concentration by measuring the absorbance at 280 nm (1 unit of absorbance of double-stranded DNA is 50 μg double-stranded DNA/ml, ~150 μM as nucleotides).

- 100 mM cupric chloride (Alfa Aesar; Cat. No. 35673) in distilled water. To prepare a 1 mM working solution, dilute the stock in distilled water.
- Hydrogen peroxide 30% (Fluka; Cat. No. H325-500). Molar concentration is about 10 M; ε_{240}=43.6 M^{-1} cm^{-1}.
- Hemoproteins. The concentration of hemoproteins, horse heart metmyoglobin (metMb; US Biologicals) and human oxyhemoglobin (oxyHb; Apex Biochemicals, Research Triangle Park, NC, USA), is adjusted spectrophotometrically by using known extinction coefficients: metMb ε_{406}=154 mM^{-1} cm^{-1} and oxyHb (Apex Biochemicals) ε_{541}=13.8 mM^{-1} cm^{-1}.
- 1 M fresh KCN in distilled water. **CAUTION: Highly toxic!**
- RAW 264.7 murine macrophage cell line (ATCC; Cat. No. TIB-71), passage 2–10 at 70–80% confluence.
- Complete culture medium: Dulbecco's modified Eagle's medium (DMEM; Invitrogen) plus 10% heat-inactivated fetal calf serum (Advantage FCS; Atlanta Biologicals).
- Ferric citrate (Sigma; Cat. No. F6129). Prepare a fresh 100 mM stock solution by dissolving the appropriate amount of powder in distilled water prewarmed to 65°C. Allow the salt to dissolve at 65°C for 1 h with occasional vortexing. Use fresh.
- 2,2′-Dipyridyl, 99+% (Aldrich; Cat. No. D216305). Prepare a 1 M stock solution in DMSO and then prepare a 100 mM working solution (100×) in distilled water.
- DMSO, anhydrous >99% (Sigma; Cat. No. D8779).
- *tert*-Butylhydroperoxide (*tert*-BOOH; Sigma; Cat. No. B2633). Prepare a 100 mM stock solution in DMSO and then a 100× working solution in $HBSS^{-}$. **CAUTION: Harmful!** Handle and dispose of following institutional safety guidelines.

For immunoanalyses

- White Maxisorp FluoroNunc 96-well microtiter plates (PGC Scientifics; Cat. No. 05-6109-00).
- Reacti-Bind DNA coating solution (Pierce; Cat. No. 17250). **CAUTION: Irritant!** Refer to instructions for proper use! A 2.5-ml volume is enough for one 96-well microtiter plate.
- Tween 20 (polyoxyethylene sorbitan monolaureate), ultrapure (USB Corp.; Cat. No. 20605).
- 10× calcium- and magnesium-free phosphate-buffered saline (CMF-PBS): 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4 7H_2O$, and 2 g KH_2PO_4 dissolved in 800 ml of distilled water. Adjust the pH to 7.4 and sterilize by filtration. One liter is enough for processing one microplate or membrane.
- Washing buffer: 1× CMF-PBS + 0.1% nonfat dry milk + 0.1% Tween 20. Five hundred milliliters is enough for washing one microtiter plate or membrane and to prepare a solution of antibodies.
- Immunoblot blocking reagent (consisting of nonfat dry milk; Upstate; Cat. No. 20-200). Ten milliliters is enough for one microplate or membrane.
- Blocking buffer: 1× CMF-PBS + 3% immunoblot blocking reagent (Pierce). Ten milliliters is enough for one microplate.
- DMPO nitrone adduct polyclonal antiserum. Prepare before use by diluting the serum to 1:10,000 (1 μl/10 ml) in washing buffer. Ten milliliters is enough for one microplate or membrane. *Note:* The anti-DMPO antiserum has been licensed to the following companies: Abcam (Cat. No. ab23702), Alexis Biochemicals (Cat. No. ALX-210-530-R100), Cayman Chemicals (Cat. No. 10006170-1), and Oxford Biomedical Research (Cat. No. RT15).
- Goat anti-rabbit IgG_{Fc} conjugated to horseradish peroxidase (HRP) (Upstate; Cat. No. 12-348). Resuspend the lyophilized powder as indicated by the manufacturer, aliquot, and keep at −80°C for up to 1 year. Avoid repeated freezing and thawing. Before use dilute 1:10,000 in washing buffer. Ten milliliters is enough for one microplate or membrane. *Note:* We have also included in this protocol the use of Immuno-Pure goat anti-rabbit IgG_{Fc} conjugated to alkaline phosphatase (Pierce; Cat. No. 31341). Follow the manufacturer's instructions to prepare this antibody.
- VisiGlo HRP Chemilu Substrate kit (Amresco; Cat. No N218-kit). Take 1 part of reagent A and 1 part of reagent B and mix by vortexing. Five milliliters of the mixture is enough for one 96-well microtiter plate and 10 ml is enough for one membrane. Let the reagents reach 15–25°C and mix immediately before use.

Instrumentation

The instrumentation used is detailed in the protocols previously published [19,20]. Any new instrumentation used in this article is listed in the respective procedure.

- Eppendorf tubes (0.65 and 1.5 ml).
- Thermomixer (Eppendorf).
- Microcentrifuge (Eppendorf).
- Automatic micropipettes (P10, P100, P1000) (Rainin).
- Multichannel micropipettes (P20 andP200) (Eppendorf).
- Infinite M200 microplate reader (Tecan, Research Triangle Park, NC, USA).
- Hydroflex microplate washer (Tecan). Optional.

- Hoefer slot-blot manifold (Amersham Pharmacia Biotech, Cat. No. PR648).
- Microplate incubator. This can be replaced by a plastic container with a lid with wet paper towels in it, placed inside a cell culture incubator.
- FluorChem HD2 Imager (AlphaInnotech, San Leandro, CA, USA) or an X-ray film developer.
- UV–Vis spectrophotometer or NanoDrop.

Instrumentation for mass spectrometry, confocal microscopy, and magnetic resonance imaging varies between laboratories. Instruments, software, and procedures are described in the corresponding sections in case the reader plans to perform the procedure in his or her own laboratory.

Protocol

Preparation of nitrone adducts in biochemical systems

Note: Among redox biochemical systems, the reaction of hemoproteins such as oxyHb or metMb with H_2O_2 is the best characterized. Hb or Mb nitrone adducts are prepared by reacting the hemoprotein (usually 1–10 μM) with a 1- or 10-fold excess of H_2O_2 in phosphate buffer and in the presence of 1–100 mM DMPO. Amine-containing buffers (for example, tris(hydroxymethyl)aminomethane, or Tris) should be avoided because of their ability to react with reactive oxygen species (ROS). Preparation of nitrone adducts in 10–50 mM ammonium bicarbonate, pH 8.0, is used in some superoxide dismutase (SOD) investigations, but not, for example, in hypochlorous (HOCl) oxidations owing to the scavenging of HOCl by the tertiary amine, forming chloramines that are also oxidants [28]. We recommend using a phosphate buffer; however, note that phosphate at high concentrations can chelate metals such as iron and copper, thus modulating Fenton chemistry. The reactions are stopped by removing excess H_2O_2 with catalase or by inhibiting the heme with cyanide or azide in peroxidase/peroxide systems or chelating agents in Fenton systems. The removal of excess reagents and DMPO by dialysis with an appropriate cut-off membrane is the safest choice. Other materials and reagents are included in the protocol.

Hb or Mb nitrone adducts are prepared as follows

Three microliters of a hemoprotein solution 100-fold more concentrated than the final heme concentration (100× stock) is added to 291 μl of 100 mM chelexed phosphate buffer, pH 7.4, containing 0.1–0.5 mM DTPA, followed by the addition of 3 μl of a 1 M DMPO solution (in water or the same buffer; the neat compound is ~10 M), and the reaction is started by adding 3 μl of freshly prepared 100× H_2O_2 solution to the reaction mixture. The reaction mixture is incubated at 37°C for 1 h with agitation in a thermomixer and stopped by removal of excess H_2O_2 with 3 μl of a catalase solution in phosphate buffer, pH 7.4 (Roche Applied Bioscience; 100 or 500 IU in 0.1 M phosphate buffer, pH 7.4). Placing the tubes containing the reaction mixture into an ice bucket will slow the radical chemistry; however, if the reaction components in excess are not removed or the reaction is not stopped, further formation of nitrone adducts continues during the analysis. Do not add Stop solution to the samples that are prepared for MS analyses. Reactions for MS analysis should be quenched by dialysis at 4°C against 10–50 mM ammonium bicarbonate buffer, pH 8.0, and then stored at −20 or −80°C until analysis (see Analysis of protein nitrone adducts). Dialysis is preferred over the addition of quenching agents as these compounds can interfere with subsequent MS analyses. DMPO is covalently bound to the atomic site of highest electron spin density. If frozen at −20 or −80°C, nitrone adducts are generally stable for years without significant alterations of the nitrone epitope(s). Representative results of ELISA and Western blot analyses of Mb–DMPO nitrone adducts and controls are shown in Figs. 2A–2D.

DNA nitrone adducts are prepared as follows

Although there are several ways to produce DNA nitrone adducts, the reaction of calf thymus DNA or DNA isolated from animal tissues with copper and H_2O_2 produces high amounts of nitrone adducts (see [19] for a detailed step-by-step protocol). DNA nitrone adducts are prepared as follows: 10 μM calf thymus DNA as nucleotide (1 unit of absorbance at 260 nm is ~50 μg DNA/ml which, in turn, is ~150 μM DNA as nucleotides) in 10 mM chelexed sodium phosphate buffer is incubated with 10 μM Cu^{2+} (chloride or sulfate), and the reaction is initiated by addition of H_2O_2 to a final concentration of 100 μM. The total volume of the reaction is usually 300 μl. After 30 or 60 min of incubation at 37°C, the reaction is stopped either by removing the excess H_2O_2 with 5 μl of catalase (10 IU), inhibiting Cu^{2+} redox cycling with 1 mM cyanide, or by chelating Cu^{2+} with DTPA (10 mM). DNA nitrone adducts can be extracted from tissues and cells exposed to oxidizing or inflammatory conditions in the presence of DMPO, with the most up-to-date and detailed protocols for production, extraction, analysis, and representative results published elsewhere [19,20].

Analysis of protein nitrone adducts

ELISA

This is a heterogeneous immunochemical technique (i.e., a procedure that requires separation between bound and unbound material in some steps) that allows simultaneous screening of many samples [29]. In this technique, 10 μl of the protein nitrone adduct solution or homogenate (1 μg/10 μl) is added to 190 μl of a 0.1 M bicarbonate buffer, pH 9.6, or PBS, pH 7.4, in 96-well microtiter plates. The protein binds to the bottom of the well through hydrophobic interactions [29]. Depending on whether the development method is chromogenic or chemiluminescent, we recommend white or transparent, respectively, high-protein-binding, flat-bottom, 96-well microplates. After coating, the nonbound material is removed by a washing step (300 μl of washing buffer per well) with a buffer containing a detergent and an inert protein to remove or avoid, respectively, any nonspecific interactions. We recommend a washing buffer composed of PBS, 0.05% Tween 20, and 0.1% nonfat milk and the use of a microplate washer. This step allows the removal of any nonprotein nitrone adducts that can interfere with the detection of protein nitrone adducts because the anti-DMPO antibody recognizes both protein-bound and free DMPO [23]. These characteristics represent one of the most important advantages of heterogeneous assays like ELISA compared to homogeneous assays.

Next, block the nonspecific binding sites with 100 μl of blocking buffer (1% nonfat milk in PBS, pH 7.4) and incubate the microplate for 40 to 60 min at 37°C. After blocking, wash the plate one time with washing buffer as before. Remove the last washing solution and add 100 μl of the rabbit anti-DMPO serum diluted in washing buffer (1:10,000). After 60 min of incubation, nonspecifically bound and unbound antiserum is removed by two or three washing steps followed by the addition of 100 μl of a goat anti-rabbit IgG conjugated to HRP. A typical dilution of the secondary antibody for ELISA is 1:10,000, and incubations are performed at 37°C for 60 min. After incubation, nonbound and weak nonspecific interactions are removed by two or three washing steps with 300 μl of washing buffer per well. Finally, immunocomplexes (nitrone adduct/anti-DMPO antibody/anti-rabbit IgG–HRP) are detected by adding 50 μl of the substrate solution, which produces luminescence (VisiGlo Chemilu HRP substrate kit; Amresco; Cat. No. 218-kit) or soluble colored products (1-Step Ultra TMB-ELISA; Pierce; Cat. No. 34028) that can be detected and measured using a microplate reader (Infinite M200; Tecan). Representative results of the analysis of Mb–DMPO nitrone adducts by ELISA are presented in Figs. 2A and 2C.

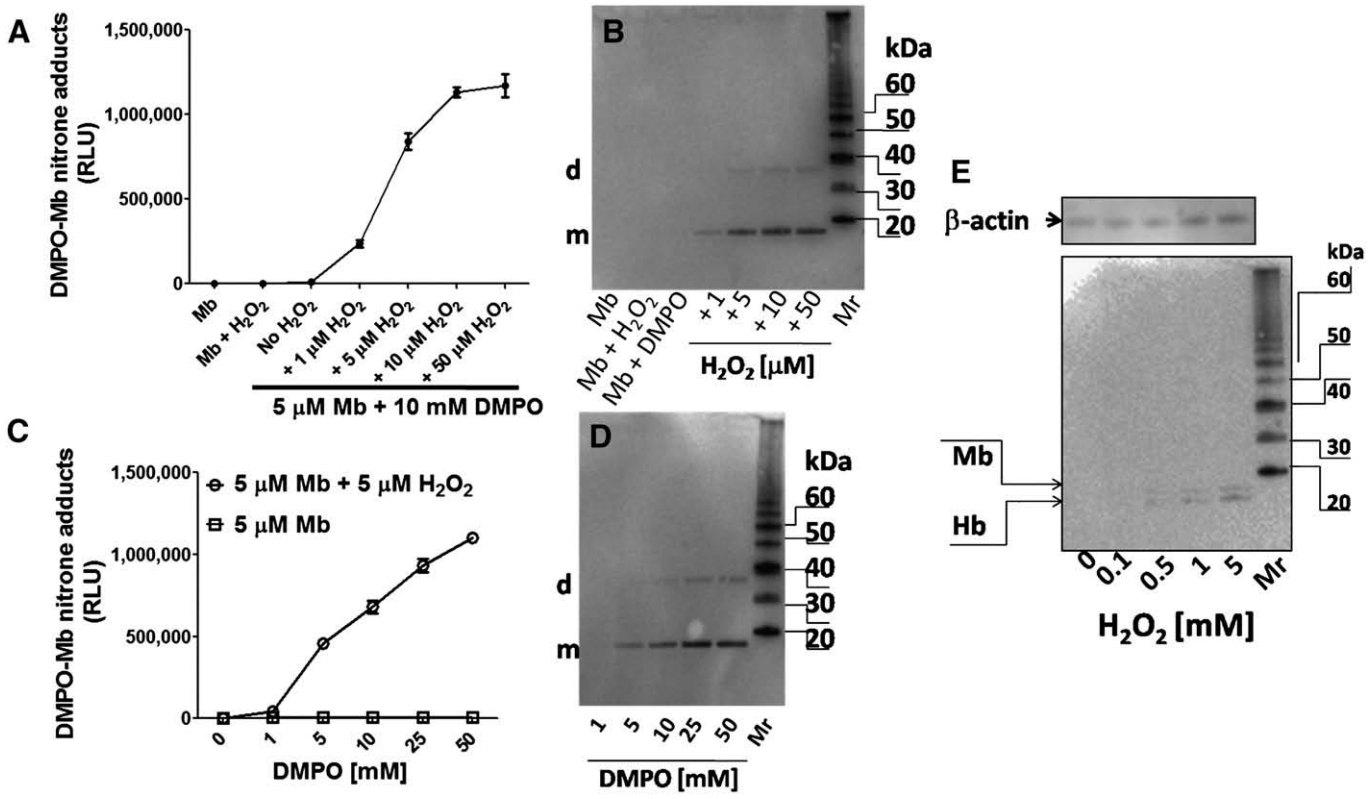


Fig. 2. Production and immuno-spin trapping with DMPO of horse and rat heart Mb-centered radicals. (A and B) ELISA and Western blot, respectively, of 5 μM horse heart Mb, which was reacted with H_2O_2 in the absence of DMPO or incubated with 10 mM DMPO in the presence of increasing concentrations of H_2O_2. (C and D) ELISA and Western blot, respectively, of 5 μM Mb and 5 μM H_2O_2 reacted in the presence of increasing concentrations of DMPO. In the Western blots m and d indicate the monomer and dimer, respectively. (E) Reproduction of results published in [23] (used by permission) showing the analysis of DMPO–protein nitrone adducts in dialyzed rat heart homogenate (0.54 mg/ml) incubated with 50 mM DMPO and various concentrations of H_2O_2. Indicated are the nitrone adducts of myoglobin (Mb) and hemoglobin (Hb) as confirmed by MS analysis [23]. β-Actin was used as a loading control and detected using a monoclonal antibody. The reactions were performed in 100 mM chelexed sodium phosphate buffer, pH 7.4, with 0.5 mM DTPA, and the total volume was 300 μl. The reagents were added from 100× more concentrated stocks in 100 mM phosphate buffer, pH 7.4. The reaction was carried out at 37°C for 1 h and stopped by adding 3 μl of a catalase solution (10 μl of catalase suspension in 990 μl of phosphate buffer containing 1 mM DTPA) and frozen until analysis. ELISAs and Western blots were performed as described. The secondary antibodies were a goat anti-rabbit IgG_{Fc} or a goat anti-mouse conjugated to HRP, and the Western blot was developed using the VisiGlo Plus Chemilu Kit and visualized in a FluorChem HD2 imager.

Western blot

To perform the Western blot analysis of protein nitrone adducts in cell or tissue homogenates, mix 30 μl of the reaction mixture or cell homogenate (0.1–1 mg/ml) with 10 μl of NuPAGE LDS sample buffer (4×) (Invitrogen; Cat. No. NP0007) and 4 μl 10× NuPAGE sample reducing agent (Invitrogen; Cat. No. NP0004). Heat the sample in a water bath at 90°C for 7–10 min, and then let it cool to room temperature. Load 10 μl of sample (1–40 μg proteins) per lane in 1.0-mm 10-well 4–12% NuPAGE Novex Bis–Tris gels (Invitrogen; Cat. No. NP0321BOX). For better standard molecular weight band visualization, we add 1 to 3 μl SeeBlue Plus2 prestained standard (Invitrogen; Cat. No. LC5925) in the first and in the middle lane of the gel. Alternatively, the use of MagicMark XP Western standard (Invitrogen; Cat. No. LC5602) helps to locate the approximate molecular weight in the developed X-ray film or imaged membrane. Perform the protein separation using an XCell SecureLock mini-cell system under continuous voltage conditions for 42 min at 200 V. Blot the separated proteins onto a nitrocellulose membrane following the manufacturer's instructions. We use a nitrocellulose membrane filter paper sandwich, 0.45-μm pore size (Invitrogen; Cat. No. LC2001), and the blotting is performed in an XCell II blot module from Invitrogen (Cat. No. EI9051).

After assembling the blot and inserting the module, blot the proteins to a nitrocellulose membrane at 40 V for 20–30 min. A semidry transfer apparatus (Bio-Rad) is advantageous with respect to time (~15 V for 20 min) and reproducibility when blotting the proteins to the membrane. After transfer is completed, the blotting efficiency can be examined by exposing the membrane to a solution of 1% Ponceau S red in 5% acetic acid for 30 s and then washing the membrane with distilled water until red bands appear in a clear background. The red bands will disappear during the blocking of the membrane.

To identify protein DMPO nitrone adducts, incubate the membrane in an appropriate, clean glass or plastic container with 10–25 ml of blocking buffer for 40–60 min at room temperature with low agitation in an orbital shaker. Blocking and washing buffer are prepared as described for the ELISA procedure. Remove the blocking buffer, add 10–25 ml of washing buffer, incubate the membrane for 10 min, and remove the washing buffer. Then add 10 ml of the anti-DMPO antiserum (1:5000 in washing buffer), and incubate for 1 h at room temperature or overnight in the refrigerator with agitation. Wash the membrane three or four times with washing buffer with 10 min of agitation each time. Remove the last washing buffer and add 10 ml of a goat anti-rabbit IgG_{Fc} conjugated to alkaline phosphatase (AP) or HRP. In either case, the dilution of the secondary antibody is 1:5000 in washing buffer. HRP produces more sensitive readings; however, some cell peroxidases and pseudo-peroxidases might react with H_2O_2 and peroxidase substrates and produce luminescence. AP is not suggested for analysis of nitrone adducts in lung tissue and macrophages, which are known to have high endogenous AP content.

Incubate the membrane for 60 min with the secondary antibody, and wash three or four times as before. Remove the last washing solution and incubate in a clean tray with 3–5 ml of the development reagent per membrane. If an AP conjugate is used as the secondary antibody, we recommend the use of CDP-Star solution (Roche; 1:1000

in Tris–HCl, pH 9.6) [20]; however, if an HRP conjugate is used, we recommend the use of a VisiGlo Plus HRP chemiluminescence kit from Amresco. In both cases, incubate the membrane with the reagent for 5 min, and then record the luminescence by using X-ray film or an imager. For a complete protocol see [20], and for representative results see Figs. 2B and 2D.

Mass spectrometry

Mass spectrometry approaches to the identification of proteins and the specific site in the primary structure of a protein with a covalently attached DMPO (amino acids at which the radical was trapped) are continually evolving [30,31]. Two ionization techniques are routinely used for the MS analysis of biomolecules: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). MALDI analysis is very fast and is routinely used for protein identification. ESI analysis is compatible with online HPLC and, therefore, is used in conjunction with HPLC for the analysis of complex mixtures. Both ionization techniques are compatible with tandem mass spectrometry (MS/MS)—the gas-phase fragmentation of biomolecules that allows for the sequence determination of peptides/proteins and the identification of specific sites of radical adduct formation.

For the identification of proteins corresponding to positive bands in Western blots of tissues or homogenates treated with an oxidant and DMPO, Western blots are compared and/or overlaid with a Coomassie-stained gel of the same sample performed in parallel [20,23,30]. After excision of the gel band and in-gel digestion, the resulting peptide mixture is analyzed by mass spectrometry. MALDI/MS analyses were used to confirm the identity of Mb from rat heart supernatant [23]. Subsequent *in vitro* reactions of horse heart metMb treated with H_2O_2 in the presence of DMPO followed by ESI/MS analyses confirmed the formation of a Mb–DMPO radical adduct (Fig. 1B) [23]. In the deconvoluted mass spectrum of the intact protein, an ion that corresponded in mass to the addition of a DMPO molecule to the protein was observed. After enzymatic digestion of the reaction mixture, LC/ESI/MS/MS was used to unequivocally assign the DMPO spin trap in horse heart myoglobin to tyrosine 103 [32,33].

We have also shown that our immuno-spin trapping approach and MS analyses are complementary. Using immuno-spin trapping, we provided the first evidence of formation of a protein radical in cells [24]. In this study we identified hemoglobin nitrone adducts in human red blood cells exposed to H_2O_2 and DMPO, and data from this study suggested that tyrosine and cysteine residues were the radical sites trapped by DMPO [30]. Later, LC/ESI/MS/MS of human oxyHb treated with H_2O_2 and DMPO confirmed these data. Using LC/MS/MS, DMPO adducts of human oxyHb were identified on cysteine 93 in the β chain and on tyrosine 24 and tyrosine 42 in the α chain. Additionally, a novel histidine residue (His 20) in the α chain was identified as a site of DMPO spin trapping (Fig. 3) [30]. In two recent studies, after Western blot analyses with the anti-DMPO antibody, myeloperoxidase was identified from the corresponding Coomassie-stained gel from HL60 cells [34,35].

Moreover, we have used mass spectrometry to study the mechanism of these free radical-mediated processes. From LC/MS/MS analyses, the identification of the primary and secondary trapping sites of radical formation in human heart Mb was determined; thereby providing insight into a potentially important mechanism of free radical damage operating in ischemia–reperfusion in the heart [36].

The MS of nitrone adducts can be analyzed from either a solution (especially in the case of *in vitro* experiments) or SDS–PAGE gels (i.e., first located by Western blot with the anti-DMPO antiserum) stained with an MS-compatible stain. For staining of the gel, we suggest Coomassie blue, Simply blue, or SYPRO ruby protein gel stain (Invitrogen; Cat. No. S12001). *Note:* Silver staining is less compatible with MS owing to a variety of reasons that, in some instances, result in the protein being covalently bound to the acrylamide gel. If possible, the samples should be free of additives such as nonvolatile salt buffers (e.g., sodium and phosphate buffers), detergents, and glycerol, as these chemicals can interfere with the analyses. We suggest that the reactions be performed in volatile buffers (e.g., 50 mM ammonium bicarbonate buffer, pH 8.0) or, alternatively, in 10 mM phosphate buffer followed by dialysis at 4°C against 10 or 50 mM ammonium bicarbonate buffer, pH 8.0. Extensive prewashing of the dialysis cassette or tube membrane with ultrapure water to eliminate preservatives is highly recommended to avoid potential interference. In biochemical systems, the reaction does not require the addition of a stopper (for example, catalase), but it does require dialysis overnight

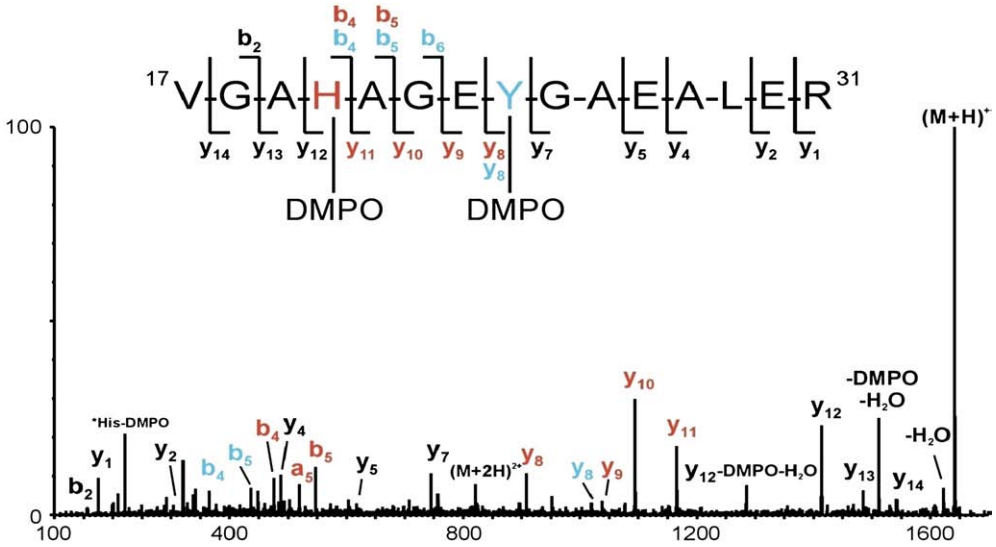


Fig. 3. Deconvoluted LC/ESI/MS/MS spectrum of tryptic peptide T4 plus one DMPO (protonated molecular ion of m/z 1640.81) of the α chain of hemoglobin. The reaction product of human hemoglobin with H_2O_2 in the presence of DMPO was digested with trypsin and analyzed by LC/MS/MS. A nearly complete series of y ions is observed as well as several b ions. Fragment ions labeled in blue correspond to the DMPO located on Tyr 24. The mass difference between the y_7 ion of m/z 745.4 and the y_8 ion of m/z 1019.5 corresponds to the mass of a tyrosine residue plus a DMPO. Fragment ions labeled in red correspond to the DMPO located on His 20. The mass difference between the y_{11} ion of m/z 1165.6 and the y_{12} ion of m/z 1413.7 corresponds to the mass of a histidine residue plus a DMPO. The ion labeled His-DMPO corresponds to the immonium ion for a histidine residue plus DMPO. Fragment ions labeled in black correspond to cleavages that can be attributed to either a His–DMPO adduct or a Tyr–DMPO adduct. From these data, it can be determined that the parent ion of m/z 1640.8 is actually a mixture of two T4 tryptic peptides, one with a DMPO located at His 20 and one with a DMPO located at Tyr 24.

at 4°C against 2 L of 10–50 mM ammonium bicarbonate buffer, pH 8.0, to eliminate excess reagents.

Example of determination of sites of DMPO spin trap: Enzymatic digestion. Hemoglobin control samples (Hb, Hb+H_2O_2, Hb+DMPO) and the hemoglobin+DMPO+H_2O_2 reaction sample are either dialyzed against or prepared in 50 mM ammonium bicarbonate buffer, pH 8.0 [30]. Samples are subjected to digestion using porcine trypsin (Promega Corp., Madison, WI, USA) at a protein:enzyme ratio of 20:1. The reactions are allowed to proceed overnight at 37°C.

Electrospray mass spectrometry. ESI mass spectra and tandem mass spectra are acquired, in our case using a Micromass Q-TOF Ultima Global (Waters/Micromass, Milford, MA, USA) hybrid tandem mass spectrometer. This instrument is equipped with a nanoflow electrospray source and consists of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer. The needle voltage is ~3000 V and the collision energy is 10 eV for the MS analyses. For the LC/MS/MS analyses, a Waters CapLC HPLC system (Waters) consisting of binary pumps and a micro autosampler is used to deliver the gradients. Injections of 1–8 nl are made on the column and a linear gradient of 5–75% acetonitrile (0.1% formic acid) over 35 min at a flow rate of ~300 nl/min is used for the chromatographic separations. The column is a 15 cm×75 μm i.d. Hypersil C18 ("pepmap") column (LC Packings, San Francisco, CA, USA).

During the LC/MS/MS experiments, we employed automated data-dependent acquisition software. For these acquisitions, the instrument can switch from the MS mode to the MS/MS mode and then return back to the MS mode based on predetermined or operator-entered parameters such as abundance and time. The advantage of this software is that both MS and MS/MS data can be acquired from a single chromatographic separation of the mixture. The collision energy for these experiments is set according to the charge state and the *m/z* of the precursor as determined from a charge state recognition algorithm. Data analysis is accomplished with a MassLynx data system, MaxEnt deconvolution software, and ProteinLynx software supplied by the manufacturer.

Immunocyto/histochemistry

The first protein radical adducts produced and trapped by DMPO inside a cell with an intact membrane were identified as hemoglobin nitrone adducts in intact red blood cells treated with H_2O_2 and DMPO [24]. The tissue [37] and subcellular [38,39] localization of DMPO–protein nitrone adducts is an extension of this study and the result of over 7 years of careful validation of the immuno-spin trapping technique in biochemical systems [19,20,23–25,40–42]. Immuno-spin trapping represents a major breakthrough for the study of biological free radical reactions because biomolecule-centered radicals are trapped by DMPO to form protein nitrone adducts, which can be detected *in situ* or isolated and characterized in detail [34,35]. For instance, with the anti-DMPO antiserum, Bonini et al. used confocal microscopy to observe that catalase–DMPO nitrone adducts were localized inside the peroxisomes of hepatocytes exposed to hypochlorite [38]. Confocal microscopy was also used to detect MPO–protein nitrone adducts in the liver in an acetone model of ketosis [43]. In this study, Stadler et al. showed that protein–DMPO nitrone adducts were located inside hepatocytes lining the centrolobular vein [43].

Production and location of protein nitrone adducts in cells by fluorescence microscopy

For fluorescence microscopy studies, we culture RAW 264.7 macrophages as previously described [19]. We use DMEM without phenol red and with 10% heat-inactivated fetal calf serum, referred to as complete medium. The cells are collected by centrifugation (200 g for 10 min at room temperature) and resuspended in complete medium at 10^3 cells/ml. A 200-μl aliquot of the cell suspension is plated onto a glass-bottomed eight-well glass-chambered slide (Lab-Tek Chamber Slide System, Cat. No. 177402; Nalge Nunc International, Rochester, NY, USA). After 24 h in a cell incubator (37°C, 5% CO_2 and 99% humidity), the monolayers are washed three times with prewarmed (37°C) PBS, pH 7.4. Then, DMPO (10–80 mM) in HBSS, pH 7.4, with Ca^{2+} and Mg^{2+} ($HBSS^+$) is typically added 15–20 min before the treatment of choice. DMPO promptly diffuses throughout cell compartments where it can trap, *in situ* and in real time, the biomolecule-centered free radicals producing nitrone adducts.

After the treatment is completed, the monolayers are washed three times with $HBSS^+$ and immediately fixed with methanol-free paraformaldehyde solution (4%) dissolved in PBS. The fixative chemistry of paraformaldehyde can be stopped by incubating the slides for 5 min with 100 mM Tris–HCl, pH 8.0. After fixation, the cells are permeabilized with cold methanol (−20°C) for 2 min. Methanol is then disposed of and the cells are washed with $HBSS^+$ four times. Paraformaldehyde is a cell fixative that produces cross-links between the proteins and the plate, and methanol dissolves the membrane lipids. After methanol permeabilization, the fixated cell structures are treated with a blocking buffer of choice for 2 h at room temperature or overnight at 4°C. We recommend the use of a source of proteins of the same origin as the secondary antibody. For example, if a horse or goat anti-rabbit IgG conjugated to HRP is used, a 1% solution of normal horse or goat serum, respectively, would be the one of choice.

After the cells are blocked and washed three times with washing buffer, the anti-DMPO antiserum, diluted 1:500 to 1:2000 in wash buffer, is added. Finally, after four washes, the Alexa Fluor 488 goat anti-rabbit IgG SFX Kit (Invitrogen; Cat. No. A31628) is used to indicate anti-DMPO binding to specific sites within the cells. Figs. 4A and 4B contain examples of DNA radical detection using immunochemistry. RAW 264.7 cells were loaded with copper or iron (100 μM) for 4 h at 37°C. The medium was removed and the monolayer was washed three times with $HBSS^+$ with 1 mM DTPA to remove the metals from the cell surface. Then 1 ml of $HBSS^+$ containing various concentrations of *tert*-BOOH was added. Fig. 4C shows the detection of nitrone adducts in macrophages treated as in Figs. 4A and 4B using immunofluorescence.

Fig. 4C shows an immunohistochemistry assay of nitrone adducts in sections of spinal cords of symptomatic $SOD1^{G93A}$ transgenic rats [37]. Interestingly, a serial injection of low amounts of DMPO (doses of 50 mg/kg) in saline 48 and 24 h before the sacrifice of symptomatic transgenic $SOD1^{G93A}$ rats has proven to restore the function of mitochondria isolated from the spinal cords of these animals [37]. Using Western blot analysis with the anti-DMPO antibody, the authors also detected increased nitrone adducts in mitochondria from the spinal cords of sick animals compared to those isolated from control animals. There were also increases in nitrone adducts in sections of the spinal cords of those sick animals injected with DMPO [37]. More recently, Stadler et al. [43] observed DMPO nitrone adducts by immunohistochemistry with the anti-DMPO antiserum in sections of livers of mice receiving acetone in their drinking water and intraperitoneal injections of DMPO (20 μl of the pure compound every 6 h for the period that the acetone treatment lasted) [43].

Localization of free radicals in the whole animal

Current methods for the *in vivo* detection of free radicals include electron paramagnetic resonance imaging (EPRI), fast EPRI, Overhauser magnetic resonance imaging (OMRI), and conventional T_1-weighted MRI. EPRI often requires the use of narrow-line spin probes, such as trityl radicals [44]. These free radical nitroxide probes are often nontoxic and biocompatible [45]. Continuous wave EPRI has a high sensitivity and general applicability for a wide range of free radicals and paramagnetic species, but it also requires long image acquisition times and, therefore, is limited in its use for most *in vivo*

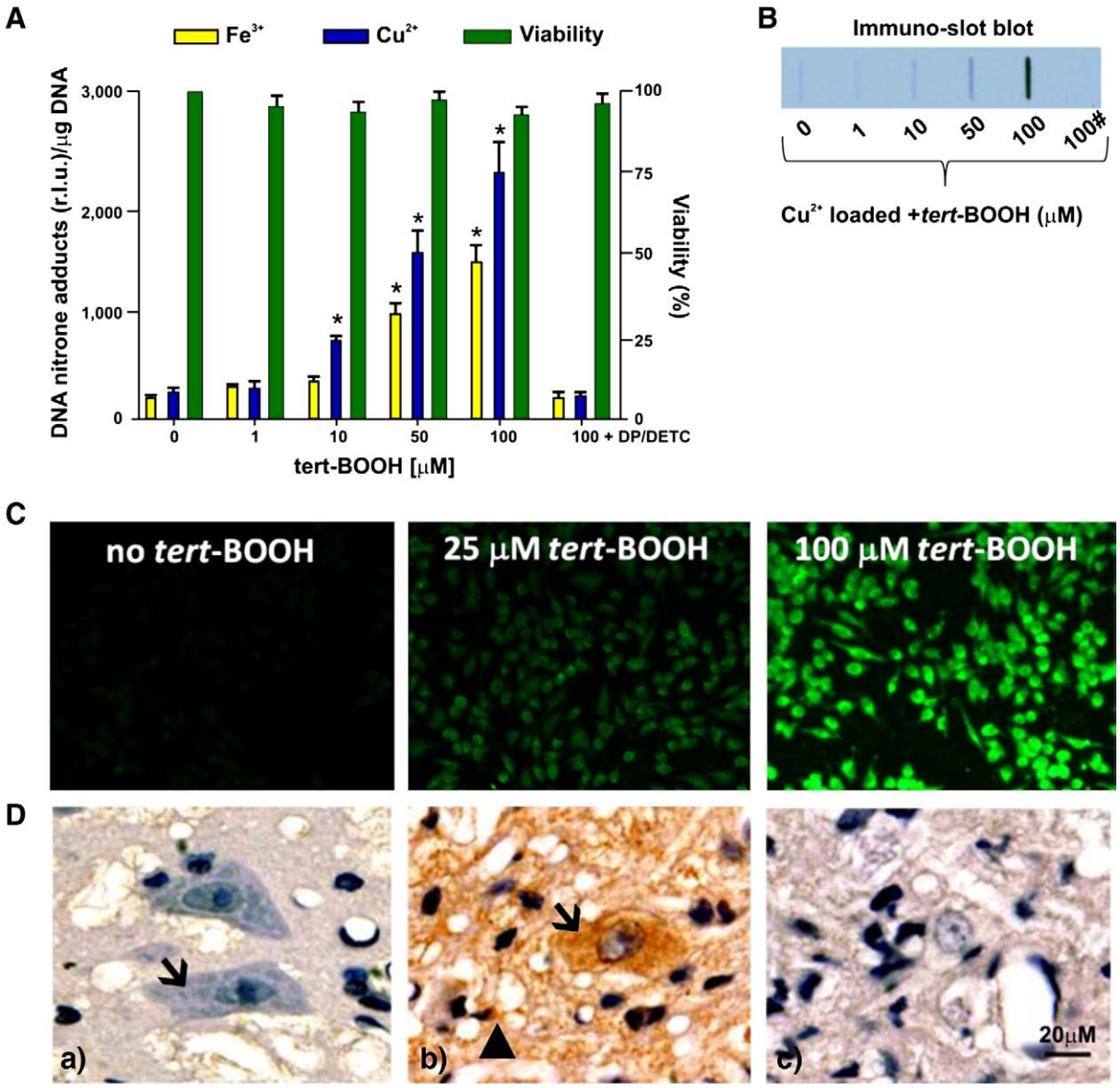


Fig. 4. Immuno-spin trapping of protein- and DNA-centered free radicals in macrophages. (A) We loaded RAW 264.7 macrophages (70% confluence) with either 100 μM ferric citrate (yellow bars) or 100 μM cupric chloride (blue bars) in complete medium (DMEM plus 10% FCS) for 18 h in an incubator. After washing with $HBSS^-$ plus 1 mM DTPA ($HBSS^-$/DTPA), we harvested and washed the cells with prewarmed (37°C) $HBSS^-$/DTPA. We counted the cells and determined that their viability (trypan blue exclusion) was above 90%. Then we divided the cells (10^6 cells/ml) in clean, clear Eppendorf tubes in 1 ml of $HBSS^-$/DTPA and added 100 mM DMPO from a 10 M stock DMPO solution. As a control, we ran experiments in which we added, at the same time as DMPO, the cell-permeative iron and copper chelators 1 mM dipyridyl (DP) and 100 μM sodium diethyldithiocarbamate (DETC), respectively. We added various concentrations of tert-BOOH (as a 100× stock in $HBSS^-$; less than 0.1% DMSO) and incubated the tubes at 37°C for 1 h. At the end we washed the cells with $HBSS^-$/DTPA, counted them, and determined their viability (green bars) using hemocytometer counting and trypan blue. We washed the cells twice with $HBSS^-$/DTPA and pulled them down for DNA extraction (for details see [19]). We loaded purified DNA samples onto two separate microtiter plates. After being coated, one of these plates was washed with phosphate buffer and used to determine bound DNA using a CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Invitrogen; Cat. No. C7026) that uses λ DNA as standard. We washed the other plate with washing buffer to determine nitrone adducts using HRP as the detection system as described in [19]. Results are mean values of DNA nitrone adducts/μg of DNA ± SEM from three experiments in triplicate (n = 9). *$p < 0.05$ compared to cells without added tert-BOOH; r.l.u., relative light units. (B) We analyzed the same samples from (A) using the immuno-slot-blot procedure as described in [19]. We show nitrone adducts from cells loaded with copper and treated with tert-BOOH and immunocomplexes detected using HRP-conjugated secondary antibodies. 100# indicates macrophages pretreated with DETC or DP before treatment with tert-BOOH. (C) Fluorescence microscopy analysis of protein nitrone adducts using the anti-DMPO antiserum and an anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). The nitrone adducts were produced as described in the protocol. Briefly, we preloaded RAW 264.7 cells with 100 μM Cu^{2+} and then treated them with tert-BOOH in the presence of DMPO. (D) Immunohistochemistry with the anti-DMPO antiserum of sections of spinal cords from wild-type and mutant $SOD1^{G93A}$ Tg rats (a model of amyotrophic lateral sclerosis) at the onset of the symptoms. (a) Control injected with DMPO, (b) mutant injected with DMPO, and (c) mutant injected with vehicle alone (see [37] for details). Note the intense punctate immunolabeling both in motor neurons (arrows) and surrounding some microglial cells (arrowhead).

applications in which rapid changes in the magnitude and distribution of spins occur [44]. Recently, a fast 3D EPRI system at L-band frequency using spiral magnetic field gradients has been developed and shows promise for *in vivo* imaging of free radicals [44].

A comparison of EPRI, OMRI, and MRI in phantom solutions was recently performed by Hyodo et al. [45]. The authors found that the sensitivity and resolution of images obtained by EPRI and OMRI greatly depended on the linewidth of the reporting nitroxides (e.g., 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3CP)), whereas images obtained by MRI were essentially independent of the EPR line width [45]. Temporal images were obtained using the three imaging modalities that monitored the reduction of 3CP by ascorbic acid [45].

OMRI is a double resonance technique that combines the advantage of MRI for spatial resolution with the sensitivity of EPR

by taking advantage of the Overhauser enhancement dependent on the linewidth of a paramagnetic agent, which is dependent on oxygen concentration [45,46]. The advantage of using standard MRI was that it provided detailed anatomical information, as well as being able to detect in vivo tissue redox status, without a dependence on the linewidth of a reporting agent [45]. The image enhancement that is observed in MR images is dependent on the effect of free radicals on the T_1 relaxation time of surrounding water protons.

Another group used the OMRI approach via dynamic nuclear polarization (DNP), which enhances the sensitivity of nuclear magnetic resonance spectroscopy and the contrast of MRI by transferring the significantly higher electron spin polarization of stable free radicals to nuclear spins [47]. Stable radicals such as 2,2,6,6-tetramethylpypiridine-1-oxyl radicals isotopically labeled with ^{15}N and deuterium are employed as the source for the DNP polarization transfer at a magnetic field relevant for MRI (0.35–1 T). These polarized molecules, in the form of an agarose-based porous material that is covalently spin-labeled with the stable radicals, are introduced to a biological system sensitive to the presence of radicals, ensuring an Overhauser effect induced by DNP without release of radicals into the biological sample [47]. Phantom solution studies indicate that to provide an optimal DNP polarization medium, it is necessary that there be high radical loading, sufficient water access to the radicals, high water mobility, and a close contact between the radicals and the water protons [47].

Molecular MRI

An extension of MRI involves the introduction of a molecular probe that is made up of a MRI contrast agent as the signaling component and an affinity component that could be a peptide or antibody for a molecular marker. We (Towner laboratory) have developed a molecular MRI approach for the *in vivo* detection of DMPO–protein adducts in disease models that involve oxidative stress processes such as amyotrophic lateral sclerosis ($SOD1^{G93A}$ transgenic mouse model) and streptozotocin-induced diabetes [48]. In this approach we synthesized a gadolinium–DTPA–albumin–biotin MRI contrast agent that is easily distributed to a tissue/organ of interest via the vascular system (iv injection via a tail-vein catheter), and anti-DMPO antibodies covalently bound to the albumin moiety, which allows *in vivo* immuno-spin trapping [48]. Specific binding of the anti-DMPO molecular MRI probe is characterized by a sustained increase in MR signal intensity in T_1-weighted images and a corresponding decrease in T_1 relaxation within affected regions [49]. A biotin component is added for *ex vivo* fluorescence labeling (streptavidin–Cy3) of an anti-DMPO mMRI probe bound to a tissue/organ that generated a high level of protein radicals (trapped by administration of DMPO) [48].

There are also numerous developments in the use of iron oxide or magnetite nanoparticles as MRI contrast agents for mMRI probes, which may increase the sensitivity of detection. The presence of the molecular-targeted magnetite nanoparticles results in a sustained decrease in MR signal intensity and a corresponding decrease in T_2 relaxation. The mMRI approach can be applied to many experimental models for diseases that involve oxidative stress processes, taking advantage of the high anatomical resolution of MRI and the specific detection of protein radicals in vivo.

Caveats

The most important components of immuno-spin trapping experiments are the controls. Among numerous factors that need to be considered are the omission of components; type, concentrations, and pH of buffers; chelators of metals and solvents; order of addition of reagents; time and temperature of incubation; target/oxidant ratio; and concentration of DMPO.

Spin trapping considerations

For some applications, DMPO is purified by vacuum sublimation at room temperature and stored under an argon atmosphere at −70°C. The vacuum-sublimation purification of DMPO and the use of DTPA in the phosphate buffer are not required for cell or animal experiments. DMPO is sensitive to light, temperature, metals, and oxygen, forming H_2O_2 via autoxidation as for any organic compound. Thus, precautions should be taken during its handling. The spin trap is available in caramel-colored flasks as a crystalline solid and requires thawing at 37°C. Once liquefied, the pure DMPO is aliquotted into 50 μl per tube, and a stream of an inert gas such as argon or nitrogen over the liquid is used to evacuate oxygen. The aliquotted tubes are kept frozen at −20 or −80°C and protected from light until use.

DMPO addition and radical trapping considerations

In spin trapping experiments, because free radicals are very reactive and hence decay very fast, DMPO is added before or during their formation. DMPO diffuses easily through all cell compartments and must be present both in high enough concentration to outcompete the mechanisms of radical decay (for example, radical–radical, radical–oxygen, and electron transfer reactions) and when and where protein and DNA free radicals are formed [19]. After the preparation of the nitrone adducts, it is convenient, but not necessary, to eliminate excess reagent (i.e., DMPO, H_2O_2, and small-molecular-weight inhibitors) by dialysis, using a membrane with a cut-off appropriate for the molecular weight of the protein or DNA under analysis.

Biomolecule, cell, or animal system considerations

To analyze protein-centered radicals in biochemical systems, it is essential to control the protein purity in the preparation, which can vary from provider to provider and from batch to batch. For example, proteins and DNA can be contaminated with bound metals that, when reacted with H_2O_2, can produce extensive fragmentation by Fenton mechanisms [25,42]. In this case, the biomolecule must be exhaustively dialyzed against chelexed buffers to eliminate the metals. Before use, metHb or metMb is passed through PD-10 desalting columns (Amersham Biosciences) to remove trace contaminants or dialyzed against 100 mM phosphate buffer, pH 7.4, using a 3.5 kDa cut-off dialysis cassette (Slide-A-Lyzer, Dialysis Cassette; Cat. No. 66330; Pierce). Observation of a pure protein in silver- or Coomassie blue-stained gel is important to avoid misinterpretations.

Typically, in chemical systems, for example peroxidases or pseudo-peroxidase/peroxide [24,50] or Fenton systems (see Table 1), a concentration of DMPO between 5 and 100 mM is used, whereas in cell culture, depending on the type of cells, 10 to 100 mM for 24 h can be used without significant cytotoxicity. High concentrations of DMPO are needed in chemical systems to outcompete the radical decay mechanisms. In immunohisto/cytochemistry, the interference by free DMPO and small DMPO–molecule nitrone adducts is not a problem owing to the fixation, permeabilization, and extensive washing carried out during the analysis before the exposure to the anti-DMPO antiserum.

When DMPO was added to cell culture with phenol red at a concentration between 50 and 100 mM, the color of the medium changed to the alkaline range (bright red). However, we have not found significant cytotoxicity in RAW 264.7 macrophages incubated for 24 h with up to 50 mM DMPO in culture medium with phenol red and 10% FCS [42]. In addition, DMPO can trap superoxide radical anion [51], but only very slowly, and therefore should not interfere with superoxide signaling or with cell development, proliferation, or physiology [1].

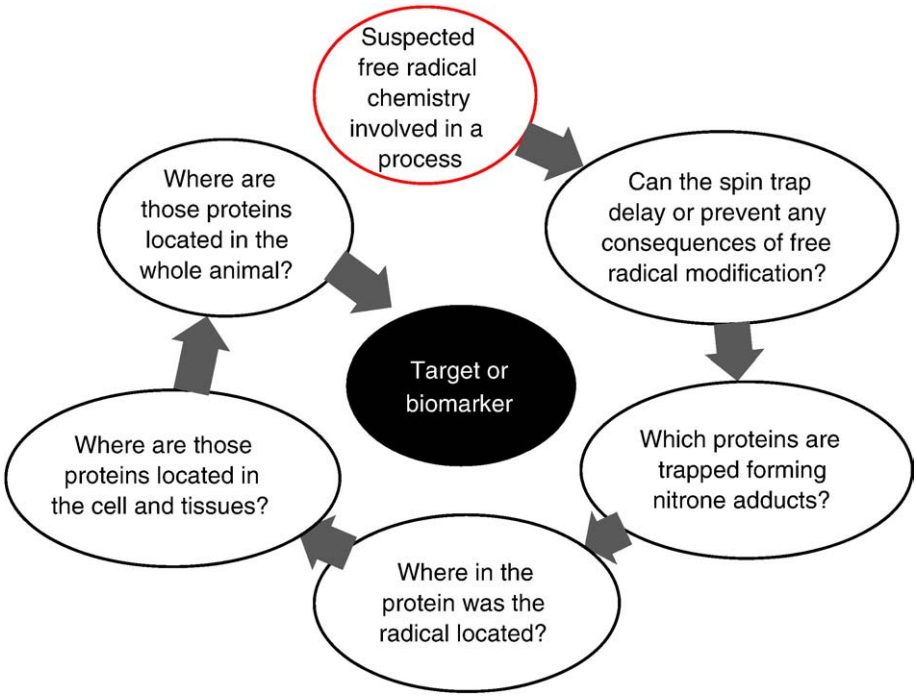
In cell experiments, it is imperative to include cells with and without treatment and with and without the spin trap. Antibiotics, normally used in cell culture, can affect redox processes and, thus, must be omitted whenever possible [8]. If antibiotics must be used, we recommend the inclusion of proper controls. We suggest a careful validation, on a case-by-case basis, of the spin trap concentration to be used in cell experiments with and without the treatment compound or condition. A series of controls has been previously published [19,20].

In addition, for in vivo experiments, the inclusion of untreated animals and those treated with injection of saline (vehicle) or DMPO alone is necessary to avoid misinterpretations. In any case, DMPO-specific immunoreactivity may be suppressed by competing DMPO and the nitrone adducts in the sample for the anti-DMPO antibody [22,23]. The control experiments must be included to enhance the significance of the immuno-spin trapping experiments.

Considerations on Western blot and MS analyses

Although less sensitive than ELISA, Western blot is the only heterogeneous immunochemical technique that allows us to investigate and separate more than one protein-centered, radical-derived nitrone adduct in the same system [20]. We can use ELISA to quantify and screen protein radical-derived nitrone adduct generation, but to study and identify any structural effect on the protein (e.g., aggregation or fragmentation), we must use Western blot analysis to detect and mass spectrometry analyses to identify the protein and the location of the DMPO in its primary structure. Although often omitted in the published literature, a Western blot of the whole homogenate should always be included. Once protein nitrone adducts are detected as positive bands in a Western blot, the corresponding bands in gels stained by Coomassie blue are cut, digested, and analyzed by mass spectrometry to identify the protein(s) that forms nitrone adducts. It should be noted that although MS is generally less sensitive than Westerns/ELISAs, it is a universal detector rather than a selective detector. Many gel bands that appear "pure" by Western blot analysis result in the detection of multiple proteins by mass spectrometry. Therefore, it is imperative to perform additional biochemical experiments to verify which protein is involved in the free radical processes. Additionally, it is quite difficult to determine the specific residue where DMPO is bound, i.e., where the radical has been trapped, especially from an *in vivo* system, owing to the low abundance of the trapped protein [9,52]. To avoid interference by DNA, which may aggregate proteins by forming a sticky net that modifies migration of proteins in the gel, we recommend treating the homogenate with benzonase (Novagen; Cat. No. 70746-3) or similar DNase enzymes to digest genomic DNA. Moreover, we add a cocktail of protease inhibitors (protease inhibitor cocktail, Amresco; Cat. No. M250-1ML) to the homogenization buffer to avoid the proteolysis of proteins by lysosomal enzymes released during cell or tissue homogenization.

In animals, for example rats and mice, a single dose of 1 to 2 g of pure DMPO/kg body wt 2 h before the sacrifice and tissue removal for analysis of nitrone adducts is well tolerated. A vehicle such as saline or PBS is used as a control. Like antibiotics, some analgesics can affect the redox process. Thus, to avoid misinterpretations, the use of analgesics in animal experiments must include appropriate controls, for example DMPO, analgesic, analgesic/DMPO, treatment/DMPO, and analgesic/treatment/DMPO.



Scheme 2. Rationale of use of immuno-spin trapping to find biomarkers and new targets of oxidation in the pathogenesis of stressor-induced oxidative stress and inflammatory diseases. The first step is to test whether DMPO prevents oxidation of cellular targets of oxidative damage. If the spin trap prevents or diminishes the oxidative damage to biomolecules, it can be because the spin trap is trapping either reactive oxygen species (e.g., superoxide radical anion, alkoxyl and peroxyl radicals, etc.) or protein radicals. DMPO binds specifically and covalently to radical sites, residues in proteins. Samples are screened using ELISA of homogenates of tissues or specific cell lineages, positive homogenates are separated in polyacrylamide gels, and protein–DMPO nitrone adducts are detected using Western blot with the anti-DMPO antiserum. To identify the proteins forming protein radicals, positive bands are identified by LC/MS/MS, and the corresponding bands are excised from a gel of the same sample run in parallel and are stained with an MS-compatible staining. The identities of the protein-centered radicals and the specific residue(s) where the radical was trapped are also important for understanding the biochemical basis of the redox process. Once the protein-forming radical is identified, its tissue and subcellular localization is analyzed using immunocyto/histochemistry with the anti-DMPO antibody. The whole-animal experiments using the powerful method of mMRI with nanoparticle–contrast agent–anti-DMPO complexes can tell us the exact site of radical formation or whether the free radical damage is in the tissue under analysis or is a consequence of a remote site of damage and generation of oxidized or bioactive mediators. Taken together, these data will help us understand the nature, targets, and mechanisms of an oxidative process and find new biomarkers of oxidative and inflammatory processes.

Concluding remarks

Immuno-spin trapping is the only technique available to study protein and DNA radicals generated inside functioning cells or animals and to determine where they are located. This technology has quickly evolved as shown by the extent of recently published data (Table 1). See Scheme 2 for a rationale for using immuno-spin trapping tools (immunochemistry, mass spectrometry, and molecular MRI) to identify targets of oxidation that may be useful biomarkers of oxidative and inflammatory diseases. Immuno-spin trapping is a straightforward technique that requires proper controls and careful considerations, some of which we have previously described [19,20] and are highlighted in this method article. Some of the advantages of using immuno-spin trapping rather than ESR and ESR-spin trapping for the detection of protein- and DNA-centered radicals are: (i) the feasibility of applying this technology in any research, clinic, or academic laboratory without requiring complicated equipment or specialists in physical chemistry and quantum mechanics, as are needed for ESR; (ii) the small quantity of sample required (micrograms), which is usually critical for exploring new systems; and (iii) the possibility of detecting, characterizing, identifying, and localizing the distribution of biomolecule-centered radicals in multiple systems (biochemical, cell, tissue, and whole animal). See Table 1 for a complete list of references.

Owing to their role in a number of biological processes and conditions, ROS and drug-induced macromolecule-centered radicals can now be detected and identified using immuno-spin trapping. Upon detection of the target(s) of oxidation by immuno-spin trapping, the identity of the protein radical and site of radical location are identified using the power of mass spectrometry techniques. This knowledge can be important in elucidating the molecular mechanisms of damage and functional consequences. We can also study the subcellular localization of macromolecule-centered radicals and infer biological consequences. *In vivo* immuno-spin trapping is still in development and may be a powerful tool to analyze the anatomical distribution of protein radicals in the living whole animal [48]. Finally, immuno-spin trapping is a powerful technique for studying oxidative modification of biomolecules by reactive chemical species and their role in posttranslational modifications in physiology and diseases.

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