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EPR spin-trapping of protein radicals to investigate biological oxidative mechanisms

Review Article

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Summary. Presently, free radicals and oxidants are considered to mediate from signaling circuits involved in physiology and pathology to cell and tissue injury. The elucidation of these many inter-related processes requires a better understanding of cellular oxidative mechanisms many of which are mediated by protein radicals. Here, we will discuss the potentialities of EPR spin-trapping of protein radicals to unravel oxidative mechanisms. An overview of the methodology and its application to identify protein residues that are the target of specific oxidants, characterize emerging oxidants, and discriminate radical from non radical mechanisms will be presented. The examples are based on work developed in our laboratories but will be discussed in a broad scenario to emphasize that simple experiments can provide relevant insights into the biological reactivity of known and emerging biological oxidants and into signaling mechanisms.

Keywords: Oxidative mechanisms – Protein radicals – EPR – EPR spin-trapping

Abbreviations: BSA, bovine serum albumin; Cu,Zn-SOD, superoxide dismutase; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DMPO, 5,5-dimethylpyrroline N-oxide; EPR, electron paramagnetic resonance; ESI, electrospray ionization; GSH, glutathione; Hb, hemoglobin; HCO_4^-, peroxycarbonate or peroxymonocarbonate; HSA, human serum albumin; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MNP, 2-methyl-2-nitrosopropane; MS, mass spectrometry; PBN, phenyl-N-t-butylnitrone; Peroxynitrite, sum of peroxynitrite anion ($ONOO^-$, oxoperoxonitrate (−1)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified; RNS, reactive nitrogen species; ROS, reactive oxygen species

Introduction

Biological oxidative mechanisms have been studied ever since Priestley, Scheele and Lavoisier discovered molecular oxygen and reported its beneficial and toxic effects to living organisms in the late 18th century. Up to the end of the 20th century, it was emphasized that biological oxidations albeit essential to aerobic organisms to obtain energy from nutrients, combat invading pathogens and eliminate xenobiotics, also produced reactive intermediates derived from molecular oxygen (reactive oxygen species, ROS). These species, if not eliminated by antioxidant defenses, would attack biomolecules producing cell and tissue injury. This view was summarized in the concept of oxidative stress that was defined as an imbalance between free radicals/oxidants and antioxidants in favor of the first (Sies and Mehlhom, 1986; Halliwell and Gutteridge, 1999; Gilbert and Colton, 1999). As a consequence, free radicals and oxidants were associated with most human diseases and many intervention studies were designed to examine the effects of antioxidant vitamins on diseases, in particular on cardiovascular diseases. These studies showed discrete results, that is, supplements of vitamin C, E or β-carotene did not influence disease outcome in most examined cases (Kris-Etherton et al., 2004). Thus, it was time to recognize that the classical concept of oxidative stress was too simplistic in encompassing only the so-called ROS and their necessary deleterious roles in vivo.

Also crucial to promoting paradigmatic changes in the field of biological oxidations in the late decades of the 20th century, was the discovery that the free radical nitric oxide is enzymatically synthesized in mammals to exert major physiological functions (Ignarro, 1990; Moncada et al., 1991). As a consequence, it became clear that free radicals and oxidants play a role in cell homeostasis and

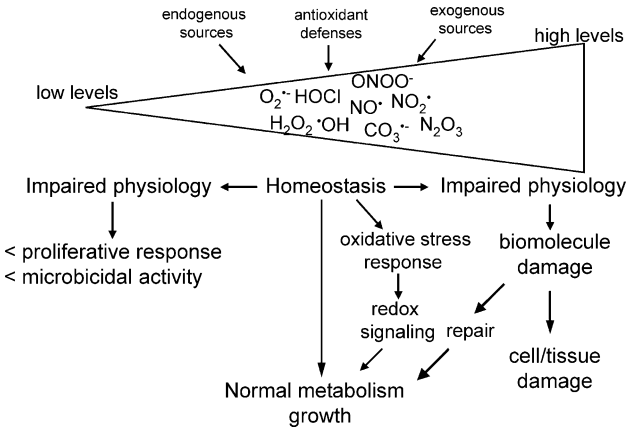

Fig. 1. Schematic representation of the present view about the multiple roles of free radicals and oxidants in physiology. See text for details

not only in cell injury as previously emphasized (Halliwell and Gutteridge, 1999; Gilbert and Colton, 1999; Aslund and Beckwith, 1999; Finkel and Holbrook, 2000; Stone, 2004; Torres and Forman, 2003). In addition, previously unsuspected oxidants were brought into biology such as peroxynitrite, nitrogen dioxide and the carbonate radical anion (Radi et al., 2000; Radi, 2004; Augusto et al., 2002a, 2004). Thus, the view of the roles of free radicals and oxidants in physiology became more complex as schematically summarized in Fig. 1. Present evidence indicates that free radicals and oxidants are constantly produced in vivo from exogenous and endogenous sources and are derived from molecular oxygen (ROS) and from nitric oxide (RNS; reactive nitrogen species). Organisms evolved enzymatic antioxidant defenses and the capability to use antioxidants from the diet to control the physiological levels of free radicals/oxidants. Indeed, high levels of some oxidants may cause dysfunction because they oxidize biomolecules that lead to cell and tissue injury if not repaired by the evolved repair systems. Low levels of certain free radicals/oxidants also compromise physiological functions that depend on them such as microbicidal activity, proliferative responses and vasodilation. On the other hand, transient and small increases of some oxidants can trigger redox-sensitive signaling pathways (Fig. 1). Thus, free radicals and oxidants are presently considered to mediate from signaling circuits involved in physiology and pathology to cell and tissue injury.

The elucidation of these many inter-related processes requires a better understanding of cellular oxidative mechanisms, including the identification of the involved oxidants, the pathways regulating their generation and their targets at molecular level. This remains a challenge requiring innovative technologies and experimental approaches to detect and quantify specific free radicals/oxidants and their products under physiological conditions. Among these products, oxidized proteins and protein radicals are particularly relevant because they may serve as biomarkers of oxidative damage and as early biomarkers, that is, as sensors of redox signaling and indicators of the transition from the stress response to injurious damage (Fig. 1). Thus, it is timely to discuss how the detection of oxidized protein products can contribute to the elucidation of biological oxidative mechanisms. To this end, we will briefly discuss the products and consequences of protein oxidation in vivo. We will emphasize detection of protein radicals by spin-trapping and its potentiality to identify protein residues that are target of specific oxidants, characterize emerging oxidants and discriminate radical from non radical mechanisms.

Protein oxidation and protein radicals

The abundance of proteins in cells and extracellular fluids argues for their importance as targets of free radicals and oxidants. Indeed, it has been estimated that proteins are likely to scavenge 50–75% of the reactive species eventually produced in vivo (Davies et al., 1999). Not surprisingly, protein oxidized products such as protein carbonylation, protein nitration (protein-3-nitrotyrosine), protein hydroxylation (protein-*o*-tyrosine, protein-*m*-tyrosine, protein-dopa) and protein halogenation (protein-cloro-tyrosine) are being examined as potential biomarkers of oxidative damage and diseases (Dalle-Donne et al., 2004 and references therein). A paradigmatic example that is likely to impact clinical research was the quantification of 3-nitrotyrosine levels in human plasma before and after statin treatment by the stable isotope-dilution LC/ESI/MS/MS methodology (Shishehbor et al., 2003). Reliable biomarkers of oxidative damage are being actively pursued by many investigators but further studies will be required to relate them to human diseases (Kadiiska et al., 2005a, 2005b; Morrow, 2005; Badouard et al., 2005; Dalle-Donne et al., 2004).

In addition to being abundant, proteins are the central players of all physiological processes and oxidized protein products participate in catalysis and signaling events. In addition to the redox changes commonly involved in metalloprotein reactions, protein radicals derived from amino acid residues such as tyrosine, tryptophan, cysteine and glycine participate in the catalytic mechanism of several enzymes (Stubbe and van der Donk, 1998). On the other hand, protein-cysteine oxidation products have been shown to play a pivotal role in cell signaling. There are

several levels of oxidation for protein-cysteine residues and those most studied so far are protein-sulfenic acids (protein-cysSOH), protein sulfinic acids (protein-cysSO_2H), protein sulfonic acids (protein-cysSO_3H), protein disulfides (protein-cysSSR; including both cystine and mixed disulfide forms) (Clairbone et al., 1993; Georgiou and Masip, 2003; Wood et al., 2003; Woo et al., 2003) and protein S-nitrosothiols (protein-cysSNO) (Stamler, 1994, 1995). Except for protein-sulfonic acid, all the other modifications appear to count with repair systems that depend on the intracellular concentration of low molecular weight thiols such as GSH in mammalian cells. Thus, all of these repairable products have been considered as mediators of protein-cysteine redox signaling. Most protein-cysteine oxidation products are usually considered to be formed by two-electron mechanisms but recent evidences indicate that they can also be produced by one-electron mechanisms through the intermediacy of protein-cysteinyl radicals (P-cys$^{\bullet}$) (see, below).

In summary, protein radicals mediate biological damage because they lead to products that appear not to be repaired in vivo such as crosslinking within themselves or with other biomolecules, protein nitration, protein hydroxylation, protein halogenation, and protein carbonylation (Davies et al., 1999; Dalle-Donne et al., 2004). Also, protein radicals mediate enzymatic catalysis (Stubbe and van der Donk, 1998) and may participate in signaling events (Augusto et al., 2004; Heo et al., 2004, 2005a, 2005b). Thus, detection and characterization of protein radicals is particularly relevant to the understanding of biological oxidative mechanisms.

Detection of protein radicals by EPR spin-trapping

The method of choice to detect and identify free radicals is EPR spectroscopy but only a few protein-tyrosyl radicals have been shown to be stable enough to be detectable by static direct EPR at room temperature and under air. As a consequence, most EPR studies of protein radicals have been performed by rapid freeze-quench EPR (Stubbe and van der Donk, 1998) and EPR spin-trapping (Davies and Hawkins, 2004). The latter methodology has been preferred in studies addressing the pathological roles of free radicals because it can be used in complex biological systems including biological fluids, cells and experimental animals (Knecht and Mason, 1993; Davies and Hawkins, 2004; Augusto et al., 2004; Mason, 2004).

The EPR spin-trapping technique relies on the reaction of a biological radical with a diamagnetic probe containing a nitroso or nitrone function, the spin trap, to form a spin trap radical adduct (a nitroxide derivative) that, being more stable than the primary radical, attains concentrations high enough to be detected by EPR (Fig. 2). If the primary radical is a protein radical, the EPR signal will usually appear as a broad spectrum that is characteristic of relatively immobilized (or high molecular weight) nitroxide. Upon proteolysis, the broad signal is converted to a sharp signal that is characteristic of mobile (or low molecular weight) nitroxide (Fig. 2).

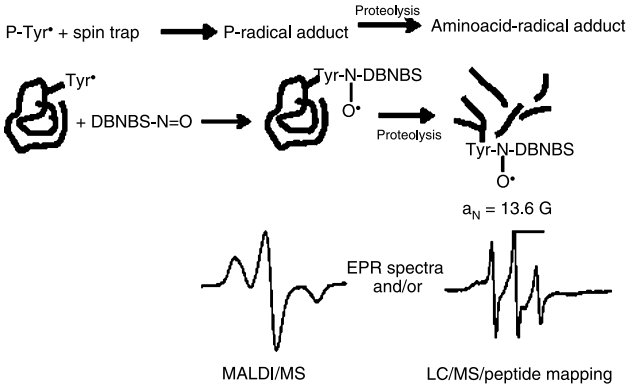

Fig. 2. Schematic representation of the spin-trapping of a protein radical and of analytical techniques employed to characterize the trapped radical. In the example, a protein-tyrosyl radical is trapped with the spin-trap DBNBS (DBNBS-N = O). The resulting P-radical adduct can be detected by EPR and/or MS spectra. To identify the trapped amino acid residue(s), the P-radical adduct is submitted to proteolysis and further EPR and LC/MS/peptide mapping analysis

EPR spin-trapping permits the EPR detection of the spin trap radical adduct and not of the primary radical and thus, it is not always easy to identify the latter. Indeed, the EPR spectrum features, the *g* value (a measure of electron delocalization onto neighboring nuclei) and the hyperfine coupling constant values (a_N and a^{β}_H resulting from electron interaction with close nitrogen and hydrogen nuclei in typical experiments without isotopic labeling) are more dependent on the spin trap than on the primary radical. The problem becomes more difficult in the case of protein radicals whose EPR spectra are broad and lack resolvable hyperfine coupling constants that help in the identification of the primary radical (Fig. 2). Consequently, a number of additional analytical methods has been employed to obtain information on the nature of trapped protein radicals (Davies and Hawkins, 2004). Methodologies that are under active development rely on the combination of spin-trapping (with or without proteolysis) with MALDI-TOF/MS and LC/MS/peptide mapping, respectively (Fig. 2) (Deterding et al., 2004). Nevertheless, the data already accumulated through many studies and methodologies provide characteristic features

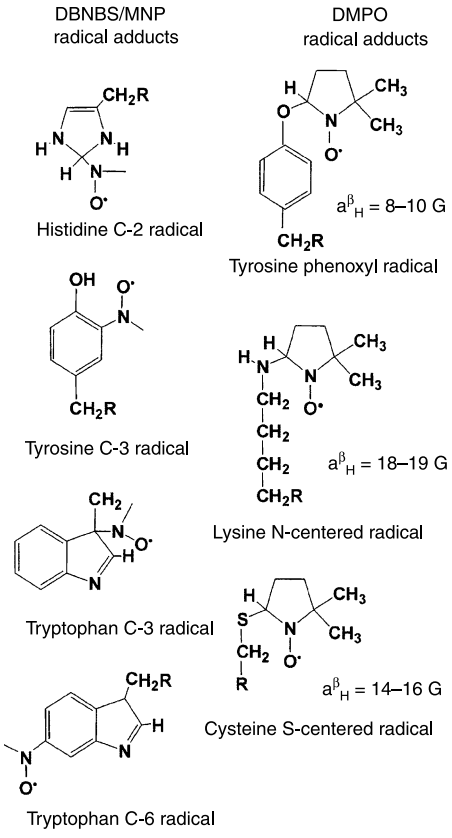

Fig. 3. Examples of the structures of characterized amino acid radical adducts of nitroso (DBNBS and MNP) and nitrone (DMPO) spin-traps. Typical a^{β}_{H} hyperfine splitting constants of DMPO radical adducts are also shown. A compilation of hyperfine splitting constants of a variety of free radical adducts can be found at http://epr.niehs.nih.gov/stdb.html

of EPR spectra of protein and amino acid radical adducts with commercial spin traps (Fig. 3). These data can be used to identify produced protein radicals by EPR spectra comparison whose application in mechanistic studies is exemplified below.

EPR spectra comparison to identify oxidant targets

Peroxynitrite, the product of the diffusion-controlled reaction between superoxide anion and nitric oxide, is an emerging biological oxidant whose chemistry and biochemistry remained controversial from 1990 up to 2000. Even before peroxynitrite reactivity towards biomolecules was fully elucidated (Radi et al., 2000; Radi, 2004; Augusto et al., 2002a, 2004), our group demonstrated that protein-cysteine residues were relevant targets of peroxynitrite in biological media by examining the features of the EPR spectra obtained in spin-trapping experiments (Fig. 4). Indeed, human erythrocytes (Augusto et al., 2002b) and plasma (Vasquez-Vivar et al., 1996) treated

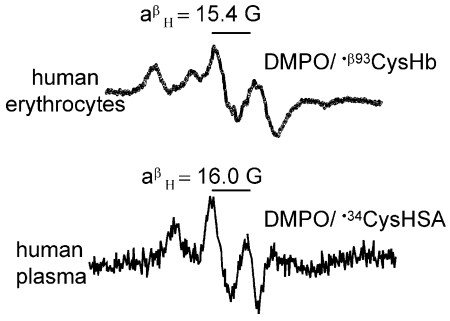

Fig. 4. EPR spectra of DMPO radical adducts of hemoglobin and albumin detected in human erythrocytes and plasma. The shown a^{β}_{H} values identified the trapped radicals as the corresponding Hb-$^{\beta 93}$cysteinyl (Augusto et al., 2002) and HSA-^{34}cysteinyl (Vasquez-Vivar et al., 1996), respectively

with peroxynitrite in the presence of DMPO showed EPR spectra with hyperfine splitting constants (a^{β}_{H} of 15–16 G) characteristic of DMPO/•cys-protein radical adducts. Human hemoglobin and albumin are the major constituents of erythrocytes and plasma, respectively, and both proteins have only one accessible reduced cysteine. Thus, the main protein radicals produced in erythrocytes and plasma were easily identified as Hb-$^{\beta 93}$cysteinyl and HSA-^{34}cysteinyl, respectively. Latter, the mechanisms involved in peroxynitrite-mediated HSA (Carballal et al., 2003; Fernandes et al., 2005) and Hb oxidation (Romero et al., 2004) were further clarified. In both cases, protein-tyrosyl radicals were also shown to be produced but remain to be fully identified.

The identification of protein residues that are oxidized to radicals is not trivial because usually each amino acid appears more than once in the protein structure. In addition, electron transfer within proteins is common and a radical produced in one residue may end up in others. Electron transfer within proteins is still poorly understood (Zhang et al., 2005; Barrows et al., 2004). Likewise, how the different spin traps compete with electron transfer is also unknown and, thus, the trapped protein radical may not be the primary radical produced. These problems are being approached by combining spin trapping with proteolysis and MS peptide mapping (Fig. 2) (Deterding et al., 2004) and by studies with model systems (Zhang et al., 2005). Further progress is expected in the near future.

Nevertheless, EPR spin-trapping studies of protein radicals can provide important information as shown by the described early studies of peroxynitrite reactions in biological media. They contributed to the view that in physiological fluids peroxynitrite is likely to act through nitrogen dioxide and carbonate radical that are produced from its reaction with carbon dioxide. As a consequence,

peroxynitrite is likely to generate biomolecule-derived radicals, including protein-cysteinyl, protein-tyrosyl and protein-tryptophanyl radicals (Radi et al., 2000; Augusto et al., 2002b). Thus, simple EPR spin-trapping experiments provided important insights into the biological reactivity of peroxynitrite and can be useful to examine known and emerging biological oxidants.

EPR spin-trapping to characterize emerging oxidants

Peroxycarbonate (HCO_4^-) can be considered as an emerging biological oxidant. Its possible formation under biological conditions was proposed on the basis of different experiments by three groups (Elam et al., 2003; Richardson et al., 2003; Bonini et al., 2004a). In the chemical literature, peroxycarbonate or peroxymonocarbonate was first described in the 1960s and recently, it has been studied as a two-electron oxidant of alkenes and organic sulfides by Richardson and co-workers (Richardson et al., 2000, 2003; Yao and Richardson, 2003).

We proposed peroxycarbonate as a potential biological oxidant based on EPR spin trapping studies and other considerations while studying BSA as a target of the oxidant produced from the peroxidase activity of Cu,Zn-SOD in the presence of bicarbonate. It has been proposed that this activity depends on the formation of a diffusible carbonate radical and is receiving much attention in the literature due to its potential relevance to familial amyotrophic lateral sclerosis (Zhang et al., 2004; Liochev and Fridovich, 2004). A diffusible carbonate radical was controversial (Elam et al., 2003) and to examine its formation we used BSA as a target. Indeed, our previous studies had shown oxidation of BSA by peroxynitrite/carbon dioxide (and thus, nitrogen dioxide plus carbonate radical) to BSA-cysteinyl and BSA-tyrosyl radicals (Gatti et al., 1994; Fernandes et al., 2005). Also, such BSA radicals were expected on the basis of the second order rate constant of reactions between carbonate radical and amino acids (Fig. 5A). However, our incubations of Cu,Zn-SOD/hydrogen peroxide/bicarbonate and BSA produced only solvent-exposed BSA-tyrosyl radicals as attested by direct EPR and EPR spin-trapping experiments (Fig. 5B). Indeed, the parameters of the EPR spectra, $g = 2.004$ and $a^{\beta}_{H} = 8.6$ G, respectively, were consistent with a protein-tyrosyl radical whose solvent-accessibility was indicated by its short half-life and reaction with both DMPO and DBNBS (Bonini et al., 2004b). A possibility to explain the non detection of BSA-cysteinyl radicals was to suppose that BSA-cysteine residues were being oxidized by a species other than the carbonate radical. This was

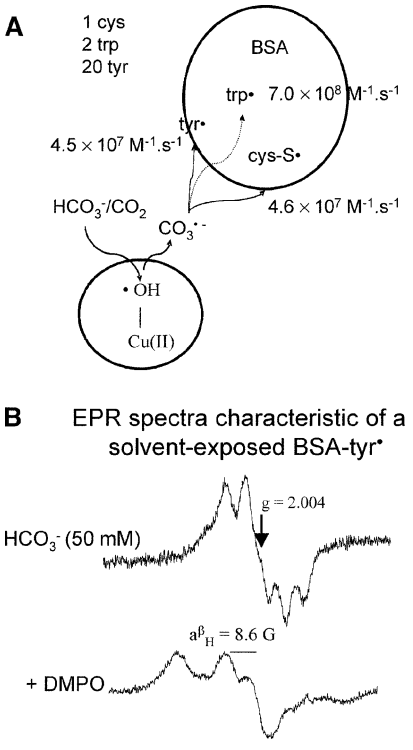

Fig. 5. Hypothetical representation (**A**) and experimental EPR spectra (**B**) of BSA attack by the oxidant produced from the peroxidase activity of Cu,Zn-SOD in the presence of bicarbonate. The main produced BSA radical was identified by the parameters of its direct EPR spectra (first) and DMPO radical adduct spectra (second). Details can be found in Bonini et al. (2004b)

confirmed by parallel experiments showing that BSA-cysteine was being rapidly oxidized by hydrogen peroxide to the corresponding sulfenic acid in a process that was considerably accelerated by bicarbonate. In view of the previous studies by Richardson and co-workers, we proposed formation of peroxycarbonate to explain the acceleration of BSA-cysteine oxidation to BSA-cysSOH. The non detection of BSA-cysteinyl radicals confirmed that peroxycarbonate was acting as a two-electron oxidant (Bonini et al., 2004b).

The possibility of peroxycarbonate formation was also useful to explain the production of the carbonate radical from xanthine oxidase turnover that was being studied in parallel in our laboratories (Fig. 6) (Bonini et al., 2004a). In summary, we proposed peroxycarbonate as a physiologically relevant species that can act either as a two-electron oxidant or as a precursor of the carbonate radical when in the presence of electron donors (Fig. 6). Recently, the latter possibility has been considered by other investigators to explain the mechanisms by which bicarbonate accelerates some transition metal ion-catalyzed peroxidations (Liochev and Fridovich, 2005; Ramirez et al., 2005;

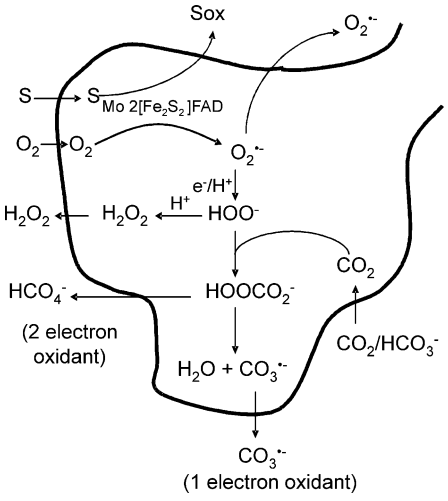

Fig. 6. Schematic representation of the mechanism proposed to explain carbonate radical production during xanthine oxidase turnover. It was proposed a peroxycarbonate intermediate whose formation and reduction is facilitated by the many xanthine oxidase redox centers. S and Sox represent a general substrate and its two-electron oxidation product, respectively. Details can be found in Bonini et al. (2004a)

Arai et al., 2005). Thus, analysis of BSA protein oxidation by spin-trapping experiments provided the basis to propose a new biological oxidant derived from the bicarbonate/carbon dioxide pair. The latter is the main physiological buffer and its derived oxidants deserve further studies.

Spin-trapping to discriminate radical and non radical mechanisms

Biological oxidations can occur by two- and one-electron mechanisms and spin-trapping is one of the few methodologies that permit their discrimination. For instance, as described above, DMPO did not trap BSA-cysteinyl radical in incubations of BSA with hydrogen peroxide/bicarbonate confirming previous studies by Richardson and co-workers that peroxycarbonate is a two-electron oxidant. Spin-trapping can be useful even when radical adduct yields are too low to be detectable by EPR ($<\mu M$) due to overall low radical production and/or radical adduct oxidation/reduction in biological media (Mason, 2004). In these cases, inhibition of product yields by spin traps provides evidence for the participation of free radicals in product formation. This approach has been extensively used in the literature and a recent and relevant example is related to the mechanisms of nitrosothiol formation.

Nitrosothiols are considered to participate in signaling circuits but the mechanisms by which they are produced in vivo remain obscure. Until recently, proposed mechanisms such as the reaction of thiols with dinitrogen trioxide (N_2O_3), dinitrosyl-iron or nitrosylheme complexes, or peroxynitrite, with a few exceptions, did not include the intermediacy of free radicals (Espey et al., 2002; Augusto et al., 2004; Bryan et al., 2004). However, the spin trap DMPO has been shown to inhibit nitrosothiol formation from glutathione and cells treated with a nitric oxide donor (spermine nonoate) (Jourd'heuil et al., 2003). Likewise, the spin trap PBN was shown to inhibit BSA-cysNO formation from treating BSA with peroxynitrite/carbon dioxide/tempol while trapping the BSA-cysteinyl radical (Fernandes et al., 2005). Also, spin-trapping experiments with model peptides/myeloperoxidase/hydrogen peroxide/nitrite have shown that intramolecular electron transfer between tyrosyl radical and cysteine residue inhibits tyrosine nitration and induce cysteinyl radical and nitrosothiol formation (Zhang et al., 2005). These studies demonstrate that nitrosothiols can be formed by recombination of nitric oxide with protein-cysteinyl radicals (Fig. 7). This mechanism is likely to operate when high nitric oxide production favors production of nitrogen dioxide and high local glutathione or reactive thiol proteins (low cysteine pKa) levels compete with nitric oxide for nitrogen dioxide. Alternatively, parallel production of nitric oxide under oxidative conditions that are prone to produce protein-cysteinyl radicals will result in nitrosothiol formation. In localized environments, the possibility of protein-cysteinyl radicals reacting with oxygen to produce sulfinyl radicals (protein-cysSO$^\bullet$) and, eventually, protein-sulfenic acid derivatives (protein-cysSOH) cannot be excluded (Augusto et al., 2004). Indeed, taken together, product analysis by Radi and co-workers (Carballal et al.,

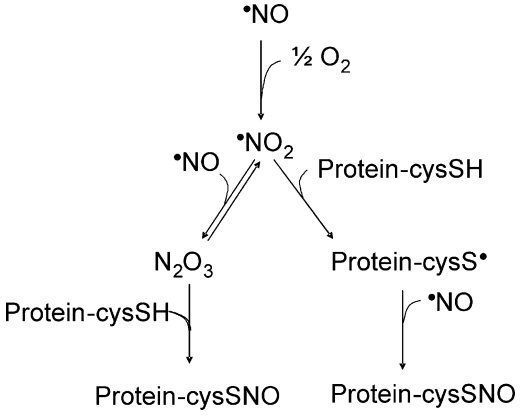

Fig. 7. Schematic representation of the production of protein-*S*-nitrosothiol by ionic (left-hand side) and radical (right-hand side) mechanisms. The occurrence of the latter was proved by spin-trap inhibition of protein-*S*-nitrosothiol yield and by spin-trapping of protein-cysteinyl radicals. See text for details and references

2003) and EPR and EPR spin trapping studies by us (Bonini and Augusto, 2001) suggest that protein-cysteinyl radicals may be intermediates in the path to protein-sulfenic acid derivatives.

Again, it is noteworthy that simple experiments relying on the well-tested reaction of spin traps with free radicals, including protein radicals, provided important information about biological oxidative mechanisms. The possibility that protein-cysteinyl radicals may intermediate the formation of nitrosothiols and protein-sulfenic acid derivatives in vivo, raises new perspectives for the understanding of cellular signaling cascades. Relevantly, recent work by Campbell and co-workers provide solid and elegant evidence for a role for Ras^{118}cysteinyl radical in Ras regulation by redox agents (Heo et al., 2004, 2005a, 2005b). This is a paradigmatic work and if protein-cysteinyl radicals become more recognized in cell signaling, there will be an increased interest in spin trapping of protein radicals.

Conclusions

The detection and characterization of free radicals under physiological conditions remains a challenge requiring new technological approaches. All the available methodologies have intrinsic drawbacks and EPR spin-trapping is no exception to the rule. Nevertheless, it can provide relevant information about oxidative mechanisms when rigorously applied. As emphasized here, EPR-spin-trapping of protein radicals can be particularly useful to unravel mechanistic details of oxidative damage and redox signaling and thus, to the understanding of the multiple roles of free radicals and oxidants in physiology and pathophysiology.

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