PROTEOMIC APPROACHES TO EVALUATE PROTEIN *S*-NITROSYLATION IN DISEASE

Laura M. López-Sánchez, Chary López-Pedrera, and Antonio Rodríguez-Ariza*
Research Unit, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Hospital Reina Sofía, Universidad de Córdoba, Spain

Received 28 November 2012; accepted 29 January 2013

Published online 15 June 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mas.21373

Many of nitric oxide (NO) actions are mediated through the coupling of a nitroso moiety to a reactive cysteine leading to the formation of a S-nitrosothiol (SNO), a process known as S-nitrosylation or S-nitrosation. In many cases this reversible post-translational modification is accompanied by altered protein function and aberrant S-nitrosylation of proteins, caused by altered production of NO and/or impaired SNO homeostasis, has been repeatedly reported in a variety of pathophysiological settings. A growing number of studies are directed to the identification and characterization of those proteins that undergo S-nitrosylation and the analysis of S-nitrosoproteomes under pathological conditions is beginning to be reported. The study of these S-nitrosoproteomes has been fueled by advances in proteomic technologies that are providing researchers with improved tools for exploring this post-translational modification. Here we review novel refinements and improvements to these methods, and some recent studies of the S-nitrosoproteome in disease. © 2013 Wiley Periodicals, Inc. Mass Spec Rev 33:7–20, 2014

Keywords: *cysteine; nitric oxide; S-nitrosothiol; S-nitrosation; S-nitrosylation; proteomics*

I. INTRODUCTION

Nitric oxide (NO) is a key regulator for physiological processes such as vasodilatation, inhibition of platelet aggregation, neurotransmission, and anti-microbial activity (Knowles et al., 1989; Moncada et al., 1991; Rees, Palmer, & Moncada, 1989; Stuehr et al., 1989). The redox state and chemistry of this simple diatomic molecule facilitate its interaction with proteins thus regulating various extracellular and intracellular events. Notably, many of NO actions are mediated through the coupling of a nitroso moiety to a reactive cysteine leading to the formation of a *S*-nitrosothiol (SNO), a process usually known as *S*-nitrosylation or *S*-nitrosation (Stamler et al., 1992b; Jaffrey et al., 2001; Foster, Hess, & Stamler, 2009; Hess & Stamler, 2012). It is well established that *S*-nitrosylation of proteins plays a regulatory role similar to phosphorylation and even if multiple cysteine residues in a protein are available for *S*-nitrosylation, few are specifically modified and are responsible for the modification of protein function (Stamler, Lamas, & Fang, 2001; Jaffrey, Fang, & Snyder, 2002; Rahman et al., 2010).

A growing number of studies are focusing on the systematic identification and characterization of those proteins that undergo *S*-nitrosylation in a particular organism, organ, or cell type. The study of these *S*-nitrosoproteomes has been fueled by recent advances in proteomic technologies that are providing researchers with improved tools for exploring this post-translational modification. In addition, our increasing knowledge of the chemistry and SNO metabolism is also contributing substantially to understanding how protein *S*-nitrosylation participates in physiological and pathophysiological processes. Thus, although the complex chemistry involving NO has complicated the investigation of SNO biochemistry, *S*-nitrosylation is emerging in redox proteomics as fundamental to the understanding of the redox processes contributing to the delicate balance between health and disease. This review will focus on the most recent advances in methodologies for the proteomic analysis of *S*-nitrosylation and how these novel technologies will advance our current knowledge of the role of deregulated *S*-nitrosylation in disease.

II. MECHANISMS PARTICIPATING IN THE S-NITROSYLATION AND DENITROSYLATION OF PROTEINS

A. Formation of SNOs and SNO-Proteins

Nitric oxide is generated by a family of enzymes termed nitric oxide synthase (NOS) through a complex reaction converting L-arginine into L-citrulline and NO (Knowles et al., 1989; Marletta, 1994). Based upon criteria including regulation of activity and inhibitor profiles, three isoforms of NOS enzymes have been described and then cloned. NOS1 (nNOS) and NOS3 (eNOS) were initially cloned in neural cells and endothelial cells, respectively, are regulated by Ca^{2+} and calmodulin and are constitutively expressed. The NOS2 (iNOS) enzyme was initially identified in macrophages, is independent on Ca^{2+} and its induction leads to the production of high levels of NO during pathophysiological conditions (Alderton, Cooper, & Knowles, 2001; Kleinert et al., 2004).

S-nitrosylation of Cys residues in target proteins was first recognized in 1992, when micromolar levels of SNO-albumin

Contract grant sponsor: Consejería de Salud de la Junta de Andalucía; Contract grant number: JA0230/09; Contract grant sponsor: Programa de Promoción de la Investigación en Salud del Ministerio de Economía y Competitividad; Contract grant number: FIS 10/00428.
**Correspondence to*: Antonio Rodríguez-Ariza, IMIBIC, Unidad de Investigación, Hospital Universitario Reina Sofía, Avda Menéndez Pidal s/n, 14004 Córdoba, Spain.
E-mail: antonio.rodriguez.exts@juntadeandalucia.es

were reported to circulate in blood (Stamler et al., 1992a). Cys are the most reactive protein residues to NO-derived reactive species at physiological pH, and they are known to be important for maintaining the conformation of proteins and essential residues at the active sites of enzymes. Some mechanisms have been proposed for SNO formation within the biological environment, as NO itself is a poor nitrosylating agent (Keszler, Zhang, & Hogg, 2010). Although the likely *S*-nitrosylating species in biological systems is dinitrogen trioxide (N_2O_3), which is formed from O_2 and NO, a significant fraction of protein *S*-nitrosylation by NO may occur in the absence of O_2 (Foster et al., 2009) or through radical-based reaction mechanisms (Hess et al., 2005). Also, *S*-nitrosylation is known to occur through ferric heme nitrosyl intermediates (Luchsinger et al., 2003). Importantly, either Metal-to-Cys or Cys-to-Cys transfer of the NO group in a transnitrosylation reaction appears to be an important enzymatic mechanism of *S*-nitrosylation and thus SNO production (Anand & Stamler, 2012; Nakamura & Lipton, 2013). Since the low-molecular-weight SNO pool, including *S*-nitrosocysteine (SNO-Cys), *S*-nitrosocysteinylglycine, and *S*-nitrosoglutathione (GSNO), exists in equilibrium with the *S*-nitrosoprotein pool, transnitrosylation reactions also have an important role in the maintenance of cellular SNO homeostasis (Lopez-Sanchez et al., 2008).

As stated above, it is commonly observed that only few of many available Cys residues in proteins undergo detectable levels of nitrosylation. A significant example is the ryanodine responsive calcium release channel of skeletal muscle (RyR1), where only 1 (Cys3635) of ~50 Cys residues were found to undergo detectable levels of *S*-nitrosylation by NO (Sun et al., 2001). The thiol microenvironment, the types and levels of NO donors, the cellular redox environment and the interaction or subcellular co-localization with NOS isoforms are among the aspects contributing to the specificity of *S*-nitrosylation. In addition, the presence of transnitrosylases and denitrosylases have been recently proposed as factors that determine selective *S*-nitrosylation of proteins (Fig. 1) (Seth & Stamler, 2011; Wu et al., 2011a).

The different compartmentalization in cells of *S*-nitrosylation protein targets, protein complex formation or direct protein–protein interaction are determinants of specific *S*-nitrosylation. Golgi apparatus and plasma membrane caveolae are the main sites of eNOS localization, and *S*-nitrosylation is concentrated on these subcellular locations in endothelial cells and other cells overexpressing this NOS isoform (Iwakiri et al., 2006; Ibiza et al., 2008). The COX-2-induced formation of a complex with iNOS is essential for the *S*-nitrosylation and activation of cPLA2a, a cytosolic enzyme involved in the biosynthesis of prostanoids in epithelial cells (Xu et al., 2008). Also, during neuronal signaling the close association with nNOS and thereby the *S*-nitrosylation of NMDA receptor and Ras-like G protein is facilitated by scaffold or adaptor proteins (Hess et al., 2005).

S-nitrosylation of Cys residues may be targeted by an acid–base motif or hydrophobic areas, which could be present either in the flanking primary sequence or emerge in tertiary structure, rendering low thiol pK_a and high sulfur atom exposure (Greco et al., 2006; Doulias et al., 2010). In addition, a revised acid–base motif, which is located more distantly to the Cys, has been suggested to play a role in protein–protein interactions that could facilitate transnitrosylation reactions, rather than being responsible for direct activation of Cys residues (Marino & Gladyshev, 2010). This would explain why apparently inert Cys residues are specifically and selectively nitrosylated within proteins. Furthermore, the significance of protein–protein interactions in targeting *S*-nitrosylation is consistent with the emerging evidence for a

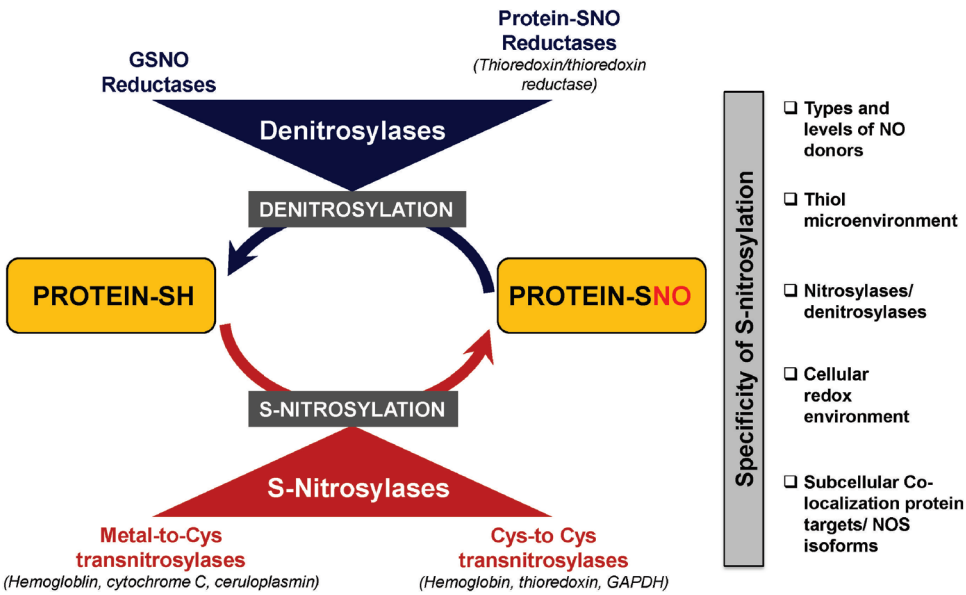

FIGURE 1. *S*-nitrosylation and denitrosylation of proteins. Mechanisms participating in *S*-nitrosylation and denitrosylation of proteins, and thus contributing to SNO homeostasis in cells, are shown. Aberrant *S*-nitrosylation of proteins from altered production of NO and/or impaired SNO homeostasis has been implicated in the pathogenesis of a number of diseases. See text for further details. GSNO, *S*-nitroglutathione; NO, nitric oxide; NOS, nitric oxide synthase; SNO, *S*-nitrosothiol.

major role of transfer of NO groups from SNO-proteins to acceptor proteins (Anand & Stamler, 2012).

B. Nitrosylases and Denitrosylases

Recent studies have led to the identification and characterization of enzyme-mediated processes in the nitrosylation and denitrosylation of proteins (Fig. 1). Proteins involved in the transfer of the NO group to a Cys residue in a protein are termed *S*-nitrosylases, and they have been classified as Cys-to-Cys (Class I) or Metal-to-Cys (Class II) transnitrosylases (Seth & Stamler, 2011; Anand & Stamler, 2012). In Metal-to-Cys nitrosylases, transfer of NO group occurs from transition metals to cysteine in intramolecular (auto-*S*-nitrosylation) or intermolecular reactions with glutathione to form nitrosoproteins or nitrosoglutathione, respectively. Thus, the transfer of NO from heme iron in mammalian hemoglobin to Cysβ93 results in auto-*S*-nitrosylation (SNOHb) (Gow & Stamler, 1998), while the transfer of metal-coordinated NO from ceruplasmin or cytochrome c to glutathione plays an important catalytic role in GSNO formation (Inoue et al., 1999; Basu et al., 2010). Cys-to-Cys (Class I) nitrosylases are SNO-proteins that transfer their NO group to target proteins through specific interactions. For instance, in the case of SNO-hemoglobin deoxygenation promotes its binding to erythrocyte membrane protein anion exchanger-1 (AE1), which is then transnitrosylated propagating a vasodilatory signal (Pawloski, Hess, & Stamler, 2001). Also, the nitrosylated form of thioredoxin 1 (SNO-Trx1) participates in the transnitrosylation of near 50 proteins with procaspase-3 being the most well characterized target (Wu et al., 2011a). Other Cys-to-Cys nitrosylase is *S*-nitrosylated glyceraldehyde 3-phosphate dehydrogenase (SNO-GAPDH), that is a nitrosylase for a subset of nuclear proteins, including DNA-PK, SIRT1, and HDAC2 (Kornberg et al., 2010).

Denitrosylases catalyze the removal of NO groups from the Cys thiol side chain of low molecular-weight thiols or proteins. *S*-nitroglutathione reductase (GSNOR) and the thioredoxin/thioredoxin reductase (Trx/TrxR) system are two enzymatic denitrosylating systems that operate under physiological settings (Benhar, Forrester, & Stamler, 2009). *S*-nitroglutathione reductase is a highly conserved NADH-dependent GSNO-metabolizing enzyme (Liu et al., 2001) that plays an important role in SNO homeostasis, as shown by the increased levels of both GSNO and SNO-proteins in GSNOR$^{-/-}$ mice, demonstrating that GSNO is in equilibrium with a pool of SNO proteins (Liu et al., 2004). *S*-nitroglutathione reductase plays an essential role for homeostatic regulation of cellular SNOs and functions to ameliorate nitrosative stress. We have demonstrated that an increase in GSNOR expression and activity is essential to return increased SNO-protein content to basal levels in L-nitrosocysteine-challenged human hepatocytes (Lopez-Sanchez et al., 2008). Moreover, inhibition of NO production during cholestasis ameliorates hepatocellular injury, improving SNOs homeostasis by reverting the reduction in GSNOR expression and activity, thereby reducing the *S*-nitrosylation of hepatic proteins (Lopez-Sanchez et al., 2010a). The complex role of this denitrosylating activity in (patho)physiology is illustrated by the differing effects observed when GSNOR is genetically deleted in mice. Thus, the genetic deletion of GSNOR in mice exerted protection from airway hyper-responsivity by increasing lung SNOs (Que et al., 2005), whereas other study reported that GSNOR$^{-/-}$ mice exhibited substantial *S*-nitrosylation and proteasomal degradation of a key DNA repair enzyme, causing a tenfold higher incidence of spontaneous hepatocellular carcinoma (Wei et al., 2010). Moreover, a recent study reported that GSNOR deficient mice exist in a persistent state of systemic vasodilatation, suggesting that aberrant protein denitrosylation resulting from altered GSNOR activity or expression, may also play a relevant role in diseases such as hypertension and heart failure (Beigi et al., 2012).

Other specific enzymatic mechanism of regulating basal and stimulus-induced protein denitrosylation is the Trx/TrxR system, that denitrosylates low-molecular-weight SNOs as well as SNO proteins, particularly caspases (Sengupta et al., 2007; Benhar et al., 2008). Increased protein nitrosylation levels are observed when cells are treated with auranofin, a highly specific TrxR inhibitor (Benhar et al., 2008; Tello et al., 2009; Lopez-Sanchez et al., 2010b), suggesting that when the protein reduction activity of Trx is impaired, it may either lose its ability to denitrosylate or increase its ability to transnitrosylate proteins (Wu et al., 2011a). In fact, it is known that oxidation of the active-site cysteines within Trx promotes *S*-nitrosylation of Cys73, which facilitates transnitrosylation of target proteins (Wu et al., 2010). Therefore, uncoupling of Trx from TrxR allows *S*-nitrosylation of oxidized Trx and may promote the function of SNO-Trx as a transnitrosylase. Studies comparing proteins that are transnitrosylated but not denitrosylated by Trx are needed to deepen in the molecular mechanisms of Trx-mediated protein denitrosylation and target specificity. In this regard, a recent proteomic study differentiated Trx transnitrosylation targets from denitrosylation target proteins in neuroblastoma cells. The authors discovered eight peptides that are solely regulated by SNO-Trx1 transnitrosylation but not Trx1 denitrosylation, and 43 peptides whose SNO-Cys residues can be regulated reversibly by Trx1-mediated trans- or denitrosylation (Wu et al., 2011b).

III. PROTEOMIC ANALYSIS OF S-NITROSYLATION

More than 10 years ago, a meeting review listed 115 proteins in which the addition of NO to protein Cys-thiol had been reported to occur in biological systems (Stamler, Lamas, & Fang, 2001). Currently, over 1,700 SNO-proteins with more than 3,000 identified SNO-Cys sites have been experimentally verified (Lee et al., 2012). Proteomic studies focused on *S*-nitrosylation, which recently have been empowered by improved methods for SNO-protein detection and identification, are important contributors to the growing number of SNO-proteins identified.

The detection of SNO-proteins is complicated by a number of factors. The weak S–N bond strength in SNOs renders high sensitivity to decomposition and NO release mediated by reducing agents, such as transition metal ions, ascorbate, anion superoxide, thiols and direct light. In addition, SNO-proteins occur at very low levels in biological samples, and sensitive and specific methods are needed for their efficient detection. Different techniques are available for the quantification of SNO and SNO-proteins in biological fluids, including spectrometry, chemiluminiscence, fluorimetry or mass spectrometry [reviewed by Giustarini et al., 2007]. Most of these methods involve the conversion of the S–NO group to nitrite, which can be determined using the Saville–Griess assay (Marzinzig et al., 1997), or its reduction to NO that can be measured by

chemiluminiscence (Rassaf, Feelisch, & Kelm, 2004). Alternatively, NO and nitrite derived from decomposed SNO can be detected using fluorescent probes (Tyurin et al., 2001). Mass spectrometry (MS)-based approaches, such as liquid chromatography (LC)-MS and gas chromatography (GC)-MS, are also among the analytical methods available for quantification of SNOs and SNO-proteins in biological samples (Tsikas et al., 1999, 2009). However, none the above-mentioned methods are feasible to accommodate in a proteomic approach. To do this, there is a need to selectively target and convert unstable SNO groups to stable conjugates for further proteomic analysis and characterization.

A. The Biotin-Switch Technique (BST)

The possibility of performing a proteomic analysis of protein nitrosylation began 10 years ago with the development by Jaffrey et al. (2001) of the biotin-switch technique (BST), an original approach that permitted the selective tagging of SNO proteins with biotin. In this breakthrough paper the authors used a proteomic approach to identify endogenously *S*-nitrosylated protein in the mouse brain and demonstrated the loss of this modification in mice deficient in nNOS. In the classic BST, the selective labeling of SNO-proteins with biotin is accomplished after three steps (Fig. 2). First, free Cys thiols in proteins are specifically blocked with the alkylating agent methyl methanethiosulfonate (MMTS). Next, protein SNO groups are selectively reduced to thiols with ascorbate, and finally these newly formed thiols are reacted with the thiol-specific biotinylating reagent *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP). The biotin-tagged proteins can be readily detected by immunodetection with anti-biotin antibodies or recognition via streptavidin, or alternatively they can be purified on streptavidin resins. The possibility that the ascorbate treatment may reduce other Cys oxidation-derived modifications, such as S-glutathionylation or *S*-oxidations, has raised concerns about specificity of the BST (Huang & Chen, 2006). However, the specificity of SNO reduction by ascorbate has been reported to rely on a transnitrosylation reaction, distinctive among other Cys oxidation products, yielding the semidehydroascorbate radical and NO (Forrester et al., 2009a). The specific removal of SNOs from the protein sample may constitute a valuable control when performing the BST. *S*-nitrosothiols decompose rapidly in the presence of mercuric ion Hg^{2+}, and $HgCl_2$ has been employed as a diagnostic indicator for the presence of SNOs when using chemiluminescence methods. However, it has been demonstrated that Hg^{2+} interferes with thiol labeling by biotin-HPDP (Zhang et al., 2005) and the pretreatment of samples with $HgCl_2$ would not be useful as control in the BST. Nevertheless, a highly advisable additional control for ascorbate specificity or incomplete blocking of thiols is the exposure of SNO proteins to a strong ultraviolet light source (SNO photolysis) prior to the BST (Forrester, Foster, & Stamler, 2007).

B. BST Improvements to Perform Proteomic Analyses

The application of BST to proteomic analysis has led to a number of significant modifications and improvements designed to facilitate the identification of modified proteins and also to identify those Cys residues that are *S*-nitrosylated in these proteins (Fig. 2). In one such modifications, the fluorophore 7-amino-4-methyl coumarin-3-acetic-acid (AMCA)-HPDP is used instead of biotin-HPDP, allowing the direct visualization of the previously *S*-nitrosylated proteins on non-reducing SDS–PAGE gels after UV exposure (Han et al., 2008). One remarkable advantage of this approach is that the AMCA tag can be easily detected in the MS spectra of the tryptic-digests from the fluorescent protein bands, and the site of the SNO-Cys residues can be accurately identified. Other fluorescent-based modification couples BST to bidimensional electrophoresis (2DE) using fluorescent maleimide reagents instead of biotin-HPDP (Tello et al., 2009). This "fluorescence switch" technique permits specific detection of the fluorescence substituting *S*-nitrosylation, while allowing also for fluorescent detection of total protein using a total protein fluorescent reagent compatible with neutral pH.

Other improvement for the proteomic analysis of *S*-nitrosylation combines difference in gel electrophoresis (DIGE) with the BST in order to examine the difference in modification before and after exposure to an experimental condition (Kettenhofen et al., 2008; Chouchani et al., 2010). In this approach the biotin label is replaced with fluorescent cyanine dyes (Cy3 or Cy5) and equal amounts of control and SNO-treated samples are mixed and separated on the same 2DE gel. Consequently, those protein thiols selectively modified by the *S*-nitrosylation treatment or condition can be detected as protein spots showing differential fluorescent intensity of the two dyes in the gel. In other DIGE-based approach biotin-HDP is replaced by DyLight maleimide sulfhydryl reactive fluors, and selecting appropriate excitation and emission wavelengths, each of the individual samples can also be visualized independently on the same 2D gel (Sun et al., 2007; Lin et al., 2009). One relevant advantage of this methodology is that since each DyLight molecule contains three to four negative charges and an approximate 1 kDa mass, DyLight labeling causes an acidic shift and a minor upwards shift in every single protein spot. Thus, those protein spots with a shifted DyLight pattern can be directly picked from the gel for MS identification. Other advantage of using DIGE-based methodologies to study protein *S*-nitrosylation is that the relative *S*-nitrosylation level for each protein may be directly inferred from the direct comparison of fluorescent intensity from each fluorophore in a single spot. This is important, because there are few quantitative data on the proportion of each protein which is *S*-nitrosylated under different physiologically relevant conditions. A recently developed quantitative BST-based approach uses a novel strategy termed fluorescence saturation (SNOFlo) to specifically label, detect, and quantify protein *S*-nitrosylation (Wiktorowicz et al., 2011). In this method protein samples are labeled to saturation with an uncharged Cys-specific fluorescent dye (BODIPY FL-maleimide) which do not modify protein isoelectric points and permits spot matching between ascorbate-treated and untreated samples in subsequent 2DE with fluorescence quantification. This quantitative proteomic approach permits to normalize for changes in protein abundance and changes in *S*-nitrosylation due to the experimental design.

1. Gel-Free Approaches

The above summarized gel-based approaches to study protein *S*-nitrosylation have a number of inherent drawbacks, such as the

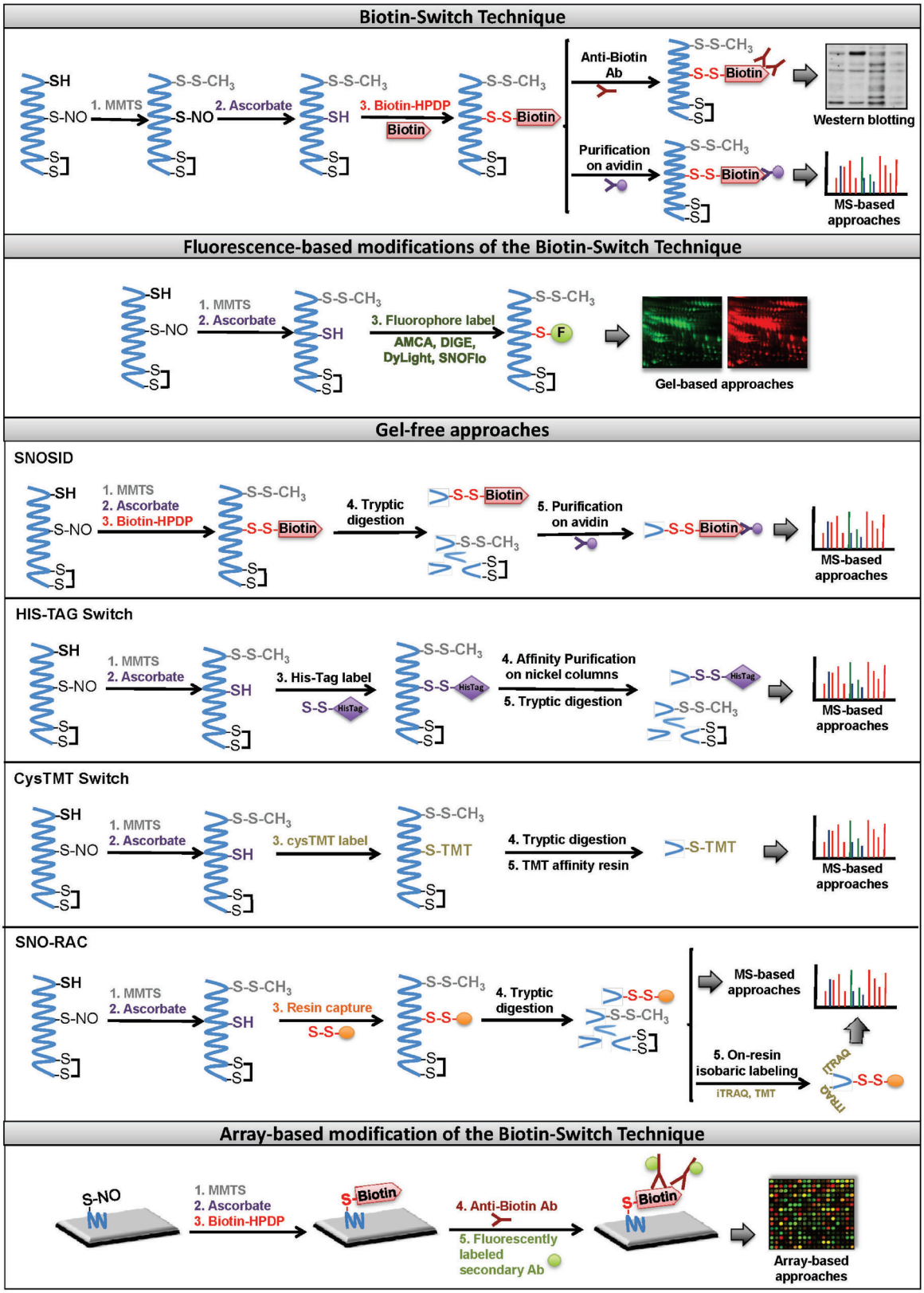

FIGURE 2. The biotin-switch technique and its modifications. During the BST assay *S*-nitrosylated proteins are selectively label with biotin and detected with anti-biotin antibodies or they can be purified on streptavidin resins for their proteomic identification. Figure shows numerous improvements and modifications have been developed from BST assay, which includes the use of fluorescent reagents and others tags instead of biotin, plus diverse MS-based methodologies to identify of SNO proteins and/or their specific sites of nitrosylation. See text for further details. Ab, antibody; AMCA, 7-amino-4-methyl coumarin-3-acetic-acid; Cys, cysteine; DIGE, difference in gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantification; MS, mass spectrometry; MMTS, methyl methanethiosulfonate; SNO, *S*-nitrosothiol; SNO-RAC, *S*-nitrosothiol resin-assisted capture; SNOFlo, *S*-nitrosothiol fluorescence saturation; SNOSID, SNO site identification; TMT, tandem mass tag.

poor detection of hydrophobic membrane proteins and the over-representation of abundant proteins. Additionally, although these approaches may be used to quantitatively analyze protein *S*-nitrosylation, the majority of them provide few information about the modified Cys residue(s) on the protein. Consequently, several BST-based gel-free methods have been developed to overcome these problems.

An important downside of the original BST is that the biotin tag is lost during the purification step, preventing the direct identification of the modified residue. Consequently, one of the first proteomic-oriented modifications of the original BST was the SNO site identification (SNOSID) method, which introduces a tryptic digestion step before biotin–streptavidin purification (Hao et al., 2006; Derakhshan, Wille, & Gross, 2007). In this manner, peptides that previously contained SNO-Cys residues are purified, instead of intact proteins, and they may be directly sequenced by LC-MS/MS to identify the modification sites (Fig. 2). Recently, the stable isotope labeling by aminoacids in cell culture (SILAC) method, which is one of the most used and accurate quantitative techniques in MS-based proteomics, has been coupled to the SNOSID method to assess the relative change in *S*-nitrosylation for hundreds of SNO-sites in a single experiment (Torta & Bachi, 2012). Using this SILAC-SNOSID approach, the authors quantitatively measured the post-translational modification level in 139 SNO-peptides out of 156 identified proteins when comparing lipopolysaccharide-treated versus control macrophages. The coupling of BST to SILAC has also been used as a quantitative proteomic strategy to identify SNO-proteins that are de-nitrosylated by Trx1 (Benhar et al., 2010).

In other variation of the BST, a His-tagged peptide conjugated to iodoacetamide (IAA) is used instead of HPDP-biotin to irreversibly label previous SNO-Cys residues, ensuring that proteins are tagged through all purification steps (Camerini et al., 2007) (Fig. 2). Moreover, the developers of this method, termed HIS-TAG switch method, designed a peptide linker which is cut away during the tryptic digestion leaving a shorter tag attached to the modified Cys residue, not only allowing the purification of previously SNO-proteins but also the identification of SNO-Cys residues by MS. In another modified BST for the site-specific identification of the *S*-nitrosoproteome the labeling of the SNO-Cys thiols is performed by reduction with ascorbate followed by irreversible biotinylation with PEO-iodoacetyl-biotin (Chen et al., 2010). After tryptic digestion, avidin enrichment and LC-MS/MS analysis the SNO site can be unequivocally determined based on the characteristic mass shift.

In a recent BST-based gel free quantitative approach SNO-proteins are purified by reduction of SNO-Cys with ascorbate, irreversible labeling with biotin-maleimide and streptavidin agarose pull-down. After in-solution digestion, SNO-peptides are analyzed by 2D-LC-MS/MS analysis using spectral counting, a label-free quantitative proteomic method which compares the number of spectra of MS/MS assigned to each protein (Zhang, Huang, & Chen, 2012). Using this method, termed SNO spectral counting (SNOSC) method, the authors quantitated the change in level of *S*-nitrosylation in 17 proteins caused by endogenous NO stimulation in RAW264.7 cells (Zhang, Huang, & Chen, 2012).

Recently, a set of novel cysteine reactive tandem mass tag (cysTMT) reagents, which confer the advantage of multiple isobaric tags with reporter ions between 126 and 131 kDa, have been employed in place of biotin for the identification and quantification of *S*-nitrosylation in pulmonary arterial endothelia cells *in vitro* and *in vivo* (Murray et al., 2012). Notably, this cysTMT-labeling method has also been performed in whole heart homogenates to measure *S*-nitrosylation occupancy in the myocardium (Kohr et al., 2012). In the same sample, free and SNO thiols were labeled with different cysTMT isobaric tags and after MS analysis, the percentage of a given Cys residue that was modified via *S*-nitrosylation was then calculated.

Other BST-based method uses a thiol-reactive resin (thiopropyl sepharose) instead of thiol-reactive HPDP-biotin, thus combining in a single step labeling and purification of SNO-proteins (Forrester et al., 2009b). This approach, which is termed resin-assisted capture (SNO-RAC), results in a covalent disulfide linkage between the SNO site and resin, and it is amenable to "on-resin" trypsinization and peptide labeling for further MS analysis. Thus, SNO peptides derived from control or SNO-treated cells were labeled on-resin with isobaric tags for relative and absolute quantification (iTRAQ) allowing the kinetic analysis of *S*-nitrosylation/denitrosylation of individual SNO sites in SNO-proteins. This combination of SNO-RAC and iTRAQ methodologies revealed varying rates of SNO turnover among individual sites of protein *S*-nitrosylation (Forrester et al., 2009b). However, the SNO-Cys residue identification is uncertain using this method, especially when more than one cysteine is present in an identified peptide sequence. For this reason, Liu and co-workers coupled the enrichment of SNO peptides using this thiol-reactive resin to the addition of two alkylation agents with different added masses to differentiate the SNO sites from the non-SNO sites (Liu et al., 2010). Other BST-based approach is the so-called d-switch method, that employs deuterated labeled *N*-ethylmaleimide to quantitate free thiol versus nitrosothiol for each modified Cys residue (Sinha et al., 2010). The above-mentioned methods with isobaric tags for relative or absolute quantification of *S*-nitrosylation are limited with regard to the number of samples that can be analyzed simultaneously. However this can be solved if label-free proteomic methods are used for the MS/MS analysis. Thus, the SNO-RAC method has been coupled to label-free proteomic analysis for the relative quantification of the increased *S*-nitrosylation observed in the ischemic-preconditioned myocardium of mice (Kohr et al., 2011).

2. *Array-Based Approaches*

One of the main limitations of the BST as a proteomic tool is that the assay is biased toward the identification of abundant proteins. Given the low abundance of endogenous *S*-nitrosylated proteins, this is a significant obstacle to proteomic analysis based on the BST and to its contribution to unravel the role of *S*-nitrosylation in disease. For that reason, some researchers have coupled BST to array-based methods for the screening of *S*-nitrosylation across a proteome (Fig. 2). In one of such studies, a fluorescently labeled secondary anti-anti-biotin antibody was used for the detection and facile identification of SNO proteins after performing the BST on a yeast protein microarray containing approximately 4,000 glutathione *S*-transferase-tagged open reading frames, after SNO treatment (Foster, Forrester, & Stamler, 2009). This high-throughput screening revealed not only that neither secondary structure nor intrinsic nucleophilicity of Cys thiols explained *S*-nitrosylation

specificity but also the importance of NO-donor stereochemistry and structure on efficiency of *S*-nitrosylation. In other array-based approach, BST was performed in cell lysates from neuroblastoma cells exposed to calcium ionophore A23187 to produce physiological concentrations of NO, and the biotin-tagged SNO-proteins were subjected to antibody array screening (Numajiri et al., 2011). Using this method 25 SNO-proteins were identified and, among them, PTEN was found to be highly sensitive to relative low concentrations of NO and negatively regulated by *S*-nitrosylation.

C. New Strategies for the Analysis of the *S*-Nitrosoproteome

The above-mentioned concerns about the specificity and/or the efficiency of the BST have encouraged the design of other methods suitable for the proteomic study of *S*-nitrosylation. The development of ascorbate-independent methods for the specific labeling of SNO-Cys residues or the direct detection of *S*-nitrosylation by MS are novel promising strategies.

1. Novel Chemical methods for Direct Labeling of SNO-Cys Residues

Recently, research has been done to explore chemical reactions that could specifically target SNO moieties and convert unstable SNO to stable and detectable products (Fig. 3). In this regard, it has been reported that gold nanoparticles (AuNPs) react with protein SNO-Cys residues to yield AuNP-protein conjugates (thiolates) and release NO (Jia et al., 2009). This property of AuNPs has been recently exploited in their use for the

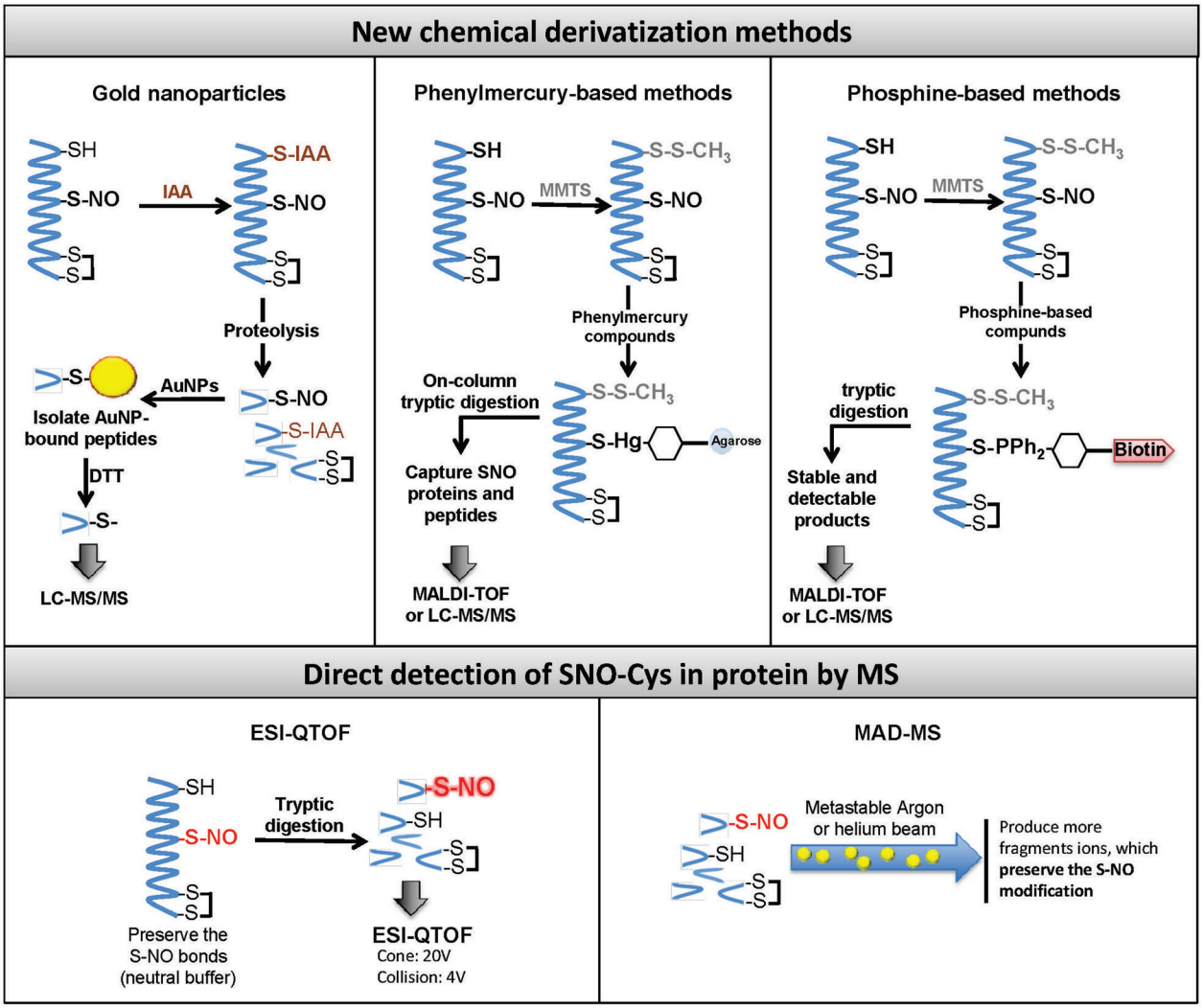

FIGURE 3. Novel tools and strategies for the identification of *S*-nitrosilated proteins. The concerns noted about the specificity and/or the efficiency of the BST has led to the development of other approaches for the detection and identification of SNO proteins such as chemical methods for direct labeling of SNO-Cys residues and novel strategies of their direct detection by MS, which are represented in the figure. See text for further details. AuNPs, gold nanoparticles; DTT, dithiothreitol; ESI, electrospray ionization; Hg, mercury; IAA, iodoacetamide; LC, liquid chromatography; MAD-MS, metastable atom-activation dissociation mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; MMTS, methyl methanethiosulfonate; MS, mass spectrometry; QTOF, quiadrupole time of flight; TOF, time of flight.

enrichment and identification of SNO sites in proteins (Faccenda et al., 2010). In this AuNP-based method free thiols are first blocked with IAA, subjected to proteolysis and incubated with AuNPs. The thiol-bound peptides are then released from AuNPs by incubation with excess dithiothreitol and analyzed by LC-MS, where SNO-Cys residues are identified and ratios of free thiol/SNO are obtained. However, a limitation of this method is that AuNPs react with both SNO-Cys and S-glutathionylated-Cys residues and, although tested in a pure protein system, has yet to be applied to complex biological mixtures.

Other alternatives to the BST have been developed for the direct labeling of SNO-Cys residues taking advantage on the distinct reactivity of the SNO group from other biological functional groups. In the Saville reaction, mercury reacts directly and efficiently with SNOs displacing thiol-bound NO and forming a relatively stable covalent bond with the thiol. Accordingly, a recently developed method employs a phenylmercury resin to capture SNO proteins and, after on-column tryptic digestion, the resulting peptides are eluted and then analyzed by LC-MS/MS to identify SNO-Cys residues (Doulias et al., 2010, 2012). Importantly, in this phenylmercury resin-based method the peptides are eluted by mild performic acid (3%), which cleaves the phenylmercury mercaptide bond and oxidizes the cysteine thiol to sulfonic acid, allowing the unequivocal identification of SNO-Cys residues by MS analysis (Doulias et al., 2010, 2012).

Other potential method for direct labeling of SNO-Cys residues in proteins is the use of photolysis of S–NO in the presence of a radical trap to specifically tag SNO-Cys residues. In contrast to cellular thiols, SNO readily homolyze to NO and thiyl radicals upon irradiation with visible light. Thus, Sengupta and co-workers were able to selectively label SNO upon irradiation by spin trapping the newly formed thiyl radical with 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The DMPO-tagged may be then detected using anti-DMPO antibodies, or pulled down if a biotinylated DMPO is used instead (Sengupta, Billiar, & Stoyanovsky, 2009).

S-nitrosothiols and phosphine-thioester substrates has been shown to undergo a reductive ligation in which unstable SNOs are converted to stable disulfides in one step under very mild conditions. This reaction has been used to identify SNO proteins by incubating cell extracts with a biotin-linked phosphine substrate to form a stable disulfide linkage with biotin (Zhang et al., 2010). In other phosphine-based approach, the water-soluble phosphine tris(4,6-dimethyl-3-sulfonatomethyl)-phosphine trisodium salt hydrate reacts rapidly and specifically with SNO-Cys, GSNO, and SNO-proteins to yield stable S-alkyl-phosphonium adducts, which are detected in MS analysis (Bechtold et al., 2010). These works clearly illustrate the ability of phosphine reagents to directly react with SNOs and form unique adducts for the detection of SNO-proteins. Additionally, these phosphines do not react with free thiols, and therefore, unlike the BST, require the SNO group functionality to form detectable S-adducts. At present, detection of these adducts relies on nuclear magnetic resonance spectroscopy and MS, but the development of novel phosphine-based reagents with fluorescent or immunologically detectable groups could provide researchers with new chemical probes for the direct detection of SNO-proteins in biological contexts (Bechtold & King, 2012; Li et al., 2012).

2. *Direct Detection of SNO-Cys in Proteins by MS*

Direct detection of SNO-proteins by MS would be preferable to BST or similar chemical derivatization methods, as the direct identification would minimize the generation of false-positives and better reflect the *in vivo* situation, thus validating the occurrence of *S*-nitrosylation under different biological conditions. Unfortunately, direct identification of SNO-sites in proteins by MS analysis is especially challenging due to the labile nature of the S–NO bond. For example, in matrix-assisted laser desorption/ionization (MALDI-TOF) MS, the laser energy required for peptide ionization causes the loss of NO from the Cys residue. However, electrospray ionization (ESI) is a more gentle ionization method, and it has been reported that in ESI-MS analysis SNO-peptides can be observed with a +29 Da mass variation with respect to the unmodified ions (Mirza, Chait, & Lander, 1995). In other approach, Gross and collaborators used ESI coupled to a triple quadrupole mass spectrometer to identify parental peptide ions that lose precisely the uncharged mass of NO (30 and 15 Da for +1 and +2 charged peptide ions, respectively) following collision (Hao, Xie, & Gross, 2004). Other LC/MS/MS strategy enabling direct identification of SNO sites in proteins by using ESI quadrupole TOF (ESI-QTOF)-MS has been recently developed (Fig. 3) (Wang et al., 2008). In their study, Wang and co-workers fine-tuned both cone and collision energy voltages in ESI-QTOF-MS to preserve the S–NO bonds, and they found that Cys73 in human Trx1 was specifically *S*-nitrosylated after GSNO treatment.

The S–NO bond is also labile under usual collision induced dissociation (CID) conditions, and the yield of fragment ions providing sequence information is too low during conventional ion trap CID MS/MS analysis. However, a new type of dissociation method using metastable atoms as energy source to induce fragmentation has been recently explored in ion trapping instruments (Fig. 3). In this method, which is termed metastable atom-activated dissociation (MAD), isolated precursor ions interact with a high kinetic energy beam of argon or helium metastable atoms to produces a high degree of peptide backbone cleavages resulting in fragment ions while retaining post-translational modifications (Cook, Collin, & Jackson, 2009). MAD has been applied for the characterization of SNO-peptides in quadrupole ion-trap MS analysis and, compared with CID, which is a dissociation method traditionally employed in ESI MS/MS, MAD produced between 66% and 86% more fragment ions, which preserved the labile NO modification (Cook & Jackson, 2011).

The above-mentioned approaches were limited to synthetic SNO-peptides or purified proteins, or were able to identify only a few SNO-sites from complex protein mixtures. Their suitability for the study of the *S*-nitrosoproteome, that is, for high-throughput analysis of unknown SNO-sites in complex biological samples, remains to be demonstrated.

3. *Computational Methods to Investigate the S-Nitrosoproteome*

In recent years, various computational approaches for classification and prediction of different functional categories of Cys residues in proteins have been developed. As a result, several bioinformatic tools for prediction of Cys involved in disulfide bonds and metal-binding sites are currently available (Marino

& Gladyshev, 2011). The ability to recognize and predict regulatory Cys residues, such as SNO-Cys, would also be extremely valuable. However, their investigation has proved difficult from a computational perspective, mainly due to the lack of large and reliable experimental datasets. Currently available proteomic approaches for the study of *S*-nitrosylation are substantially improving this aspect, and recently developed computational methods for prediction of SNO-Cys represent a major advance in this direction. However, a potential weakness of computational methods is that, due to the absence of endogenous SNO-Cys proteomes, they rely on datasets from studies using exogenous NO or *S*-nitrosylating agents to discover SNO-proteins and their sites of modification, but many of them have not been confirmed *in vivo* (Marino & Gladyshev, 2010).

Recently, an improved algorithm (GPS 3.0) has been released and implemented in a web accessible software (GPS-SNO 1.0, http://sno.biocuckoo.org) for prediction of SNO sites (Xue et al., 2010). Their developers used scientific literature and public databases to obtain data from 504 experimentally verified SNO sites in 327 unique proteins. To evaluate the predictive value they also collected 485 potentially *S*-nitrosylated substrates from large- or small-scale studies, in which the exact SNO sites had not been experimentally determined, and GPS 3.0 predicted 359 (74%) of these targets with at least one potential SNO site (Xue et al., 2010). A set of supervised learning algorithms (support vector machines) has been recently used to develop other automated computational method (CPR-SNO) to predict *S*-nitrosylation sites from protein sequences (Li et al., 2011). Their developers claim that CPR-SNO has a superior predictive performance compared to GPS-SNO. The predictions from these and other computational methods might be of use for annotating large-scale potential SNO sites and for further experimental verification. In this regard, a new database (dbSNO) containing the experimentally verified SNO sites from research articles has been created and made freely accessible (http://dbsno.mbc.nctu.edu.tw) to facilitate the functional analysis of SNO-proteins (Zhang et al., 2012b).

IV. PROTEOMIC ANALYSIS OF DEREGULATED S-NITROSYLATION IN DISEASE

A broad number of studies have shown that *S*-nitrosylation processes play a crucial role in the pathophysiology of a plethora of human diseases including cardiovascular (Lima et al., 2010), pulmonary (Ckless et al., 2008), immunological (Hernansanz-Agustin et al., 2013), and neurological dysfunctions (Nakamura & Lipton, 2011), as well as in cancer (Aranda et al., 2012). In many cases, pathophysiology correlates with hypo- or hyper-*S*-nitrosylation of specific protein targets, rather than a general cellular insult due to loss of or enhanced NOS activity (Foster, Hess, & Stamler, 2009). Accordingly, recent studies pinpoint the key role of nitrosylases and denitrosylases in governing levels of *S*-nitrosylation under both physiological and pathophysiological conditions (Anand & Stamler, 2012). As reviewed above, researchers now have access to a variety of novel proteomic tools to study protein *S*-nitrosylation. Certainly, the use of such nitrosoproteomic methods will suggest novel mechanisms for the dysregulation of protein *S*-nitrosylation in disease and add new potential therapeutic targets. However, the overwhelming majority of the studies on deregulated *S*-nitrosylation and disease have not been based on proteomic approaches, and as a result the number of studies of "pathological" *S*-nitrosoproteomes published to date is still very limited.

Dysregulation of protein *S*-nitrosylation appear to be prevalent in neurodegenerative disorders characterized, in particular, by the accumulation of misfolded proteins (Foster, Hess, & Stamler, 2009; Nakamura & Lipton, 2011). Thus, debilitating neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, can be attributed to neuronal cell damage in specific brain regions. An important hallmark of these diseases is increased oxidative and nitrosative stress that occurs via overproduction of highly reactive oxygen and nitrogen species. *S*-nitrosylation of an increasing number of proteins compromises important cellular functions, including mitochondrial dynamics, endoplasmic reticulum protein folding, and signal transduction, thereby promoting synaptic damage, cell death, and neurodegeneration (Akhtar et al., 2012). However, to date there are no comprehensive proteomic studies of *S*-nitrosylation in neurodegenerative diseases. To date, only a recent study, by Riederer and co-workers, identified differences in nitrosylated proteins between aging and Alzheimer's samples by using a proteomic approach (Riederer et al., 2009). After separating proteins by 1D- and 2D-gel electrophoresis and immunodetection of SNO-proteins with a polyclonal anti-*S*-nitrosyl-cysteine antibody, the authors identified a number of glial fibrillary proteins in nitrosylated state.

Other researchers have employed animal experimental models to gain a proteomic insight into the connection between deregulated *S*-nitrosylation of proteins and neuropathology. Thus, a recent report identified major *S*-nitrosylated proteins in vivo during the course of inflammatory demyelination in mice with acute and chronic experimental autoimmune encephalomyelitis (EAE), which serves as a model for the human disease multiple sclerosis (Bizzozero & Zheng, 2009). *S*-nitrosylated proteins were isolated from spinal cord of mice with acute and chronic EAE by using the BST and immunodetected with antibodies against previously identified major *S*-nitrosylated substrates of spinal cord tissue. The *S*-nitrosylation state of several proteins, particularly neuronal specific enolase and glyceraldehyde-3-phosphate dehydrogenase, were found increased in EAE, although further research is needed to establish the pathological consequences of increased *S*-nitrosylation of these and other proteins in demyelinating disorders (Bizzozero & Zheng, 2009).

Recently, Scheving and co-workers analyzed the spinal cord *S*-nitrosoproteome in a murine nerve-injury model to assess whether direct *S*-nitrosylation of proteins may contribute to the pathological adaptations leading to chronic neuropathic pain (Scheving et al., 2012). They used BST coupled to DIGE as a method for the screening of SNO-proteins in spinal cord after nerve injury, time dependent *S*-nitrosylation and effect of NOS inhibition. Additionally, the SNOSID method was used directly to identify SNO-Cys and to compare different techniques. SNO site identification (SNOSID)[***] and BST-DIGE provided complementary information, revealing 53 proteins with altered *S*-nitrosylation state and the modification sites in 17 proteins. The authors of this study suggest that SNO-modifications in the identified SNO-proteins, which are involved in mitochondrial functions, protein folding, redox control and cytoskeletal

remodeling, contribute to the development of neuropathic pain (Scheving et al., 2012).

The long recognized role of NO as important mediator in liver physio(patho)logy suggest that deregulation of *S*-nitrosylation may be the underlying cause of a variety of hepatic disorders. Our group has recently reported that enhanced *S*-nitrosylation of hepatic proteins during induced cholestatic liver injury in rats is related to down-regulation of GSNOR expression and activity (Lopez-Sanchez et al., 2010a). Important enzymes responsible for energy production and metabolism, as well as molecular chaperones and proteins involved in the structural integrity of the cells, were found to be *S*-nitrosylated in cholestatic livers, as revealed by BST-based proteomic analysis. Notably, the *S*-nitrosylation during cholestasis of important enzymes involved in the methionine cycle, such as methionine synthetase and betaine-homocysteine S-methyltransferase, suggest novel therapeutic targets in the treatment of cholestatic disorders (Lopez-Sanchez et al., 2010a). *S*-nitroglutathione reductase deficiency in the liver has also been recently involved in the promotion of hepatocarcinogenesis through *S*-nitrosylation and proteasomal degradation of the key DNA repair enzyme *O*(6)-alkylguanine-DNA alkyltransferase (Wei et al., 2010). Further proteomics research on hepatic *S*-nitrosylation/denitrosylation and their role in liver disease will be essential to decipher the intricate role of this NO-related post-translational modification in liver disease.

The SNO-mediated actions of NO are relevant to a wide range of cardiovascular disease processes, including angiogenesis, diabetes, atherosclerosis, or heart failure (Lima et al., 2010). In the cardiovascular system protein *S*-nitrosylation have been involved in vascular and cardiac signaling (Lima et al., 2010; Maron, Tang, & Loscalzo, 2013) and also in cardioprotective processes (Sun & Murphy, 2010). One of the mechanisms proposed for the cardioprotective effects of *S*-nitrosylation is that this modification not only leads to changes in protein structure and function, but also prevents these thiol(s) from further irreversible oxidative/nitrosative modification (Sun & Murphy, 2010). In this regard, Kohr and co-workers have recently reported the use of the SNO-RAC method for the simultaneous measurement of protein thiol oxidation and *S*-nitrosylation during preconditioning and ischemia/reperfusion (I/R) injury (Kohr et al., 2011). In this study, protein *S*-nitrosylation induced by ischemia preconditioning (IPC) shielded critical Cys residues against oxidation. A total of 47 SNO sites in 33 unique SNO proteins were identified in mice hearts after IPC. Notably, the majority of these proteins exhibited reduced or no oxidation at the same site following ischemia and early reperfusion, supporting the notion that *S*-nitrosylation provides a direct protective effect against Cys oxidation following I/R injury (Kohr et al., 2011). This same group has recently developed the above described cysTMT-labeling method as a novel approach for the measurement of SNO occupancy in the myocardium (Kohr et al., 2012). This technique, which was validated using GSNO-treated whole-heart homogenates, was used to confirm that IPC-induced *S*-nitrosylation is sufficient to shield Cys residues against excessive oxidation. The protection afforded by *S*-nitrosylation may prevent the degradation of proteins that are excessively oxidized, Interestingly, several of the protein targets previously known to be degraded to a lesser extent following IPC also showed high levels of *S*-nitrosylation in this model of cardioprotection (Kohr et al., 2012). The developers of the SNOFlo method applied this quantitative proteomic approach to study *S*-nitrosylation in ischemic brain injury (Wiktorowicz et al., 2011). In their study, Wiktorowicz and colleagues identified 41 proteins with differential *S*-nitrosylation status, and 21 of them showed the greatest changes upon hypoxia induction, while 19 showed the greatest changes upon reperfusion. The differentially *S*-nitrosylated proteins were involved in apoptosis, branching morphogenesis of axons, cortical neurons and sympathetic neurites, neurogenesis, and calcium signaling (Wiktorowicz et al., 2011).

Pre-eclampsia is a pregnancy-specific hypertensive disorder which is an important cause of maternal and fetal morbidity and mortality worldwide. Nitric oxide is known to play a crucial role in lowering vascular resistance of the uterus and placenta unit throughout pregnancy (Rosselli, Keller, & Dubey, 1998), and inappropriate placental hypoxia and hypoxia-reoxygenation injury is considered among the possible mechanisms participating in the pathogenesis of pre-eclampsia (Gilbert, Nijland, & Knoblich, 2008). A recent study employed the BST coupled with the CyDye/2D-DIGE method to assess whether deregulated *S*-nitrosylation may participate in the pathogenesis of pre-eclampsia (Zhang, Wang, & Chen, 2011). Comparing the *S*-nitrosoproteomes in normotensive and pre-eclamptic human placentas the authors identified 41 differentially *S*-nitrosylated proteins, including enzymes critical to protein synthesis, folding, post-translational modification and degradation. Although the differential analysis of the two *S*-nitrosoproteomes suggests that protein *S*-nitrosylation seems to play a critical role in regulating normal placental physiology and pathophysiology, further research is warranted to evaluate whether changes in SNO-proteins contribute to the pathogenesis of pre-eclampsia.

V. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The *S*-nitrosylation of a growing number of proteins has been reported over the past decade (Lee et al., 2012), and in many cases this reversible post-translational modification is accompanied by altered protein function. Recent research has highlighted the key role of enzyme-mediated processes in SNO homeostasis and their participation as factors that determine the site specificity of *S*-nitrosylation. Albeit purification and identification of SNO-proteins is still a challenging methodological matter, methods currently available for the proteomic analysis of *S*-nitrosylation surpass those available only a few years ago (Lopez-Sanchez et al., 2009). If appropriate controls are included, many of the proteomic approaches here described may be sensitive enough to detect endogenous *S*-nitrosylation, although this is highly dependent on the biological sample under analysis. Although it is difficult to indicate the depth of analysis for each approach, it is worth to note that the BST was first described to be able to identify endogenously *S*-nitrosylated protein in the mouse brain and the loss of this modification in mice deficient in nNOS. On the other hand, the false identification rates for the majority of these methods are unknown, and additional direct approaches (i.e., genetic approaches) must be required to unequivocally establish the *S*-nitrosylation status of the protein/s of interest.

New recent refinements and improvements to these methods have been directed to a more direct detection, shortening preparative protocols, addressing reagents selectivity and avoiding the actual loss of the functional group of interest. Consequently, there is a growing contribution of proteomic advances to the analysis of the nitrosoproteome and to our knowledge of how this post-translational modification affects protein function of each protein. Aberrant *S*-nitrosylation of proteins, caused by altered production of NO and/or impaired SNO homeostasis, has been repeatedly reported in numerous pathophysiological settings. Therefore, the analysis of nitrosoproteomes under pathological conditions is beginning to be reported, and the development of novel therapeutic targets will be inspired by this type of studies in the near future. Also, progress in new diagnostic or prognostic biomarkers of disease will be greatly helped by the currently available or further developed methods to detect and quantify SNO-proteins from tissues or biological fluids. In this regard, new exciting direct methods for the chemical labeling of SNOs emerged within the last 5 years and they will undoubtedly facilitate new chemical probes for the direct detection of protein *S*-nitrosylation in complex biological samples. We can envisage that our knowledge concerning deregulated protein *S*-nitrosylation and disease will certainly be boosted by some of the proteomic tools reviewed here.

VI. ABBREVIATIONS

2DE	bidimensional electrophoresis
AMCA	7-amino-4-methyl coumarin-3-acetic-acid
AuNPs	gold nanoparticles
Biotin-HPDP	*N*-[6-(biotinamido)hexyl]-3'-(2'pyridyldithio) propionamide
BST	biotin-switch technique
CID	collision induced dissociation
cysTMT	cysteine reactive Tandem Mass Tag
DIGE	difference in gel electrophoresis
DTT	dithiothreitol
EAE	experimental autoimmune encephalomyelitis
ESI	electrospray ionization
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gas chromatography
GSNO	*S*-nitrosoglutathione
GSNOR	*S*-nitrosoglutathione reductase
I/R	ischemia/reperfusion
IAA	iodoacetamide
IPC	ischemia preconditioning
iTRAQ	isobaric tags for relative and absolute quantification
LC	liquid chromatography
MAD	metastable atom-activated dissociation
MALDI-TOF	matrix-assisted laser desorption/ionization
MMTS	methyl methanethiosulfonate
MS	mass spectrometry
NO	nitric oxide
NOS	nitric oxide synthase
SILAC	stable isotope labeling by aminoacids in cell culture
SNO	*S*-nitrosothiol
SNO-Cys	*S*-nitrosocysteine
SNOFlo	SNO fluorescence saturation
SNO-RAC	SNO resin-assisted capture
SNOSC	SNO spectral counting
SNOSID	SNO Site Identification
Trx	thioredoxin
TrxR	thioredoxin reductase

ACKNOWLEDGMENTS

Supported by the Andalusian Government (Grant JA0230/09 from the Consejería de Salud de la Junta de Andalucía) and Spanish Government (Grant FIS 10/00428 from the Programa de Promoción de la Investigación en Salud del Ministerio de Economía y Competitividad).

REFERENCES

Akhtar MW, Sunico CR, Nakamura T, Lipton SA. 2012. Redox regulation of protein function via cysteine *S*-nitrosylation and its relevance to neurodegenerative diseases. Int J Cell Biol 2012:463756.

Alderton WK, Cooper CE, Knowles RG. 2001. Nitric oxide synthases: Structure, function and inhibition. Biochem J 357:593–615.

Anand P, Stamler JS. 2012. Enzymatic mechanisms regulating protein *S*-nitrosylation: Implications in health and disease. J Mol Med (Berl) 90:233–244.

Aranda E, Lopez-Pedrera C, De La Haba-Rodriguez JR, Rodriguez-Ariza A. 2012. Nitric oxide and cancer: The emerging role of *S*-nitrosylation. Curr Mol Med 12:50–67.

Basu S, Keszler A, Azarova NA, Nwanze N, Perlegas A, Shiva S, Broniowska KA, Hogg N, Kim-Shapiro DB. 2010. A novel role for cytochrome c: Efficient catalysis of *S*-nitrosothiol formation. Free Radic Biol Med 48:255–263.

Bechtold E, King SB. 2012. Chemical methods for the direct detection and labeling of *S*-nitrosothiols. Antioxid Redox Signal 17:981–991.

Bechtold E, Reisz JA, Klomsiri C, Tsang AW, Wright MW, Poole LB, Furdui CM, King SB. 2010. Water-soluble triarylphosphines as biomarkers for protein *S*-nitrosation. ACS Chem Biol 5:405–414.

Beigi F, Gonzalez DR, Minhas KM, Sun QA, Foster MW, Khan SA, Treuer AV, Dulce RA, Harrison RW, Saraiva RM, Premer C, Schulman IH, Stamler JS, Hare JM. 2012. Dynamic denitrosylation via *S*-nitrosoglutathione reductase regulates cardiovascular function. Proc Natl Acad Sci USA 109:4314–4319.

Benhar M, Forrester MT, Hess DT, Stamler JS. 2008. Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins. Science 320:1050–1054.

Benhar M, Forrester MT, Stamler JS. 2009. Protein denitrosylation: Enzymatic mechanisms and cellular functions. Nat Rev Mol Cell Biol 10:721–732.

Benhar M, Thompson JW, Moseley MA, Stamler JS. 2010. Identification of *S*-nitrosylated targets of thioredoxin using a quantitative proteomic approach. Biochemistry 49:6963–6969.

Bizzozero OA, Zheng J. 2009. Identification of major *S*-nitrosylated proteins in murine experimental autoimmune encephalomyelitis. J Neurosci Res 87:2881–2889.

Camerini S, Polci ML, Restuccia U, Usuelli V, Malgaroli A, Bachi A. 2007. A novel approach to identify proteins modified by nitric oxide: The HIS-TAG switch method. J Proteome Res 6:3224–3231.

Chen YJ, Ku WC, Lin PY, Chou HC, Khoo KH, Chen YJ. 2010. S-alkylating labeling strategy for site-specific identification of the *S*-nitrosoproteome. J Proteome Res 9:6417–6439.

Chouchani ET, Hurd TR, Nadtochiy SM, Brookes PS, Fearnley IM, Lilley KS, Smith RA, Murphy MP. 2010. Identification of *S*-nitrosated mitochondrial proteins by *S*-nitrosothiol difference in gel electrophoresis (SNO-DIGE): Implications for the regulation of mitochondrial function by reversible *S*-nitrosation. Biochem J 430:49–59.

Ckless K, Lampert A, Reiss J, Kasahara D, Poynter ME, Irvin CG, Lundblad LK, Norton R, van der Vliet A, Janssen-Heininger YM. 2008. Inhibition of arginase activity enhances inflammation in mice with allergic airway disease, in association with increases in protein *S*-nitrosylation and tyrosine nitration. J Immunol 181:4255–4264.

Cook SL, Jackson GP. 2011. Characterization of tyrosine nitration and cysteine nitrosylation modifications by metastable atom-activation dissociation mass spectrometry. J Am Soc Mass Spectrom 22:221–232.

Cook SL, Collin OL, Jackson GP. 2009. Metastable atom-activated dissociation mass spectrometry: Leucine/isoleucine differentiation and ring cleavage of proline residues. J Mass Spectrom 44:1211–1223.

Derakhshan B, Wille PC, Gross SS. 2007. Unbiased identification of cysteine *S*-nitrosylation sites on proteins. Nat Protoc 2:1685–1691.

Doulias PT, Greene JL, Greco TM, Tenopoulou M, Seeholzer SH, Dunbrack RL, Ischiropoulos H. 2010. Structural profiling of endogenous *S*-nitrosocysteine residues reveals unique features that accommodate diverse mechanisms for protein *S*-nitrosylation. Proc Natl Acad Sci USA 107:16958–16963.

Doulias PT, Raju K, Greene JL, Tenopoulou M, Ischiropoulos H. 2012. Mass spectrometry-based identification of *S*-nitrosocysteine in vivo using organic mercury assisted enrichment. Methods [Epub ahead of print]. doi: 10.1016/j.ymeth.2012.10.009.

Faccenda A, Bonham CA, Vacratsis PO, Zhang X, Mutus B. 2010. Gold nanoparticle enrichment method for identifying *S*-nitrosylation and S-glutathionylation sites in proteins. J Am Chem Soc 132:11392–11394.

Forrester MT, Foster MW, Stamler JS. 2007. Assessment and application of the biotin switch technique for examining protein *S*-nitrosylation under conditions of pharmacologically induced oxidative stress. J Biol Chem 282:13977–13983.

Forrester MT, Foster MW, Benhar M, Stamler JS. 2009a. Detection of protein *S*-nitrosylation with the biotin-switch technique. Free Radic Biol Med 46:119–126.

Forrester MT, Thompson JW, Foster MW, Nogueira L, Moseley MA, Stamler JS. 2009b. Proteomic analysis of *S*-nitrosylation and denitrosylation by resin-assisted capture. Nat Biotechnol 27:557–559.

Foster MW, Forrester MT, Stamler JS. 2009. A protein microarray-based analysis of *S*-nitrosylation. Proc Natl Acad Sci USA 106:18948–18953.

Foster MW, Hess DT, Stamler JS. 2009. Protein *S*-nitrosylation in health and disease: a current perspective. Trends Mol Med 15:391–404.

Foster MW, Liu L, Zeng M, Hess DT, Stamler JS. 2009. A genetic analysis of nitrosative stress. Biochemistry 48:792–799.

Gilbert JS, Nijland MJ, Knoblich P. 2008. Placental ischemia and cardiovascular dysfunction in preeclampsia and beyond: Making the connections. Expert Rev Cardiovasc Ther 6:1367–1377.

Giustarini D, Milzani A, le-Donne I, Rossi R. 2007. Detection of *S*-nitrosothiols in biological fluids: a comparison among the most widely applied methodologies. J Chromatogr B Analyt Technol Biomed Life Sci 851:124–139.

Gow AJ, Stamler JS. 1998. Reactions between nitric oxide and haemoglobin under physiological conditions. Nature 391:169–173.

Greco TM, Hodara R, Parastatidis I, Heijnen HF, Dennehy MK, Liebler DC, Ischiropoulos H. 2006. Identification of *S*-nitrosylation motifs by site-specific mapping of the *S*-nitrosocysteine proteome in human vascular smooth muscle cells. Proc Natl Acad Sci USA 103:7420–7425.

Han P, Zhou X, Huang B, Zhang X, Chen C. 2008. On-gel fluorescent visualization and the site identification of *S*-nitrosylated proteins. Anal Biochem 377:150–155.

Hao G, Xie L, Gross SS. 2004. Argininosuccinate synthetase is reversibly inactivated by *S*-nitrosylation in vitro and in vivo. J Biol Chem 279:36192–36200.

Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. 2006. SNOSID, a proteomic method for identification of cysteine *S*-nitrosylation sites in complex protein mixtures. Proc Natl Acad Sci USA 103:1012–1017.

Hernansanz-Agustin P, Izquierdo-Alvarez A, Garcia-Ortiz A, Ibiza S, Serrador JM, Martinez-Ruiz A. 2013. Nitrosothiols in the immune system: Signaling and protection. Antioxid Redox Signal 18:288–308.

Hess DT, Stamler JS. 2012. Regulation by *S*-nitrosylation of protein post-translational modification. J Biol Chem 287:4411–4418.

Hess DT, Matsumoto A, Kim SO, Marshall HE, Stamler JS. 2005. Protein *S*-nitrosylation: Purview and parameters. Nat Rev Mol Cell Biol 6:150–166.

Huang B, Chen C. 2006. An ascorbate-dependent artifact that interferes with the interpretation of the biotin switch assay. Free Radic Biol Med 41:562–567.

Ibiza S, Perez-Rodriguez A, Ortega A, Martinez-Ruiz A, Barreiro O, Garcia-Dominguez CA, Victor VM, Esplugues JV, Rojas JM, Sanchez-Madrid F, Serrador JM. 2008. Endothelial nitric oxide synthase regulates N-Ras activation on the Golgi complex of antigen-stimulated T cells. Proc Natl Acad Sci USA 105:10507–10512.

Inoue K, Akaike T, Miyamoto Y, Okamoto T, Sawa T, Otagiri M, Suzuki S, Yoshimura T, Maeda H. 1999. Nitrosothiol formation catalyzed by ceruloplasmin. Implication for cytoprotective mechanism in vivo. J Biol Chem 274:27069–27075.

Iwakiri Y, Satoh A, Chatterjee S, Toomre DK, Chalouni CM, Fulton D, Groszmann RJ, Shah VH, Sessa WC. 2006. Nitric oxide synthase generates nitric oxide locally to regulate compartmentalized protein *S*-nitrosylation and protein trafficking. Proc Natl Acad Sci USA 103:19777–19782.

Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. 2001. Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. Nat Cell Biol 3:193–197.

Jaffrey SR, Fang M, Snyder SH. 2002. Nitrosopeptide mapping: A novel methodology reveals *S*-nitrosylation of dexras1 on a single cysteine residue. Chem Biol 9:1329–1335.

Jia HY, Liu Y, Zhang XJ, Han L, Du LB, Tian Q, Xu YC. 2009. Potential oxidative stress of gold nanoparticles by induced-NO releasing in serum. J Am Chem Soc 131:40–41.

Keszler A, Zhang Y, Hogg N. 2010. Reaction between nitric oxide, glutathione, and oxygen in the presence and absence of protein: How are *S*-nitrosothiols formed? Free Radic Biol Med 48: 55–64.

Kettenhofen NJ, Wang X, Gladwin MT, Hogg N. 2008. In-gel detection of *S*-nitrosated proteins using fluorescence methods. Methods Enzymol 441:53–71.

Kleinert H, Pautz A, Linker K, Schwarz PM. 2004. Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol 500: 255–266.

Knowles RG, Palacios M, Palmer RM, Moncada S. 1989. Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. Proc Natl Acad Sci USA 86:5159–5162.

Kohr MJ, Sun J, Aponte A, Wang G, Gucek M, Murphy E, Steenbergen C. 2011. Simultaneous measurement of protein oxidation and *S*-nitrosylation during preconditioning and ischemia/reperfusion injury with resin-assisted capture. Circ Res 108:418–426.

Kohr MJ, Aponte A, Sun J, Gucek M, Steenbergen C, Murphy E. 2012. Measurement of *S*-nitrosylation occupancy in the myocardium with cysteine-reactive tandem mass tags: Short communication. Circ Res 111:1308–1312.

Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JV, Snowman AM, Law L, Hester LD, Snyder SH. 2010. GAPDH mediates nitrosylation of nuclear proteins. Nat Cell Biol 12:1094–1100.

Lee TY, Chen YJ, Lu CT, Ching WC, Teng YC, Huang HD, Chen YJ. 2012. dbSNO: A database of cysteine *S*-nitrosylation. Bioinformatics 28: 2293–2295.

Li YX, Shao YH, Jing L, Deng NY. 2011. An efficient support vector machine approach for identifying protein *S*-nitrosylation sites. Protein Pept Lett 18:573–587.

Li S, Wang H, Xian M, Whorton AR. 2012. Identification of protein nitrosothiols using phosphine-mediated selective reduction. Nitric Oxide 26:20–26.

Lima B, Forrester MT, Hess DT, Stamler JS. 2010. *S*-nitrosylation in cardiovascular signaling. Circ Res 106:633–646.

Lin J, Steenbergen C, Murphy E, Sun J. 2009. Estrogen receptor-beta activation results in *S*-nitrosylation of proteins involved in cardioprotection. Circulation 120:245–254.

Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. 2001. A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. Nature 410:490–494.

Liu L, Yan Y, Zeng M, Zhang J, Hanes MA, Ahearn G, McMahon TJ, Dickfeld T, Marshall HE, Que LG, Stamler JS. 2004. Essential roles of *S*-nitrosothiols in vascular homeostasis and endotoxic shock. Cell 116:617–628.

Liu M, Hou J, Huang L, Huang X, Heibeck TH, Zhao R, Pasa-Tolic L, Smith RD, Li Y, Fu K, Zhang Z, Hinrichs SH, Ding SJ. 2010. Site-specific proteomics approach for study protein *S*-nitrosylation. Anal Chem 82:7160–7168.

Lopez-Sanchez LM, Corrales FJ, Gonzalez R, Ferrin G, Munoz-Castaneda JR, Ranchal I, Hidalgo AB, Briceno J, Lopez-Cillero P, Gomez MA, de la MM, Muntane J, Rodriguez-Ariza A. 2008. Alteration of *S*-nitrosothiol homeostasis and targets for protein *S*-nitrosation in human hepatocytes. Proteomics 8:4709–4720.

Lopez-Sanchez LM, Muntane J, de la MM, Rodriguez-Ariza A. 2009. Unraveling the *S*-nitrosoproteome: Tools and strategies. Proteomics 9:808–818.

Lopez-Sanchez LM, Corrales FJ, Barcos M, Espejo I, Munoz-Castaneda JR, Rodriguez-Ariza A. 2010a. Inhibition of nitric oxide synthesis during induced cholestasis ameliorates hepatocellular injury by facilitating *S*-nitrosothiol homeostasis. Lab Invest 90:116–127.

Lopez-Sanchez LM, Corrales FJ, Lopez-Pedrera C, Aranda E, Rodriguez-Ariza A. 2010b. Pharmacological impairment of *S*-nitrosoglutathione or thioredoxin reductases augments protein *S*-nitrosation in human hepatocarcinoma cells. Anticancer Res 30:415–421.

Luchsinger BP, Rich EN, Gow AJ, Williams EM, Stamler JS, Singel DJ. 2003. Routes to *S*-nitroso-hemoglobin formation with heme redox and preferential reactivity in the beta subunits. Proc Natl Acad Sci USA 100:461–466.

Marino SM, Gladyshev VN. 2010. Structural analysis of cysteine *S*-nitrosylation: A modified acid-based motif and the emerging role of tran*S*-nitrosylation. J Mol Biol 395:844–859.

Marino SM, Gladyshev VN. 2011. Redox biology: Computational approaches to the investigation of functional cysteine residues. Antioxid Redox Signal 15:135–146.

Marletta MA. 1994. Nitric oxide synthase: Aspects concerning structure and catalysis. Cell 78:927–930.

Maron BA, Tang SS, Loscalzo J. 2013. *S*-nitrosothiols and the *S*-nitrosoproteome of the cardiovascular system. Antioxid Redox Signal 18:270–287.

Marzinzig M, Nussler AK, Stadler J, Marzinzig E, Barthlen W, Nussler NC, Beger HG, Morris SM, Jr., Bruckner UB. 1997. Improved methods to measure end products of nitric oxide in biological fluids: Nitrite, nitrate, and *S*-nitrosothiols. Nitric Oxide 1:177–189.

Mirza UA, Chait BT, Lander HM. 1995. Monitoring reactions of nitric oxide with peptides and proteins by electrospray ionization-mass spectrometry. J Biol Chem 270:17185–17188.

Moncada S, Rees DD, Schulz R, Palmer RM. 1991. Development and mechanism of a specific supersensitivity to nitrovasodilators after inhibition of vascular nitric oxide synthesis in vivo. Proc Natl Acad Sci USA 88:2166–2170.

Murray CI, Uhrigshardt H, O'Meally RN, Cole RN, Van Eyk JE. 2012. Identification and quantification of *S*-nitrosylation by cysteine reactive tandem mass tag switch assay. Mol Cell Proteomics 11:M111.

Nakamura T, Lipton SA. 2011. Redox modulation by *S*-nitrosylation contributes to protein misfolding, mitochondrial dynamics, and neuronal synaptic damage in neurodegenerative diseases. Cell Death Differ 18:1478–1486.

Nakamura T, Lipton SA. 2013. Emerging role of protein–protein transnitrosylation in cell signaling pathways. Antioxid Redox Signal 18:239–249.

Numajiri N, Takasawa K, Nishiya T, Tanaka H, Ohno K, Hayakawa W, Asada M, Matsuda H, Azumi K, Kamata H, Nakamura T, Hara H, Minami M, Lipton SA, Uehara T. 2011. On-off system for PI3-kinase-Akt signaling through *S*-nitrosylation of phosphatase with sequence homology to tensin (PTEN). Proc Natl Acad Sci USA 108:10349–10354.

Pawloski JR, Hess DT, Stamler JS. 2001. Export by red blood cells of nitric oxide bioactivity. Nature 409:622–626.

Que LG, Liu L, Yan Y, Whitehead GS, Gavett SH, Schwartz DA, Stamler JS. 2005. Protection from experimental asthma by an endogenous bronchodilator. Science 308:1618–1621.

Rahman MA, Senga T, Ito S, Hyodo T, Hasegawa H, Hamaguchi M. 2010. *S*-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. J Biol Chem 285:3806–3814.

Rassaf T, Feelisch M, Kelm M. 2004. Circulating NO pool: Assessment of nitrite and nitroso species in blood and tissues. Free Radic Biol Med 36:413–422.

Rees DD, Palmer RM, Moncada S. 1989. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci USA 86:3375–3378.

Riederer IM, Schiffrin M, Kovari E, Bouras C, Riederer BM. 2009. Ubiquitination and cysteine nitrosylation during aging and Alzheimer's disease. Brain Res Bull 80:233–241.

Rosselli M, Keller PJ, Dubey RK. 1998. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 4:3–24.

Scheving R, Wittig I, Heide H, Albuquerque B, Steger M, Brandt U, Tegeder I. 2012. Protein *S*-nitrosylation and denitrosylation in the mouse spinal cord upon injury of the sciatic nerve. J Proteomics 75:3987–4004.

Sengupta R, Ryter SW, Zuckerbraun BS, Tzeng E, Billiar TR, Stoyanovsky DA. 2007. Thioredoxin catalyzes the denitrosation of low-molecular mass and protein *S*-nitrosothiols. Biochemistry 46:8472–8483.

Sengupta R, Billiar TR, Stoyanovsky DA. 2009. Studies toward the analysis of *S*-nitrosoproteins. Org Biomol Chem 7:232–234.

Seth D, Stamler JS. 2011. The SNO-proteome: Causation and classifications. Curr Opin Chem Biol 15:129–136.

Sinha V, Wijewickrama GT, Chandrasena RE, Xu H, Edirisinghe PD, Schiefer IT, Thatcher GR. 2010. Proteomic and mass spectroscopic quantitation of protein *S*-nitrosation differentiates NO-donors. ACS Chem Biol 5:667–680.

Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, Loscalzo J. 1992a. Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. Proc Natl Acad Sci USA 89:7674–7677.

Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J. 1992b. *S*-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci USA 89:444–448.

Stamler JS, Lamas S, Fang FC. 2001. Nitrosylation. The prototypic redox-based signaling mechanism. Cell 106:675–683.

Stuehr DJ, Gross SS, Sakuma I, Levi R, Nathan CF. 1989. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. J Exp Med 169:1011–1020.

Sun J, Murphy E. 2010. Protein *S*-nitrosylation and cardioprotection. Circ Res 106:285–296.

Sun J, Xin C, Eu JP, Stamler JS, Meissner G. 2001. Cysteine-3635 is responsible for skeletal muscle ryanodine receptor modulation by NO. Proc Natl Acad Sci USA 98:11158–11162.

Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. 2007. Preconditioning results in *S*-nitrosylation of proteins involved in regulation of

mitochondrial energetics and calcium transport. Circ Res 101:1155–1163.

Tello D, Tarin C, Ahicart P, Breton-Romero R, Lamas S, Martinez-Ruiz A. 2009. A "fluorescence switch" technique increases the sensitivity of proteomic detection and identification of *S*-nitrosylated proteins. Proteomics 9:5359–5370.

Torta F, Bachi A. 2012. Quantitative analysis of *S*-nitrosylated proteins. Methods Mol Biol 893:405–416.

Tsikas D, Sandmann J, Holzberg D, Pantazis P, Raida M, Frolich JC. 1999. Determination of *S*-nitrosoglutathione in human and rat plasma by high-performance liquid chromatography with fluorescence and ultraviolet absorbance detection after precolumn derivatization with *o*-phthalaldehyde. Anal Biochem 273:32–40.

Tsikas D, Dehnert S, Urban K, Surdacki A, Meyer HH. 2009. GC-MS analysis of *S*-nitrosothiols after conversion to *S*-nitroso-*N*-acetyl cysteine ethyl ester and in-injector nitrosation of ethyl acetate. J Chromatogr B Analyt Technol Biomed Life Sci 877:3442–3455.

Tyurin VA, Liu SX, Tyurina YY, Sussman NB, Hubel CA, Roberts JM, Taylor RN, Kagan VE. 2001. Elevated levels of *S*-nitrosoalbumin in preeclampsia plasma. Circ Res 88:1210–1215.

Wang Y, Liu T, Wu C, Li H. 2008. A strategy for direct identification of protein *S*-nitrosylation sites by quadrupole time-of-flight mass spectrometry. J Am Soc Mass Spectrom 19:1353–1360.

Wei W, Li B, Hanes MA, Kakar S, Chen X, Liu L. 2010. *S*-nitrosylation from GSNOR deficiency impairs DNA repair and promotes hepatocarcinogenesis. Sci Transl Med 2:19ra13.

Wiktorowicz JE, Stafford S, Rea H, Urvil P, Soman K, Kurosky A, Perez-Polo JR, Savidge TC. 2011. Quantification of cysteinyl *S*-nitrosylation by fluorescence in unbiased proteomic studies. Biochemistry 50:5601–5614.

Wu C, Liu T, Chen W, Oka S, Fu C, Jain MR, Parrott AM, Baykal AT, Sadoshima J, Li H. 2010. Redox regulatory mechanism of transnitrosylation by thioredoxin. Mol Cell Proteomics 9:2262–2275.

Wu C, Parrott AM, Fu C, Liu T, Marino SM, Gladyshev VN, Jain MR, Baykal AT, Li Q, Oka S, Sadoshima J, Beuve A, Simmons WJ, Li H. 2011a. Thioredoxin 1-mediated post-translational modifications: Reduction, transnitrosylation, denitrosylation, and related proteomics methodologies. Antioxid Redox Signal 15:2565–2604.

Wu C, Parrott AM, Liu T, Jain MR, Yang Y, Sadoshima J, Li H. 2011b. Distinction of thioredoxin transnitrosylation and denitrosylation target proteins by the ICAT quantitative approach. J Proteomics 74:2498–2509.

Xu L, Han C, Lim K, Wu T. 2008. Activation of cytosolic phospholipase A2alpha through nitric oxide-induced *S*-nitrosylation. Involvement of inducible nitric-oxide synthase and cyclooxygenase-2. J Biol Chem 283:3077–3087.

Xue Y, Liu Z, Gao X, Jin C, Wen L, Yao X, Ren J. 2010. GPS-SNO: Computational prediction of protein *S*-nitrosylation sites with a modified GPS algorithm. PLoS ONE 5:e11290.

Zhang Y, Keszler A, Broniowska KA, Hogg N. 2005. Characterization and application of the biotin-switch assay for the identification of *S*-nitrosated proteins. Free Radic Biol Med 38:874–881.

Zhang J, Li S, Zhang D, Wang H, Whorton AR, Xian M. 2010. Reductive ligation mediated one-step disulfide formation of *S*-nitrosothiols. Org Lett 12:4208–4211.

Zhang HH, Wang YP, Chen DB. 2011. Analysis of nitroso-proteomes in normotensive and severe preeclamptic human placentas. Biol Reprod 84:966–9975.

Zhang X, Huang B, Chen C. 2012. SNO spectral counting (SNOSC), a label-free proteomic method for quantification of changes in levels of protein *S*-nitrosation. Free Radic Res 46:1044–1050.

Zhang X, Huang B, Zhang L, Zhang Y, Zhao Y, Guo X, Qiao X, Chen C. 2012. SNObase, a database for *S*-nitrosation modification. Protein Cell. 3:929–933.