DE GRUYTER Biol. Chem. 2017; 398(12): 1267–1293

Review

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Regulation of protein function by *S*-nitrosation and *S*-glutathionylation: processes and targets in cardiovascular pathophysiology

https://doi.org/10.1515/hsz-2017-0150
Received April 26, 2017; accepted August 7, 2017; previously published online August 19, 2017

Abstract: Decades of chemical, biochemical and pathophysiological research have established the relevance of post-translational protein modifications induced by processes related to oxidative stress, with critical reflections on cellular signal transduction pathways. A great deal of the so-called 'redox regulation' of cell function is in fact mediated through reactions promoted by reactive oxygen and nitrogen species on more or less specific aminoacid residues in proteins, at various levels within the cell machinery. Modifications involving cysteine residues have received most attention, due to the critical roles they play in determining the structure/function correlates in proteins. The peculiar reactivity of these residues results in two major classes of modifications, with incorporation of NO moieties (*S*-nitrosation, leading to formation of protein *S*-nitrosothiols) or binding of low molecular weight thiols (*S*-thionylation, i.e. in particular *S*-glutathionylation, *S*-cysteinylglycinylation and *S*-cysteinylation). A wide array of proteins have been thus analyzed in detail as far as their susceptibility to either modification or both, and the resulting functional changes have been described in a number of experimental settings. The present review aims to provide an update of available knowledge in the field, with a special focus on the respective (sometimes competing and antagonistic) roles played by protein *S*-nitrosations and *S*-thionylations in biochemical and cellular processes specifically pertaining to pathogenesis of cardiovascular diseases.

Keywords: cardiovascular diseases; glutathione; mixed disulfides; nitric oxide; RNS; ROS; *S*-glutathionylation; *S*-nitrosation.

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Introduction

The role of nitric oxide (NO) in several signaling routes has been clearly established (Grisham et al., 1999; Pacher et al., 2007). NO, one of the most important substances produced by the endothelium, plays a key role in homeostasis maintenance and has gained recognition as a crucial modulator in vascular pathophysiology. NO has a number of intracellular effects, e.g. vasorelaxation, endothelial regeneration, inhibition of leukocyte chemotaxis, and platelet adhesion. The physiological chemistry of NO is complex, for it encompasses numerous potential reactions. Increasing attention has been paid to its ability to produce covalent post-translational protein modifications, and among these, the modification of cysteine residues has received more attention due to the functional relevance of many of them. The cysteine thiol may be modified by incorporation of a NO moiety (*S*-nitrosation) or of a glutathione moiety (*S*-glutathionylation). Both modifications may ensue from different reactions induced by nitric oxide-related species, especially *S*-nitrosoglutathione (GSNO). GSNO is one of the most important intracellular *S*-nitrosothiols (Martínez-Ruiz and Lamas, 2007), and as will be detailed below, a number of studies have investigated its potential use as a therapeutic agent in selected conditions, including cardiovascular dideases (Hornyák et al., 2011).

In the present review, the first part will focus on the role of NO as a signaling molecule, by analyzing the main post-translational modifications (PTMs) it can induce in cellular proteins. The second part will explore the similarities, differences and commonalities between the processes of protein *S*-nitrosation and *S*-glutathionylation. In fact, both modifications share many mechanistic and functional features, making them solid candidates as general mechanisms for intracellular signal transduction. Finally, the third section will focus on the identification of proteins individually modified by *S*-nitrosation and *S*-gluthathionylation. The most abundant protein thiol is the free cysteine of albumin, which makes of this abundant protein an inevitable target of both processes. *S*-nitrosylation of albumin has been recognized since the beginning as a mechanism involved in the bioactivity of endothelium-derived NO, at that time denoted as EDRF (Stamler et al., 1992; Keaney et al., 1993). Our review will be focused on the resulting effects of these PTMs on other protein targets critical in cardiovascular pathophysiology. The competition of the two processes on the same protein targets will also be discussed, taking into account the dual ability of GSNO to both *S*-nitrosate as well as *S*-glutathionylate proteins.

NO signaling in the cardiovascular system

NO is a gaseous radical with a short half-life, continuously synthesized from L-arginine by the nitric oxide synthase (NOS) (Palmer et al., 1988). There are three distinct isoforms of NOS that differ in structure and function (Stuehr, 1997). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and are referred as Ca^{2+}-dependent enzymes (Ayajiki et al., 1996) and generate small amounts of NO for signaling. The third type is the inducible isoform (iNOS), Ca^{2+}-independent and inducible by immunological stimuli (Schulz et al., 1992). This latter is activated in response to inflammation and generates high amounts of NO (Knowles and Moncada, 1994).

From a functional point of view, NO acts as a chemical messenger particularly in vascular and immune systems, where it participates in the regulation of a wide range of physiological processes. Its production within the cell is finely adjusted to ensure appropriate effects. Indeed, in physiological conditions, the low concentrations of NO (10 nM) produced by eNOS can act both as a vasodilator and an inhibitor of platelet aggregation. Through nitrosylation, NO released from the endothelium stimulates soluble guanylyl cyclase (sGC), producing increased concentrations of cyclic guanosine monophosphate (cGMP). Cyclic GMP interacts with three types of intracellular proteins: cGMP-dependent protein kinases (PKGs), cGMP-regulated ion channels, and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs). Thus, cGMP can alter cell function through mechanisms dependent or independent of protein phosphorylation. Depending on the sites of NO release and cGMP activation, different biological effects can be observed. In vascular smooth muscle cells (VSMCs), increased cyclic GMP concentrations will activate cGMP-dependent kinases capable of decreasing intracellular calcium, thus producing relaxation (Moncada et al., 1991), whereas increased cGMP in platelets will decrease platelet activation and adhesion to the surface of the endothelium (Radomski and Moncada, 1993).

Conversely, the activation of iNOS, induced during pathophysiological processes such as inflammation, produces much higher concentrations of NO (>1 μM). iNOS is expressed physiologically, but is induced by certain inflammatory cytokines (IL-1, INFγ, TNF-α), LPS and oxidizing agents. The effects of NO will depend on the site of its formation, concentrations achieved, as well as the type of targeted tissue.

Generally, modulation of protein function by NO can occur through three main reactions:

1. Nitrosylation, a reversible coordination of NO to transition metal ions in enzymes, such as ferrous (Fe^{2+}) heme prosthetic groups within the sGC enzyme, leading to enzyme activation and increased formation of cGMP.
2. Protein nitrosation, with formation of a covalent bond between NO and cysteine (*S*-nitrosation) or tryptophan (*N*-nitrosation) residues. Modifications of free cysteine residues present at active sites of effector proteins and peptides will change the activity or function of these proteins. This configures a mode of post-translational protein modification as important as phosphorylation (Lima, 2010; Heikal, 2011). A number of studies have focused on the mechanistic aspects of protein nitrosation (see e.g. Wolhuter and Eaton, 2017), however it has been shown that de-nitrosation reactions also play critical roles in the control of nitrosated proteins levels as well as of NO release. In the specific case of *S*-nitrosation, the formed *S*-nitrosothiols (RSNOs) can undergo spontaneous or assisted trans-nitrosations, resulting in the transfer of the NO moiety from high molecular weight (protein) thiols to low molecular ones, and inversely.
3. Nitration, i.e. the addition of a nitro group (NO_2) covalently bound to the aromatic ring of tyrosine

or tryptophan residues. This often results in a loss of protein function, due to the involvement of functionally important residues, as well as in proteolytic degradation of damaged proteins. Nitration mainly consists in the formation of nitrotyrosine, occurring either via peroxynitrite or via the one-electron oxidation of nitrite by peroxydases in the context of heavy tissue inflammation and oxidative stress (van der Vliet et al., 1997; Wu et al., 1999). Nitrotyrosine is identified as an indicator or marker of cell damage, of inflammation as well as of high NO production. Previous studies have demonstrated that nitrotyrosine is enriched in human atherosclerotic lesions and low-density lipoprotein (LDL) obtained from human atheromas (Shishehbor et al., 2003).

Decreased levels of endogenously produced NO have been linked to a number of cardiovascular diseases (CVD). Decreased NO bioavailability can have different origins, e.g. (i) the impairment of endothelial membrane receptors that mediate the release of NO in response to agonists or mechanical stimuli, (ii) decreased levels or impaired utilization of L-arginine substrate, or cofactors for NOS (such as BH_4), (iii) decreased concentration or activity of NOS, (iv) enhanced degradation of NO by oxygen free radicals, (v) impaired diffusion from endothelium to smooth muscle cells (SMCs), (vi) impaired interaction of NO with soluble guanylate cyclase and limitation of the increase in intracellular cGMP levels, (vii) generalized decrease in smooth muscle cell sensitivity to vasodilators (Briasoulis et al., 2012). Decreased NO bioactivity is a consequence of endothelial dysfunction, and may thus contribute to the development of atherosclerosis and its clinical expression in several CVD forms (Tousoulis et al., 2012).

Conversely, excess NO produced during inflammation and oxidative stress has also been repeatedly involved at the basis of cardiovascular pathologies. Such relationships can be summarized as follows: (i) NO is released during inflammation processes (mainly following the activation of inducible NO synthase), (ii) it induces positive or negative effects on vascular homeostasis, depending on its concentration and other concurrent factors, with deleterious impacts during oxidative stress and inflammation, and (iii) the released NO can itself modulate the production and action of inflammatory mediators. The reasons for such multiplicity of effects lie in concentrations, duration of exposure, and origin of increased NO: i.e. direct production from NOSs, as opposed to release of NO from the endogenous reservoir represented by *S*-nitrosothiols (RSNOs). The role of the latter as an important storage form of NO will be discussed below.

A summary of NO effects related to its concentration and cardiovascular conditions is reported in Figure 1.

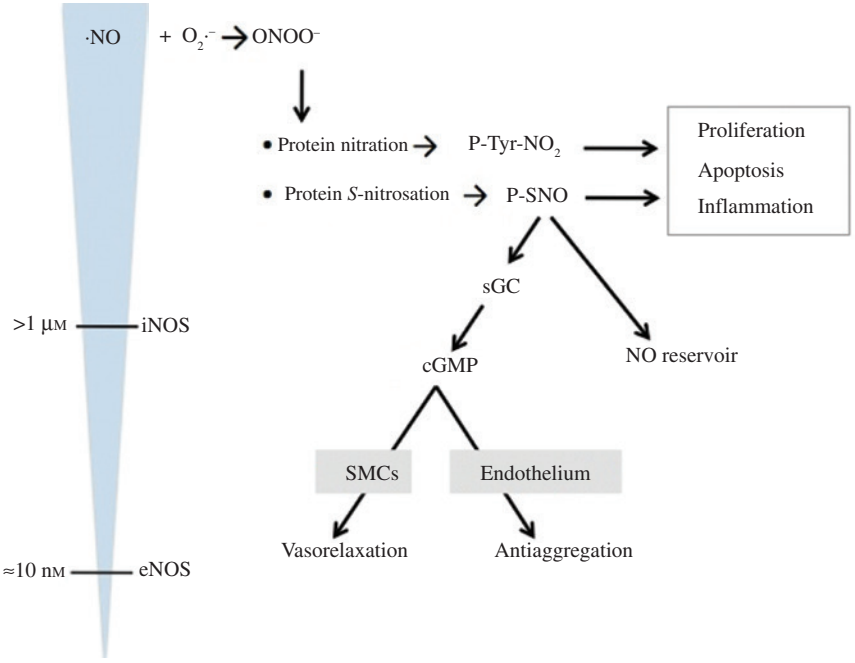


Figure 1: Pathophysiological effects of protein modifications induced by increasing concentrations of NO in the cardiovascular setting. sGC, soluble guanylyl cyclase; cGMP, cyclic guanosine monophosphate.

S-nitrosothiols: post-translational protein modifications and nitric oxide donors

The delivery of supplementary NO in the form of NO-donor drugs represents an attractive therapeutic option for the treatment of cardiovascular diseases as well as other pahologies. NO donors, i.e. agents capable to release NO, are often complex chemicals such as nitrosamines, organic nitrates and metal-NO complexes, but simpler molecules such as *S*-nitrosothiols have recently come center stage. Over time, several comprehensive reviews have appeared in the literature to which the reader is addressed for more details (see e.g. Feelisch and Stamler, 1996; Wang et al., 2002). Basically, three NO release mechanisms were proposed, depending on the chemical structure of the specific NO donor. The first type is spontaneous NO donation, which releases NO through thermal or photochemical self-decomposition (e.g. *S*-nitrosothiols, *N*-diazeniumdiolates, oximes). The second type is a chemically catalized NO release triggered by acid, alkali, metals and thiols. This is the case for organic nitrates, nitrites, sydnonimines and *S*-nitrosothiols. The third type is an enzymatically catalized NO donation depending on enzymatic oxidation or reduction: e.g. N-hydroxyguanidines, following the action of NOS's or oxidases, or *S*-nitrosothiols, following the action of redoxins (Perrin-Sarrado et al., 2016). Peptidases are also implicated in NO release from *S*-nitrosothiols, as shown by γ-glutamyltransferase (GGT) implication in NO release from GSNO, thus mediating its rapid vasorelaxant effects (Dahboul et al., 2012).

Some NO-donor drugs are already in widespread clinical use, in particular organic nitrates (e.g. nitroglycerin, isosorbide dinitrate or isosorbide-5-mononitrate), organic nitrites (e.g. amyl nitrite), the ferrous nitro-complexes (e.g. sodium nitroprusside) and the sydnonimines (e.g. molsidomine) (Tullett and Rees, 1999).

Conversely, Stamler and co-workers postulated the existence of an endogenous NO reserve in the plasma, where NO is in equilibrium with *S*-nitrosoproteins and/or *S*-nitrosopeptides characterized by a covalent *S*-NO bond and serving in NO transport, signal transduction pathways and regulation of gene expression (Stamler et al., 1992; Stamler, 1994). Indeed, part of the action of the endothelium-derived relaxing factor (EDRF) was suggested to be effected through a protein-SNO adduct with serum albumin (Keaney et al., 1993).

The formation of *S*-nitrosothiols may also play an important role in leukocyte adhesion to microvascular endothelium. *S*-nitrosothiols are in fact known to inhibit leukocyte adhesion to microvascular endothelial cells *in vivo*, presumably via the release of NO. Sulfhydryl groups are essential for normal leukocyte-endothelial cell adhesion (Grisham et al., 1998). *S*-nitrosation of these critical -SH groups on the surface of endothelial cells and/or neutrophils could therefore decrease adhesion, thereby limiting leukocyte infiltration. Furthermore, the formation of endogenous antiadhesive *S*-nitrosothiols may be inhibited by $O_2^{\bullet-}$ due to formation of peroxynitrite, suggesting that enhanced $O_2^{\bullet-}$ production during inflammation may promote the adhesion of neutrophils to endothelium (Wink et al., 1997). Indeed it is well established that exogenous NO donors are very effective at inhibiting the adhesion of neutrophils *in vivo* (Granger and Kubes, 1996; Grisham et al., 1998).

In contrast to classic NO donors used in pharmacotherapy, *S*-nitrosothiols could be promising substitutes as they do not induce the development of tolerance or cyanide poisoning (Al-Sa'doni and Ferro, 2000; Belcastro et al., 2017). *S*-nitrosothiols proved effective in a variety of cardiovascular disorders, including e.g. myocardial infarction (Lima et al., 2009). Alterations of NO homeostasis are implicated in atherosclerosis and the potential therapeutic benefit of NO donors (e.g. organic nitrates, nicorandil and sydnonimines) or drugs increasing the availability of endogenous NO (e.g. statins, angiotensin-converting enzyme inhibitors, L-arginine and tetrahydrobiopterin) has been proposed (Gori et al., 2001; Herman and Moncada, 2005; De Meyer et al., 2012). The rationale for this implication lies in the potential effects of NO on macrophages, SMCs and endothelium, the main cellular components of atherosclerotic plaque. However, it should also be considered that NO by itself (Chang et al., 1994; Wang et al., 1994) or in combination with superoxide anions (Darley-Usmar et al., 1992) can also stimulate the oxidation of LDL, which constitutes a critical triggering event in atherogenesis and makes the picture more complex.

Endothelium and SMCs are two critical components in the pathogenesis of atherosclerotic plaques, forming the basis for a number of cardiovascular diseases. The effects of *S*-nitrosothiols in normal endothelial cells and SMCs were first analyzed by comparing the effects on native LDL oxidation of *S*-nitroso-N-acetyl-penicillamine (SNAP), a RSNO, and two other known NO donors, SIN-1 and sodium nitroprusside (Jaworski et al., 2001). These authors demonstrated that sodium nitroprusside strongly oxidizes LDL in medium alone, as well as in endothelial or smooth muscle cell cultures, and it should therefore be used carefully in therapy for vascular conditions, especially in case of high concentrations and prolonged administration. SIN-1 also oxidized LDL in the absence of cells, and clearly enhanced the LDL oxidation in cultures

(Darley-Usmar et al., 1992; Mital et al., 1999). At variance, SNAP was not able to oxidize LDL either in acellular systems or in the presence of cells, showing that the amount of superoxide and other reactive oxygen species released by these cells did not suffice, contrary to those liberated by macrophages, to combine with NO and produce prooxidant effects. Conversely, it has been shown that other *S*-nitrosothiols such as GSNO could protect endothelial cells from the toxic effect of oxidized LDL (Struck et al., 1995). In addition, SNAP was reported to be useful in the treatment of heart failure, by reducing myocardial oxygen consumption (Mital et al., 1999), inducing vasodilation in rat femoral arteries (Megson et al., 1997) and inhibiting platelet activation and aggregation (Salas et al., 1994; Gordge et al., 1998). Taken together, these observations suggest that *S*-nitrosothiols could limit the progression of atherogenesis without increasing oxidative stress, and thus encourage the pharmacological application of these compounds.

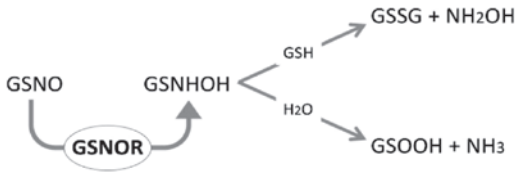
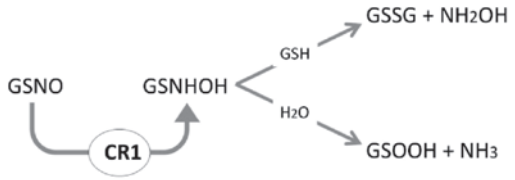
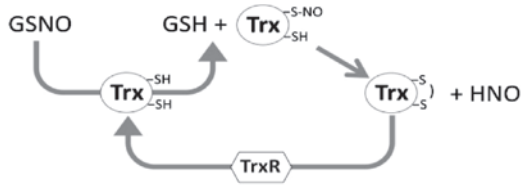
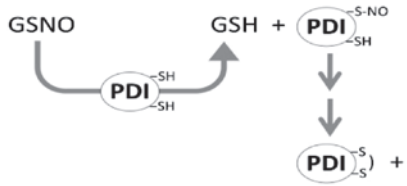
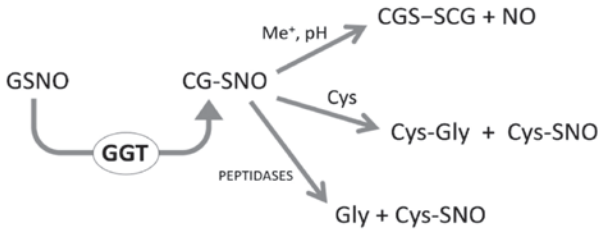
S-Nitrosoglutathione (GSNO): the main endogenous NO donor

Among endogenous RSNOs, the attention has been mainly focused on *S*-nitrosoglutathione (GSNO) and *S*-nitrosocysteine (Cys-NO), the most represented low-molecular weight (lmw) *S*-nitrosothiols *in vivo*, because of their potential pharmacological and therapeutic applications.

GSNO, formed by the *S*-nitrosation of reduced glutathione, is the main lmw form for storage and transport of NO. It exhibits higher stability than NO, mediates protein *S*-nitrosation processes and is thought to play an important role in vascular signaling and protection, especially in a context of inflammation (Khan et al., 2006). The biological activity of GSNO and particularly its vasorelaxant effect have been reported in *ex vivo* isolated vessel models (Sogo et al., 2000; Alencar et al., 2003; Dahboul et al., 2012; Perrin-Sarrado et al., 2016), and is directly linked to its decomposition resulting in the release of NO. Generally, RSNOs are quite stable *in vitro* at 37°C and pH 7.4 (McAninly et al., 1993) but are degraded *in vivo*, especially by enzymatic activities. Indeed, GSNO decomposition *in vitro* is promoted by factors such as pH, temperature and metal ions, while *in vivo* it is effected by enzyme activities such as GSNO reductase (GSNOR), carbonyl reductase 1 (CR1), protein dislfide isomerase (PDI), thioredoxin system (Trxs) and γ-glutamyltransferase (GGT). Cellular mechanisms of GSNO degradation are widely described in the literature (Broniowska et al., 2013; Corti et al., 2014), and are summarized in Table 1.

In view of its primary relevance as endogenous RSNO, as well as of the interest it has raised as potential therapeutic agent in cardiovascular diseases and other conditions, GSNO will represent the main focus of this review. Cellular responses to GSNO have in fact received great attention during the last few years (Broniowska et al., 2013). Indeed, GSNO does not induce any tolerance or oxidative stress (Al-Sa'doni and Ferro, 2000; Belcastro et al., 2017). To date, there have been nearly 20 clinical trials investigating the therapeutic efficacy of GSNO in multiple pathological contexts, and most of them have focused on its effects in cardiovascular diseases (Gaucher et al., 2013). The best-characterized effects of GSNO in humans are its direct and selective actions on platelets (Langford et al., 1994; Kaposzta et al., 2002). GSNO has been shown to decrease embolism from symptomatic carotid plaques and after carotid endarterectomy or angioplasty (Kaposzta et al., 2002) or vein graft (Salas et al., 1998) by limiting platelet activation. Its beneficial effects in the vasculature extend to cardiac left ventricular function and systemic vasodilation (Rassaf et al., 2002), and preeclampsia (Lees et al., 1996) (Table 1). Although with some discrepancies related to the analytical method used (Giustarini et al., 2006; Bramanti et al., 2009), GSNO was found at nano- to low micromolar concentrations in extracellular fluids and tissues (Gaston et al., 1993; Kluge et al., 1997; Giustarini et al., 2006), and due to its nature of endogenous compound and its important role in NO signaling and *S*-nitrosation, it has been considered an attractive candidate for therapeutic applications (Snyder et al., 2002; Corti et al., 2014). However, despite its powerful antiplatelet activity, vasodilator effects, antimicrobial and antithrombotic effects, this molecule is not yet present in any pharmaceutical composition for the treatment of vascular diseases. Considering that its stability is limited by enzymatic and non-enzymatic degradations, and therefore is too low to provide a long-lasting effect and to deliver appropriate NO concentrations to target tissues for clinical application, GSNO (like RSNOs in general) has to be protected. GSNO encapsulation is an interesting response to provide protection from degradation, but its hydrophilic nature and the instability of the *S*-NO bond during the formulation process raise difficulties for encapsulation. The direct encapsulation of GSNO within polymeric nanoparticles and nanocomposite particles has been recently described, with preservation of the activity of this fragile molecule after the formulation process (Wu et al., 2015a,b). As far as protection against degradation effected by γ-glutamyltransferase, useful hints could derive from previous studies on glutathione. In *in vitro* experiments, it was in fact shown that complexation of

Table 1: Cellular mechanism of GSNO degradation.

Enzymatic activities	Features	Reactions	References
GSNO reductase (GSNOR)	Located in the cytosol NADH-dependent enzyme	 In either case, nitric oxide is not liberated during GSNO catabolism and the nitroso moiety is reduced, and in this way removed from the NO pool	Jensen et al. (1998)
Carbonyl reductase 1 (CR1)	Located in the cytosol NADPH-dependent enzyme	 CR1 does not release NO in its catalytic action	Staab et al. (2008); Corti et al. (2014)
Thioredoxin system (Trxs)	Located in the cytosol Consists of Trx, NADPH and thioredoxin reductase (TrxR) Involved in denitrosation and transnitrosation reactions		Mitchell and Marletta (2005); Benhar et al. (2008); Sengupta and Holmgren (2012); Gaucher et al. (2013)
Protein disulfide isomerase (PDI)	Located in the cytosol and on cell membrane surface (transferred to the membrane during oxidative stress) Involved in thiol-disulfide-exchange and in denitrosation of RSNO (catalyzed by the same active-site thiols involved in the thiol-disulfide-exchange)		Sliskovic et al. (2005)
γ-Glutamyltransferase (GGT)	Located on cell membrane surface Involved in hydrolysis and trans-peptidation reactions GSNO is a specific substrate for GGT		Tate and Meister (1981); Hogg et al. (1997); Angeli et al. (2009)

GSH with α-cyclodextrin (α-CyD) could afford 50% protection against GGT at 2 h (Garcia-Fuentes et al., 2006).

These formulations for chronic oral treatments with GSNO showed a sustained *in vitro* release of GSNO (Wu et al., 2015b) and a storage of NO in the vascular wall 17 h after oral administration to wistar rats (Wu et al., 2016). Other formulations developed for subcutaneous long lasting delivery of GSNO also showed potential for stroke treatment (Parent et al., 2015). Even if the potential of GSNO for NO supplementation is limited by its poor stability and high hydrophilicity, causing NO release to not be sustained long enough for a chronic *in vivo* therapeutic effect, these studies however represent a starting point for the implementation of chronic oral treatments with GSNO, e.g. for the treatment of vascular diseases.

S-Nitrosation vs. *S*-glutathionylation: differences, similarities and commonalities

NO can affect cardiovascular physiology in different ways. In addition to the classic NO-mediated activation of the cGMP-dependent pathway, in recent years increasing attention has been paid to the ability of NO to produce covalent post-translational protein modifications, in conjunction with other reactive oxygen (ROS) and nitrogen species (RNS). Among these, the modification of cysteine residues has been shown to be of particular importance due to the functional relevance of many of them. In this second part we are focussing on the modification of the cysteine thiol by the incorporation of a NO moiety (*S*-nitrosation) or the formation of a mixed disulfide between a protein thiol and a lmw thiol (*S*-thionylation). Levels of glutathione (GSH) are remarkably high at intracellular level, and *S*-glutathionylation, the reversible addition of glutathione to thionylate anions of cysteines in target proteins, is the most abundant type of thionylation, and likely the most important (Chai et al., 1994a; Ravichandran et al., 1994; Schuppe-Koistinen et al., 1994).

Both *S*-nitrosation and *S*-thiolation can indeed result from the action of nitric oxide-related species. Conversely, as the intrinsic instability of the nitrosothiol bond facilitates its rapid reaction with other species to generate more stable disulfide bonds, it has been suggested that protein (mixed) disulfides formed in this way, via a nitrosothiol intermediate redox state, likely represent the end modifications actually mediating functional alterations in target proteins (Wolhuter and Eaton, 2017). There are emerging data suggesting that both PTMs play an important role in cardioprotection, for they not only lead to changes in protein structure and function but also protect these thiol(s) from further irreversible oxidative/nitrosative modification. A better understanding of these mechanisms and their roles is needed, in view of new therapeutic opportunities and targets for interventions in cardiovascular diseases.

The perturbation of thiol-disulfide homeostasis is a critical process in many diseases. The reduced/oxidized glutathione (GSH/GSSG) redox couple is of great importance in maintaining cellular redox status, including modulation of protein thiols. The estimated *in vivo* redox potential for the GSH/GSSG couple ranges from –260 mV to –150 mV depending on the conditions (Jones, 2002). Thus, changes in the GSH/GSSG ratio are fundamental in the fine tuning of signal transduction related, for example, to cell cycle regulation (Schafer and Buettner, 2001). Under oxidative stress, the concentration of GSH decreases leading to irreversible cell degeneration and death. In fact, shifting the GSH/GSSG redox toward the oxidizing state activates several signaling pathways including protein kinase B, calcineurin, NF-κB, c-Jun N-terminal kinase, apoptosis signal-regulated kinase 1, and mitogen-activated protein kinase, thereby reducing cell proliferation and increasing apoptosis (Sen, 2000). In recent years, additional roles of GSH related to signal transduction have emerged.

The most common covalent protein PTMs of cysteine residues are those involving NO, i.e. *S*-nitrosation, or a thiol, by incorporation for example of a glutathione moiety, *S*-glutathionylation (Figure 2).

S-Nitrosation is a redox-dependent, thiol-based, reversible post-translational protein modification (Gow et al., 2002; Gaston et al., 2006; Paige et al., 2008; Foster et al., 2009) and it is analogous to phosphorylation, *S*-glutathionylation, palmitoylation, acetylation and other physiological modifications of proteins. When compared to other signaling mechanisms such as phosphorylation, it presents unique features, e.g. the fact that *S*-nitrosothiols can be also formed and degraded depending on spontaneous chemical reactions without the aid of enzymatic catalysis. Thus, some concepts like specificity are based on different biochemical principles, and hence this may represent a new paradigm in signal transduction (Martínez-Ruiz and Lamas, 2004).

Conversely, *S*-glutathionylation provides protection of protein cysteines from irreversible oxidation and serves to transduce a redox signal by changing structure/function of the target protein (Ghezzi, 2005, 2013). This process

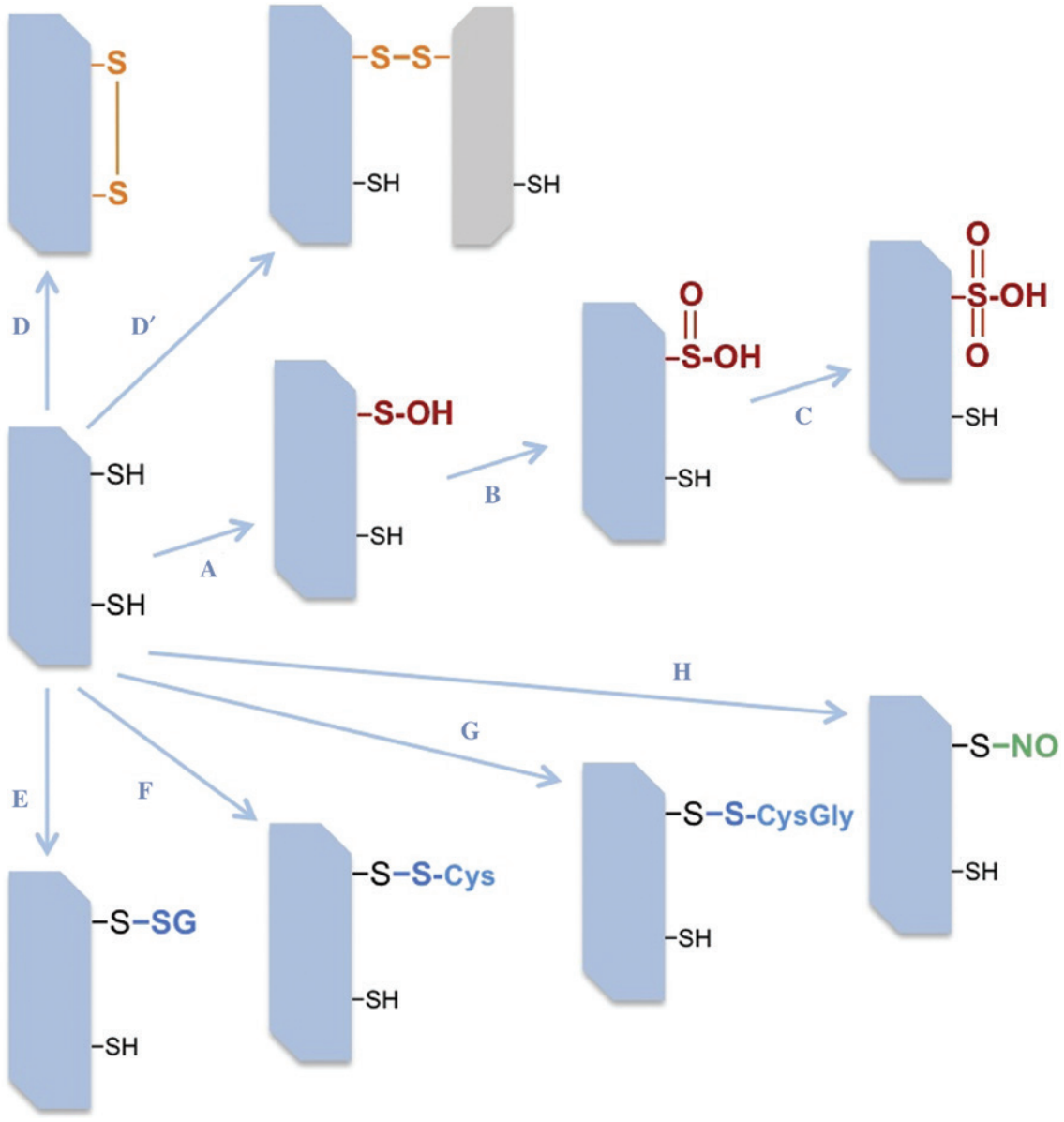


Figure 2: Chemical pathways leading to the formation of protein *S*-nitrosation and *S*-glutathionylation.
The thiol group in side chain of a cysteine residue is susceptible to several oxidative modifications. Its oxidation can result in the formation of a cysteinyl radical (P–S, not shown) or a sulfenic (PSOH) (A), sulfinic (PSO_2H) (B) or sulfonic (PSO_3H) acid (C). The formation of PSOH can be reversed, for example by GSH, while the two other species are usually irreversible. Alternatively, oxidation can result in the formation of disulfide bridges (D, D′). Disulfides can form between two adjacent proteins (intermolecular cystine, or interprotein disulfide) (D) or between two adjacent sulphydryl groups within the same protein (intramolecular cystine, or intraprotein disulfide) (D′), causing changes in protein aggregation and conformation. Reaction between protein -SH grous and lmw. thiols such as GSH and free cysteine can yield protein-glutathione (E), protein-cysteine (F) or protein-cysteinylglycine mixed disulfides (G), respectively (i.e. *S*-glutathionylated, *S*-cysteinylated or *S*-cysteinylglycinylated proteins). Protein thiols found in consensus motifs wherein the cysteine is adjacent to basic and acid residues or aromatic residues can serve as sites of *S*-nitrosation, which can be mediated by NO, *S*-nitrosothiols (i.e. GSNO) or several higher N-oxides (H), or catalyzed by transition metals.

is observed both under physiological redox signaling or oxidative stress. Protein *S*-glutathionylation involves the reaction of protein cysteine residue, or an oxidized derivative such as *S*-nitrosyl (*S*-NO), sulfenic acid (*S*-OH), thiyl radical ($S^{\bullet}$), with GSH. The reversal of *S*-glutathionylation (i.e. de-glutathionylation) is catalyzed by glutaredoxin (Grx) at the expense of GSH as a cosubstrate (Kalinina et al., 2014).

The PTMs described above can alter protein function, by regulating the activities of several redox-sensitive

proteins. This has been discussed for both sets of modifications (Nathan, 2003), although each modification has special and unique features. In this regard, *S*-nitrosation shows some parallels with sulfenic acid formation, as it is easily reversible (Poole et al., 2004). Sulfinic and sulfonic acid formation are more stable modifications, which have been considered as irreversible in cell systems until the description of reversion enzymatic mechanisms for sulfinic acid (Biteau et al., 2003; Woo et al., 2003). Disulfide bond formation is a well-known redox modification in proteins, largely dependent not only on the redox environment but also on protein structure. However, *S*-glutathionylation can be considered as the most important thionylation, as it has been also shown to be the most abundant (Chai et al., 1994a; Schuppe-Koistinen et al., 1994).

Numerous reports have shown the importance of oxidative cysteine modification in proteins in modulating cardiovascular function, and both protein *S*-nitrosation and *S*-glutathionylation are emerging as a critical signaling mechanism in cardiovascular diseases. Both PTMs in fact appear to regulate several physiological processes involved in cardiovascular homeostasis, including myocyte contraction, oxidative phosphorylation, protein synthesis, vasodilation, glycolytic metabolism and response to insulin. Thus, perturbations in protein nitrosation/glutathionylation status may contribute to the etiology of many cardiovascular diseases, such as myocardial infarction, cardiac hypertrophy and atherosclerosis.

There are several chemical mechanisms that can lead to both *S*-nitrosation and *S*-glutathionylation in protein-cysteine thiols. First, it is necessary to assume that the formation of both modifications does not occur directly by addition of the species, nitric oxide (·NO) or glutathione (GSH), to the cysteine thiol (P–SH), but a redox reaction is needed by losing one electron per sulfur atom in the case of nitrosation, and two electrons in the case of *S*-glutathionylation.

A direct reaction is only possible if a thiyl radical is formed on the cysteine thiol (P–S$^{\cdot}$), that would easily react with nitric oxide (a) or with glutathione thiyl radical (b):

$$P-S^{\cdot} + {\cdot}NO \rightarrow P-S-NO \quad (a)$$

$$P-S^{\cdot} + GS^{\cdot} \rightarrow P-S-SG \quad (b)$$

Moreover, oxidized species are the most important candidates for modifying a reduced thiol, and N_2O_3, the higher oxidation state among nitrogen oxides (c), is considered the *S*-nitrosating species par excellence (Wink et al., 1993; Hogg, 2002). Oxidized glutathione (GSSG) would be the equivalent for glutathione (d):

$$N_2O_3 + RSH \rightarrow RSNO + H^+ + NO_2^- \quad (c)$$

$$P-SH + GS-SG \rightarrow P-S-SG + GSH \quad (d)$$

Another mechanism, sharing some common features, is the transfer of the modification between two distinct thiols. In the case of *S*-nitrosation, this is termed transnitrosation (e), a fast reaction likely to occur quite easily in cells (Rossi et al., 1997; Liu et al., 1998). The disulfide exchange between glutathione and another thiol (including *S*–glutathionylation by GSSG) could be called 'transglutathionylation' (f), and is a slow, thermodynamically unfavored reaction basing on typical redox potentials for cysteine residues (Gilbert, 1995):

$$R-SH + R'-S-NO \rightarrow R-S-NO + R'-SH \quad (e)$$

$$R-SH + R'-S-SG \rightarrow R-S-SG + R'-SH \quad (f)$$

Transnitrosations are likely to occur promptly, but can be easily reverted by other thiols, a circumstance that may explain in part the high lability of protein *S*-nitrosation. In contrast, *S*-glutathionylation by GSSG is less likely if not assisted by enzymes, such as those described below, but may become more stable in the presence of other thiols, e.g. glutathione itself.

An alternative mechanism for protein mixed disulfide formation derives from the intriguing observation that this modification may occur in intact cells in various experimental models of ROS generation, even without any detectable changes in the GSH/GSSG ratio (Chai et al., 1994a,b). This has been explained by direct oxidation of protein cysteines by ROS generating a reactive protein thiol intermediate such as a thiyl radical or sulfenic acid (g), which further reacts with GSH to form the mixed disulfide (h) (Poole et al., 2004):

$$P-SH + H_2O_2 \rightarrow P-SOH + H_2O \quad (g)$$

$$P-SOH + GSH \rightarrow P-S-SG + H_2O \quad (h)$$

In support of this hypothesis, it was demonstrated that the active-site Cy*S*-215 of protein tyrosine phosphatase (PTP-1B) can be oxidized by superoxide and H_2O_2 to a short-lived sulfenate (Denu and Tanner, 1998; Lee et al., 1998), which is rapidly converted into the relatively more stable mixed disulfide by GSH (Barrett et al., 1999a,b), preventing it from being overoxidized. These findings are important for interpretation of previous work (reviewed in Claiborne et al., 1999) proposing that protein sulfenates may represent a stable and functionally relevant oxidized

form of a protein thiol. However, although there is evidence that some protein sulfenates may be stable oxidation end products when they are stabilized and protected against reaction with other thiols by the protein structure, the possibility should be considered that sulfenates can only survive in unnatural oxidizing conditions *in vitro*, i.e. in experiments carried out in the absence of GSH (Claiborne et al., 1993).

Finally, an emerging concept suggests that *S*-nitrosation could be considered as an intermediate step to *S*-glutathionylation (Wolhuter and Eaton, 2017). Indeed, the *S*-nitrosothiol itself represents another activated form of the protein cysteine thiol, and can react with GSH leading to *S*-glutathionylation (i):

$$PS-N=O+GSH \rightarrow PS-SG+HNO \quad \text{(i)}$$

which is chemically equivalent to the *S*-glutathionylation produced by GSNO (j):

$$PSH+GSNO \rightarrow PS-SG+HNO \quad \text{(j)}$$

Individual analysis of each single mechanism is very difficult, especially if we consider the fast transnitrosation reaction between the two types of nitrosothiols (k):

$$PSNO+GSH \leftrightarrows PSH+GSNO \quad \text{(k)}$$

Thus, the role of *S*-nitrosothiols as primers for *S*-glutathionylation should be considered as a whole, with special emphasis given to GSNO as a reagent that can induce not only *S*-nitrosation but also *S*-glutathionylation in proteins.

Although *S*-nitrosation and *S*-glutathionylation in cells can be explained by mere chemical reactions, some enzymatic systems have been described as capable of accelerating some of the steps involved. Regarding *S*-nitrosation, and apart from the fact that certain protein targets are specifically assisted in their modification (Hess et al., 2005), the main enzymatic mechanism described so far is the one assisted by GSNOR activity. The latter is otherwise known as the GSH-dependent formaldehyde dehydrogenase activity (Jensen et al., 1998) and would presumably have a function in controlling the levels of intracellular GSNO (and, indirectly, of protein *S*-nitrosothiols as well) as it promotes the formation of GSNHOH, a non-NO donating molecule.

Although GSNOR is the most ubiquitous and well-studied enzyme in GSNO metabolism, other enzymes are also involved, such as plasma membrane γ-glutamyltransferase (GGT). GGT is able to hydrolize GSNO to the less-stable dipeptide *S*-nitroso-cysteinylglycine (CGSNO), which rapidly releases NO in presence of trace metal ions (Hogg et al., 1997; Angeli et al., 2009). Although GGT activity resides on the outer aspect of cell membrane, it can be envisaged that, by cleaving extracellular GSNO, GGT may facilitate SNO uptake and *S*-nitrosation of intracellular proteins (Hogg et al., 1997; Zhang and Hogg, 2005). In this regard for instance, it was demonstrated that GSNO-induced Akt kinase activity and the subsequent downstream HIF-1α stabilization are blocked by acivicin, a GGT inhibitor (Carver et al., 2007). Many studies have provided evidence in favor of a crucial role of plasma membrane-bound GGT enzymatic activity in the modulation of the redox status of cellular protein thiols, with special reference to proteins of the cell surface. Dominici et al. found that GGT activity is associated with the transient loss of free protein thiols on the surface of U937 lymphoma cells (Dominici et al., 1999), suggesting that this occurs through GGT-dependent *S*-glutathionylation. The mechanism by which GGT induces protein thionylation may involve H_2O_2 and reactive thiol metabolites (thiyl radicals), both generated as by-products of GGT mediated GSH metabolism. It was in fact shown that reactive cystinyl-glycine, produced during GGT-dependent GSH metabolism, can bind protein thiols thus producing a protein *S*-cysteinyl-glycinylation (Corti et al., 2005). In this way the redox status of protein thiols can be modulated, with potential reflections on cell proliferation as well as on the resistance against drugs such as chemoterapeutics (Corti et al., 2010). Interestingly, GGT expression levels vary considerably among cells types (Hanigan and Frierson, 1996; Pompella et al., 2006) and may increase upon cell activation (Khalaf and Hayhoe, 1987; Corti et al., 2010).

Other important enzymes involved in this context and their corresponding mechanisms of action are described in Table 2.

As far as disulfide exchange with *S*-thionylation, this is a slow chemical reaction that is accelerated in the intracellular environment by several enzyme activities helping to maintain the reduced state of intracellular thiols by transferring the oxidizing equivalents to an acceptor cosubstrate. Trx, Grx and PDI have been involved in catalyzing the breaking of protein-GSH mixed disulfides (Shelton et al., 2005; Biswas et al., 2006). More recently, sulfiredoxin, the enzyme that reverts protein sulfinic acid, has been shown to potentially play the same role (Findlay et al., 2006). In addition, glutathione *S*-transferase P has also been involved in *S*-glutathionylation (Tew, 2007), and a role has been recently described for glutathione transferase omega-1 (GSTO1-1) (Menon and Board, 2013).

Another important factor affecting *S*-nitrosation and *S*-glutathionylation is subcellular compartmentation. This is specially important due to submolecular and hence multimolecular specificites, derived from diffusibility and structural features of individual compounds (Nathan, 2003). Indeed, the probability of the reaction between

Table 2: *S*-nitrosated and/or *S*-glutathionylated protein targets, as assessed after treatment of cells or purified proteins with nitric oxide, GSNO or other RNS.

Protein	Stimulus	*S*-Nitrosation	*S*-Glutathionylation	References
Hb	GSNO, diamide, superoxide, GSSG	+	+	Klatt et al. (2000)
Creatine kinase	GSNO, diamide, GSSG	+	+	Klatt et al. (2000); Giustarini et al. (2005)
Alcohol dehydrogenase	GSNO, diamide	+	+	Klatt et al. (2000); Giustarini et al. (2005)
Ryanodine receptor	GSNO	+	+	Aracena et al. (2003); Sun et al. (2006b)
Papain	GSNO	+	+	Giustarini et al. (2005)
GAPDH	GSNO, diamide, superoxide, GSSG	+	+	Klatt et al. (2000); Giustarini et al. (2005); Mohr et al. (1999); Tao and English (2004)
Actin	GSNO	+	+	Dalle-Donne et al. (2003)
c-Jun	GSNO, NO/GSH, GSSG	+	+	Klatt et al. (1999, 2000)
Caspase-3	GSNO, SNAP + GSH	+	+	Klatt et al. (2000); Mitchell and Marletta (2005); Zech et al. (1999)
H-Ras	GSNO, ONOO-	+	+	Ji et al. (1999)
BSA	Decomposed GSNO (diamide)	+	+	Tao and English (2004); Klatt et al. (2000)
Myosin	GSNO	+	+	Buttkus (1971); Pastore and Piemonte (2013)
p50	GSNO, GSSG		+	Klatt et al. (2000)
Cathepsin K	GSNO		+	Percival et al. (1999)
Aldose reductase	GSNO, GSSG		+	Chandra et al. (1997); Srivastava et al. (2003)
Glycerol phosphate dehydrogenase	GSNO, diamide		+	Klatt et al. (2000)
Glutaredoxin	GSNO, diamide		+	Klatt et al. (2000)
Thioredoxin	GSNO, diamide		+	Klatt et al. (2000)
Carbonic anhydrase	GSNO, diamide		+	Klatt et al. (2000); Ji et al. (1999)
Glycogen phosphorylase b	GSNO, diamide		+	Klatt et al. (2000); Ji et al. (1999)
Cu, Zn-SOD	GSNO, diamide		+	Klatt et al. (2000); Tao and English (2004)
Ca^{2+}-ATPase (SERCA)	$ONOO^-$		+	Viner et al. (1999)
Glutathione reductase	GSNO	+		Becker et al. (1995)
Mitochondrial complex I/IV	GSNO	+		Burwell et al. (2006); Zhang and Hogg (2005)
Citocrome c oxidase	GSNO	+		Reynaert et al. (2004)
NF-KB	GSNO	+		Reynaert et al. (2004)
Trx	GSNO	+		Haendeler et al. (2002)
HIF-1α	GSNO	+		Marshall et al. (2004); Reynaert et al. (2004)
Actin, Aortic smooth muscle	GSNO	+		Belcastro et al. (2017)
Myosin light chain RCL-A	GSNO	+		Belcastro et al. (2017)
Transgelin	GSNO	+		Belcastro et al. (2017)
Transgelin-2	GSNO	+		Belcastro et al. (2017)
Tubulin β-2 chain	GSNO	+		Belcastro et al. (2017)
Filamin-A	GSNO	+		Belcastro et al. (2017)
Pdz and Lim domain protein 5	GSNO	+		Belcastro et al. (2017)

certain reactive species and certain proteins will be higher if they have the same subcellular localization. In the case of *S*-nitrosation, this has been studied mainly in terms of the NOS enzymes, as they are the initial source for NO. Undoubtedly, the most important mode of regulation of eNOS in endothelial cells is related to its acylation and interaction with a significant number of proteins, determining its topological fate. Acylation and other PTMs of eNOS – such as phosphorylation and *S*-nitrosation – can in fact dynamically regulate eNOS activity by altering its subcellular localization. Iwakiri et al. (2006) showed that *S*-nitrosation is preferentially taking place at cell sites where transfected eNOS is located, and can alter Golgi trafficking function by altering *S*-nitrosation sensitive proteins.

Conversely, *S*-glutathionylation is related to a different subcellular localization/distribution of GSH. Early experiments detected an important presence of glutathione in the nucleus (Hjelle et al., 1994; Voehringer et al., 1998) while a more recent study has highlighted the presence of GSH in perinuclear sites including mitochondria, with an irregular distribution in nuclear, perinuclear and discrete membrane localizations (Söderdahl et al., 2003).

Other factors are involved in modulation of S-nitrosation and S-glutathionylation. The half-life of Cys-NO *in vitro* was shown to be shorter than that of GSNO across the entire pH range tested (Kuo et al., 2003). The stability of GSNO was higher in basic conditions, while mildly acidic pH can favor *S*-nitrosation of target proteins *in vitro* (Hornyák et al., 2012). *S*-Nitrosation of cysteamine by peroxxynitrite was shown to be maximal at strongly acidic pH (Mbiya, 2013). The acid dissociation constant (p*K*a) of protein cysteines involved is critical in facilitation of *S*-glutathionylation. In carbonic anhydrase, distinct Cys residues were shown to possess higher or lower pKa values, and corresponding *S*-glutathionylation rates, depending on their position with respect to specific adjacent aminoacid residues in the polypeptide chain (Kim and Levine, 2005).

S-Nitrosation vs. *S*-glutathionylation in the cardiovascular system: a matter of competition?

When ROS/RNS levels are modified (increased), changes are observed in protein activity, stability, conformation and/or ability to interact with other molecules, resulting in modulation of cellular function. A significant number of clinical disorders or pathophysiological entities have been in fact related to redox PTMs, and often the same protein targets may be affected by either *S*-nitrosation or *S*-glutahionylation, depending on several factors. In the heart, redox modified proteins include proteins involved in calcium handling and contractile function, e.g. calmodulin kinase II, calcium channels, ryanodine receptor, sarco/endoplasmatic reticulum calcium ATPase (SERCA) and phospholamban (Burgoyne et al., 2012; Steinberg, 2013; Tullio et al., 2013), as well as proteins involved in various signaling pathways and/or transcriptional activities.

The last section of this review is focused on the identification of proteins individually modified by both *S*-nitrosation and *S*-glutathionylation, and on their status in cardiovascular disesases. The competition between these two PTMs on the same protein targets will be also discussed, keeping in mind the dual ability of GSNO to promote both types of modification.

Protein *S*-nitrosation

S-Nitrosation of proteins plays important roles both in normal physiology and in a broad spectrum of human diseases. As regards the regulation of vascular tone, the balance between angiotensin II (Ang-II) and NO appears crucial in the cardiovascular and renal systems, where homeostasis is lost when the effects of Ang-II predominate over those of NO (Schulman et al., 2006). Pathophysiology often correlates with hypo- or hyper-*S*-nitrosation of specific protein targets, rather than with a loss of or enhanced NOS activity (Foster et al., 2009). Dysregulated *S*-nitrosations are usually the result of a modification of NO availability, in dependence of the specific context in which it is released, e.g. in concomitance with reactive oxygen, H_2S and/or other reactive species (Cortese-Krott et al., 2017). In turn, NO availability results not only from alterations of the expression, compartmentation and/or activity of NOSs, but also reflects the contribution of denitrosylases, including GSNO-metabolizing enzymes such as GSNOR, producing the non-bioactive molecule GSNHOH, or GGT, producing instead reactive *S*-nitroso-cysteinylglycine, CGNO (Dahboul et al., 2012). The irreversible oxidation of thiols can prevent physiologic modifications by *S*-nitrosation or *S*-glutathionylation and thereby interfere with normal physiologic signaling (Adachi et al., 2004a). However, it has been suggested that nitrosation can also protect cells from oxidative stress (Sun et al., 2006a). Protein *S*-nitrosation not only leads to changes in protein structure and function, but may also prevent those thiols from further irreversible oxidative/nitrosative modification. The direct antioxidant properties of NO itself are well

established (Wink et al., 2001). It is not surprising thus that the loss or inhibition of NOS can sometimes enhance oxidative stress.

High spatial and temporal specificities have been shown to affect protein *S*-nitrosation and its redox reversibility. A determinant governing its specificity is in fact provided by the colocalization of NO sources and target proteins, and is based at least in part on specific protein–protein interactions with NO synthases. In addition, *S*-nitrosation dependent signaling is also subject to temporal constraints, which depend on the timing of formation of NO and other nitrosylating equivalents. Many Cys-containing proteins in signal transduction cascades are potential targets, as they can undergo a range of RO*S*- or RN*S*-dependent oxidative and nitrosative modifications. Several and diverse cellular responses have been described. *S*-nitrosation can have antioxidant effects by inhibiting NADPH oxidase activity (Selemidis et al., 2007), or by promoting the ROS scavenging activity of thioredoxin-1 via *S*-nitrosation on Cys-69 residue (Haendeler et al., 2002; Liu et al., 2004a,b). Indeed, endothelial cells subjected to physiological shear stress produce NO (Gaucher et al., 2007; Gaucher-Di Stasio et al., 2009) and increase protein *S*-nitrosation independently of cGMP-dependent signaling (Hoffmann et al., 2003; Huang et al., 2009). In contrast, after TNF-α or mild oxidized LDL treatment endothelial cells present with decreased *S*-nitrosation (Hoffmann et al., 2001). Early studies demonstrated that the activity of AP-1 [an important transcription factor regulating gene expression in response to a variety of stimuli and involved in several cellular processes including differentiation, proliferation, and apoptosis (Ameyar et al., 2003)] was altered by *S*-nitrosation (Marshall et al., 2000) or oxidation of Cys residues (Xanthoudakis et al., 1992). Indeed, transcriptional activators NF-κB, AP-1 and p53 all contain reactive thiols in their DNA binding regions, whose modification, e.g. by *S*-nitrosation, can alter their binding to DNA. For example in neuronal development, brain-derived neurotrophic factor can activate nNOS, with subsequent *S*-nitrosation of histone deacetylase 2 (HDAC2, on cysteines 262 and 274) causing its dissociation from chromatin (Nott et al., 2008). This increases histone acetylation, permitting transcription of target genes required for regulation of dendritic growth (Nott et al., 2008). Additionally, *S*-nitrosation and denitrosation of critical proteins appear to affect cell cycle regulation and are involved in the mitotic process (Marozkina and Gaston, 2012; Rychter et al., 2016).

A number of protein interactions at nuclear level are indeed modulated by *S*-nitrosation. For example, the hypoxia-inducible factor 1-α (HIf1-α) is stabilized by physiological levels of *S*-nitrosothiols following *S*-nitrosation of von Hippel Lindau protein (VHL, C162), preventing its degradation (Pawloski et al., 2001). Then HIf1-α can interact with HIF1-β to bind to hypoxia-responsive elements in gene promoter regions, resulting in transcription of genes such as vascular endothelial growth factor (VEGF). HIF1-α can itself undergo both *S*-nitrosation (Li et al., 2007) and *S*-glutathionylation (Watanabe et al., 2016), both resulting in its direct stabilization.

S-nitrosation also affects NF-κB activity, as it can involve both p50 (C 62) and p65 (C 38) subunits, as well as IκB kinase, IKK (Marshall et al., 2004; Reynaert et al., 2004). The net effect of these reactions is generally an increased cytosolic NF-κB-IκB interaction, preventing nuclear translocation and binding of NF-κB to inflammatory gene promoters, with overall anti-inflammatory action. As with other cellular effects, however, the understanding of the role of *S*-nitrosation signaling in regulation of gene expression is just beginning.

Altogether, modulation of *S*-nitrosation can offer, in principle, novel therapeutic opportunities in a wide range of pathologies, firstly in cardiovascular diseases associated with oxidative stress. As mentioned above, in a therapeutic perspective *S*-nitrosothiols (GSNO in the first place) may represent promising NO-donor molecules, compared to other NO-related therapeutics limited by tolerance phenomena and oxidant stress (Al-Sa'doni and Ferro, 2000; Hornyák et al., 2011).

Protein *S*-glutathionylation

Protein *S*-glutathionylation is emerging as a critical signaling mechanism in cardiovascular diseases, as it regulates several relevant physiological processes such as oxidative phosphorylation, protein synthesis, myocyte contraction, vasodilation, glycolytic metabolism, and response to insulin (Mieyal et al., 2008; Pimentel et al., 2012). Thus, perturbations in protein *S*-glutathionylation status may contribute to the etiology of cardiovascular diseases such as atherosclerosis, cardiac hypertrophy and myocardial infarction. Protein *S*-glutathionylation is increased following ischemia-reperfusion (IR), with the majority of the *S*-glutathionylation events occurring early in the reperfusion period (Eaton et al., 2002). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was identified as a cardiac protein *S*-glutathionylated during IR with loss of enzyme function, suggesting that GAPDH *S*-glutathionylation is likely inhibitory *in vivo*. The effects of this inhibition may be multifold: (i) a block of

glycolysis, otherwise characteristic of ischemic injury, (ii) an interference with its nuclear translocation, resulting in increased apoptosis, or (iii) a homeostatic answer to the excess of oxidants generated at reperfusion. Of note, GAPDH activity is restored at the end of reperfusion, thus suggesting that *S*-glutathionylation may constitute a temporary protection of catalytic cysteines from irreversible oxidation. Additional studies highlighted the *S*-glutathionylation of actin in a rat model of *in vivo* IR (Chen and Ogut, 2006). Studies on isolated G-actin indicated that its *S*-glutathionylation can delay its rate of polymerization and decrease the cooperativity of its binding to tropomyosin, suggesting that the phenomenon may contribute to the decline in cardiac contractility observed during ischemia (Wang et al., 2001a,b).

Other targets of *S*-glutathionylation are the mitochondrial complexes I and II. The latter seems to be deglutathionylated during IR (Chen et al., 2007), indicating that a single oxidative stimulus can affect *S*-glutathionylation in different directions. The potential critical role of Grx in myocardial infarction was highlighted through experiments in a mouse model of embryonic Grx1 knock-out, suggesting a cardioprotective role for Grx isoforms (Malik et al., 2008). *S*-Glutathionylation of the mitochondrial complex I, with associated increased superoxide production, would be expected to increase cytochrome *c* release and caspase activation, contributing to infarct size and cardiac dysfunction (Malik et al., 2008).

The precise role of *S*-glutathionylation in the development and progression of atherosclerosis is unknown. However, conditions existing within atherosclerotic plaques (i.e. hypoxia, oxidative stress, oxLDL accumulation and inflammation) have been shown to promote *S*-glutathionylation in other contexts (Eaton et al., 2002; Wang et al., 2006), and Grx has been reported to associate with areas of oxidative stress within the vasculature (Okuda et al., 2001). Protein *S*-glutathionylation increases in human macrophages exposed to oxLDL, a major component of atherosclerotic plaques (Wang et al., 2006) and, together with GSH depletion, increased *S*-glutathionylated proteins seem to be implicated in oxLDL-induced macrophage death *in vitro* (Kher and Marsh, 2004). The role of specific *S*-glutathionylated proteins in macrophage cell death is not yet determined, nor is it known whether global protein *S*-glutathionylation increases in other cells types exposed to oxLDL. Patients with atherosclerosis obliterans (ASO) exhibit increased *S*-glutathionylation of serum proteins, and a positive correlation between disease progression and the level of protein *S*-glutathionylation was found (Nonaka et al., 2007). ApoB100, the major component of LDL, was identified as a target for increased *S*-glutathionylation in ASO, but whether the *S*-glutathionylated apoB-100 represents a disease marker or contributes to the pathogenesis of ASO remains an open issue (Nonaka et al., 2007).

Another target of *S*-glutathionylation is the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA), a key protein regulating the intracellular storage of Ca^{2+}. SERCA glutathionylation represents a physiological, cGMP-independent mechanism of vessel relaxation, which is disrupted during atherosclerosis (Adachi et al., 2004b). Molecular biology and mass spectroscopic analysis suggested that *S*-glutathionylation of Cys-674 was responsible for SERCA activation. The irreversible oxidation (i.e. sulfonic acid formation) at Cys-674 during atherosclerosis prevents its reversible *S*-glutathionylation, and may thus contribute to impaired vasodilation of atherosclerotic smooth muscle. Analysis of cysteine modifications in atherosclerotic vs. normal rabbit aortas showed increased sulfonate formation, corresponding to decreased *S*-glutathionylation, as well as reduced NO-induced relaxation and Ca^{2+} reuptake (Adachi et al., 2004b).

Oncoprotein Ras, one of the major regulators in growth factor signaling in the vasculature, can also undergo *S*-glutathionylation, which could contribute to vascular hypertrophy associated with atherosclerosis and hypertension in rat (Adachi et al., 2004a). Indeed, treatment *in vitro* of VSMCs with angiotensin II, known to induce vascular hypertrophy, led to *S*-glutathionylation and activation of Ras, resulting in increased phosphorylation of p38 and Akt and increased protein synthesis. These effects were dependent upon NADPH oxidase activation and ROS formation, and were blocked by overexpression of Grx1 or mutation of Ras at the site of *S*-glutathionylation (Cys-118) (Wang et al., 2001a,b; Landmesser et al., 2002). *S*-Glutathionylated Ras may contribute to atherosclerosis by mediating the response to oxLDL in endothelial cells. Treatment of bovine aortic endothelial cells with peroxynitrite led to Ras *S*-glutathionylation and activation of both ERK and Akt pathways (Clavreul et al., 2006). Akt is emerging as a signaling molecule within the heart and vasculature, implicated in various pathological signaling events as well as in normal development and homeostasis. Deglutathionylation by Grx could participate in regulating the balance between physiological and pathophysiological Akt activation; overall, a complex relationship exists between protein *S*-glutathionylation, Grx and Akt activity within the cardiovascular system (Wang et al., 2007).

S-Nitrosation vs. *S*-glutathionylation: competition for the same protein targets?

In some proteins, a competition between *S*-nitrosation and *S*-glutathionylation has been described, and the suceptibility to either PTM has been compared. It was thus shown that there are sensible differences as regards the tendency of a protein to undergo one or the other of such modifications (Ji et al., 1999; Mallis et al., 2001; Giustarini et al., 2005). GSNO is known for its dual ability to *S*-nitrosate as well as *S*-glutathionylate proteins. As previously described, GSNO-dependent protein *S*-nitrosation occurs through the direct transfer of a nitroso group from GSNO or another lmw. *S*-nitrosothiol to a protein thiol through transnitrosation. Despite a plethora of studies examining the effects of GSNO both *in vitro* and *in vivo*, few to date have identified the compound that actually effects *S*-nitrosation of a specific protein thiol, i.e. whether it may be GSNO or rather another lmw. *S*-nitrosothiol, e.g. L-Cys-NO. Nevertheless, GSNO is the source of the nitroso moiety and a likely intermediate in the protein *S*-nitrosation process.

S-nitrosation of specific proteins has been proposed to play a role in several disease states. Some proteins are *S*-nitrosated in their native state, and variations of basal *S*-nitrosation may control cellular responses with both deleterious or beneficial effects. Loss of *S*-nitrosation (hypo-nitrosation) in natively *S*-nitrosated proteins has been found in association with selected pathologies, and the same was reported for hyper-nitrosation of other proteins that are not natively *S*-nitrosated. Attempts to identify a so-called *S*-nitrosoproteome, and the specific role for GSNO in the process *in vivo*, have dealt with comparing *S*-nitrosated proteins from different organs in wild-type and NOS-knockout mice. These studies have revealed the existence of tissue- and context-specific protein targets of *S*-nitrosation, with conflicting results as to pathophysiology. NO*S*-ko mice were in fact protected from heart failure (Lima et al., 2009) and asthma (Que et al., 2005), but suffered from increased mortality in models of septic shock (Liu et al., 2004a,b).

In addition to transnitrosation, GSNO can slowly form disulfides with protein thiols, resulting in *S*-glutathionylation. In acellular systems, selected protein thiols appear to have a preference for GSNO-dependent *S*-thionylation over *S*-nitrosation. For example, bovine serum albumin showed extensive *S*-nitrosation after incubation with both SNAP and GSNO, whereas creatine kinase favored *S*-thionylation when exposed to GSNO, but *S*-nitrosation when exposed instead to SNAP (Konorev et al., 2000). In a thermodynamic perspective, it is likely that in these contexts disulfide formation is the eventual end-point, as the reaction is actually irreversible (at least in the *in vitro* setting). The preference for *S*-nitrosation or *S*-thionylation is likely due to tuning of reaction kinetics by the thiol environment and, as in the example above, the nature of the initial *S*-nitrosothiol. There is evidence that it is not GSNO *per se* but a product of its degradation, glutathione disulfide-*S*-oxide, that actually effects protein *S*-glutathionylation (Li et al., 2001; Tao and English, 2004). This may limit the biological relevance of the reaction, as it is not clear if this oxide can be formed from GSNO *in vivo*.

Once ascertained that both modifications are possible if GSNO is produced in the cell, it will become critical to discriminate which of the cellular proteins will be more easily or more stably modified by one or the other PTM. The functional relevance of the modifications can also become significantly different depending on the site of modification. For example, in transcription factors that bear in their DNA binding domain a Cys residue that needs to be in the reduced state to allow transcriptional activity, both modifications can impair this functionality, as it is the case for c-Jun in AP-1 or p50 in NF-κB (Klatt et al., 1999; Marshall and Stamler, 2001). Altogether, these PTMs likely represent a general mechanism for transducing oxidative stress into a repression of gene expression. Differences between the two PTMs will gain importance in terms of stability or potential reversibility. A few examples of protein targets modified by both PTMs, likely in a competitive way, are reported below.

Ryanodine receptor channel (RyR2)

It was established that different reactive thiols are present in the RyR2 protein, with preferential selectivity for *S*-nitrosation (Sun et al., 2001) or *S*-glutathionylation (Aracena et al., 2003), each leading to specific functional consequences. RyR2 is a large ligand-activated intracellular Ca^{2+} release channel located at the endoplasmic and sarcoplasmic reticulum (SR). Calcium release through the cardiac RyR2 triggers heart muscle contraction, and therefore RyR2 plays a crucial role in mediating excitation contraction coupling after an action potential. The regulatory role of *S*-glutathionylation is not surprising, considering that RyR2 contains 364 cysteines with about 84 of them having free thiol groups. Basal *S*-glutathionylation of RyR2 was discovered in microsomal fractions enriched in SR vesicles isolated from dog cardiac ventricular muscle (Sanchez et al., 2005). Under physiological conditions,

such as tachycardia and exercise, RyR2 *S*-glutathionylation increases, and this suggests that cardiac cells utilize this redox modification to increase RyR2 activity when the functional demand is increased (Sanchez et al., 2008). The RyR2 activity is enhanced also in heart failure, which likely contributes toward decreasing Ca^{2+} content in SR and inducing the Ca^{2+} release abnormalities observed in heart failure. The physiological sources of ROS responsible for RyR2 redox modifications are poorly understood. With both exercise and tachycardia, the administration of the NADPH oxidase (Nox) inhibitor, apocynin, prevented RyR2 *S*-glutathionylation and attenuated Ca^{2+} release from the SR (Donoso et al., 2011). Greater understanding of RyR2 redox modulation is necessary to counteract the deleterious consequences of its deregulation caused by oxidative stress.

However, *S*-nitrosation of cardiac RyR2 has been shown to increase its channel opening probability. Conversely, the decreased *S*-nitrosation of RyR2 in hearts of nNOS knock-out mice has been suggested to expose its Cys residues to oxidation under oxidative stress, leading to Ca^{2+} leakage from SR and arrhythmogenesis (Gonzalez et al., 2007).

The cardiac L-type Ca^{2+} channel, acting as a RyR2 activator, is also a target of *S*-nitrosation, which is reported in the literature to decrease the channel activity in hearts under adrenergic stimulation (Sun et al., 2006b), oxidative stress during ischemia (i.e. preconditioning) (Sun et al., 2007) and atrial fibrillation (Carnes et al., 2007).

Sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA)

SERCA, the key protein regulating the intracellular storage of Ca^{2+} as it enhances refilling of Ca^{2+} in sarcoplasmic and endoplasmic reticula, is another common target of both PTMs. Two major SERCA2 splice variants have been characterized: the muscle-specific isoform, SERCA2a, is expressed in the SR of the heart and slow-twitch skeletal muscle. SERCA2b is found instead in the ER of most cell types, and is considered the housekeeping isoform.

A key finding was the observation that SERCA may be modified by different post-translational mechanisms in the presence of NO-related species (Viner et al., 1999, 2000). NO can stimulate calcium uptake in SR, thereby decreasing intracellular Ca^{2+} concentration, facilitating the uncoupling of the contractile machinery and favoring vasorelaxation (Cohen et al., 1999).

Adachi et al. showed that NO-dependent *S*-glutathionylation of SERCA2a (occurring via peroxynitrite and therefore dependent on superoxide generation, beyond NO and GSH) results in an increased activity (Adachi et al., 2004b). Similarly, in preconditioned (ischemic) hearts, Sun et al. found that SERCA activity was also increased by *S*-nitrosation (Sun et al., 2007). Peroxynitrite is able to activate SERCA by promoting its *S*-thionylation in the context of arterial relaxation, an effect recapitulated by eNOS agonists (Eu et al., 2000). A specific target (Cy*S*-674) seems to be present, whose *S*-thionylation results in the suppression of NO-mediated increase of activity. Such modification occurs *in vivo*, and a decrease in its levels due to irreversible -SH oxidation was found in a rabbit model of atherosclerosis, where SERCA *S*-glutathionylation in abdominal aorta was increased, resulting in decreased relaxation (Adachi et al., 2004b). In the heart, stimulation of SERCA activity and cardiomyocyte contractility by the nitroxyl anion generator, Angeli's salt, also is accompanied by *S*-glutathionylation (Lancel et al., 2009).

Overall, these data suggest a potential pathophysiological role of SERCA *S*-nitrosation and *S*-glutathionylation in vascular disease. However, whether *S*-nitrosation of SERCA can increase activity *per se*, or rather it does so by enhancing *S*-glutathionylation, remains to be clarified.

Oncoprotein Ras

The oncogenic Ras protein may become *S*-nitrosated, and this may convert it to its biologically active GTP-bound state (Lander et al., 1997). Heo et al. have contributed to explain the structure-functional relationships of Ras *S*-nitrosation in Cy*S*-118, hence gaining mechanistic insight into the process of NO-mediated Ras guanine nucleotide dissociation in the presence of O_2 (Heo and Campbell, 2006). Conversely, Ras may also undergo *S*-glutathionylation at the same cysteine residue (Ji et al., 1999; Mallis et al., 2001), and it was suggested that this modification may mediate signaling by Ang-II in vascular SMCs (Adachi et al., 2004a). Peroxynitrite, albeit at moderately high concentrations, is able to promote *S*-thionylation of Ras in endothelial cells, and this has been correlated with the activation of its downstream signaling effectors (Clavreul et al., 2006). Of interest, modifications of Ras cysteines (not yet identified) in myocites are potentially reversible by Trx (Kuster et al., 2005). In terms of pathophysiology, one of the most interesting observations situates ROS-induced *S*-thionylation of Ras as a mediator of cardiac myocyte hypertrophy in a strain-stimulated model. The modification of Ras likely occurs at Cy*S*-118 and consists in a *S*-nitrosation followed by an exchange reaction, leaving Ras *S*-glutathionylated (Pimentel et al., 2006).

Hemoglobin (Hb)

Hb is a target for *S*-nitrosation and this modification may play a physiological role. NO binds to the most reactive thiol in human Hb, Cys-93, which led to hypothesize the ability of the resulting *S*-nitroso-Hb to serve as a storage and transport form of NO participating in the control of vascular tonus (Jia et al., 1996). Following this observation, a number of studies have investigated in detail the roles potentially played by *S*-nitrosohemoglobin, actually fueling a long controversy. On the one hand, experimental results were confirming the ability of *S*-nitrosohemoglobin to serve as a generator of NO, thus providing a precious source of the mediator in the vascular system (Stamler et al., 1997; Allen et al., 2009). On the other hand, numerous studies also highlighted that the prompt reaction of hemoglobin with NO would rather configure its opposite role as a NO scavenger/depletor, especially in conditions of inflammation (reviewed in Roche et al., 2013). The dispute was also fueled by divergences in the blood levels of *S*-nitrosohemoglobin reported by different laboratories, possibly a result of methodological issues (Giustarini et al., 2004). Presently, the interpretation has eventually prevailed that *S*-nitrosohemoglobin indeed participates in stabilization and delivery of NO in the vascular bed, contributing to the regulation of vascular tone, blood flow and platelet aggregability (Zhang et al., 2016).

The binding of GSH to Cys-93 of Hb β chain has been known for more than 20 years, and was associated with inhibition of Hb S polymerisation, increased oxygen affinity and reduced alkaline Bohr effect (Garel et al., 1986). This made it an interesting candidate to combat sickle cell anemia; however, neither the modification of Hb by GSH nor by NO have translated into effective treatments for such disease (Head et al., 1997). In contrast, *S*-glutathionylated Hb has been widely documented in disease states such as uremia or diabetes (Niwa, 2006), and it is currently believed to be a consequence of the oxidative stress underlying these diseases. At least three different types of glutathionylated Hb can exist in erythrocytes: a mixed disulfide between GSH and normal Hb; a disulfide bond between GSH and the Cy*S*-93 of metHb β-chain; and a disulfide bond between GSH and other cysteine residues of met-Hb α- and/or β-chain (Mawatari and Murakami, 2004; Regazzoni et al., 2009). Rat erythrocytes treated with diamide, a thiol oxidizing agent, also presented Hb *S*-glutathionylation (Kosower et al., 1977). Besides Cys-93, Hb glutathionylation also occurs at Cys-112 in the β-chain (Metere et al., 2014). These authors found that CO treatment of whole blood increases the GSH concentration in red blood cells cytosol, a result of significant Hb deglutathionylation. This process does not activate glycolytic metabolism, boosts the pentose phosphate pathway, increases glutathione reductase activity and decreases GSSG concentration, thus highlighting a CO-dependent redox signaling in human erythrocytes driven by Hb *S*-glutathionylation.

Caspase-3

Several years ago the antiapoptotic effect of NO was related to a potential inhibition of caspase-3 activation, through both cGMP-dependent and independent mechanisms (Kim et al., 1997). It has been documented that Fas ligand is under the control of the NO pathway, and that *S*-nitrosation/denitrosation of caspase-3, probably linked to subcellular locallization, may be the mechanism by which this ligand controls the general process of apoptosis (Mannick et al., 1997, 1999). In immune-response lineage cells, procaspase-3 basally presents with *S*-nitrosation on its catalytic Cy*S*-163 (Mannick et al., 1999), and other caspases, such as caspase-9, could also undergo *S*-nitrosation (Mannick et al., 2001). Interestingly, Trx is involved in the process of *S*-nitrosation of caspase-3 (Mitchell and Marletta, 2005) and *S*-nitrosation of the catalytic site Cys residue might be assisted by a nitrosopeptide that mimics the substrate configuration, thus providing an elegant example for a specific transnitrosation mechanism (Mitchell et al., 2006).

Yin et al. (2013) investigated the effects of GSNO pretreatment on the *S*-nitrosation of Fas and subsequent events in the Fas pathway, revealing the correlation between Fas *S*-nitrosation and nNOS activation in rat hippocampus after global cerebral ischemia. Results showed that GSNO pre-treatment not only facilitated the survival of hippocampal CA1 pyramidal neurons, but also abolished the activation of pro-apoptotic caspase-3, 8 and 9, as well as of the pro-apoptotic protein Bid. Consistently, a moderate dose of GSNO would confer neuroprotection against ischemic injury via inhibiting Fa*S*-dependent apoptosis. Fas *S*-nitrosation could represent the critical regulation hinge in such a neuroprotective mechanism. Actually, GSNO-generated NO *S*-nitrosates and inactivates nNOS itself, resulting in the inhibition of endogenous NO production and a decrease in Fas *S*-nitrosation. This insight into GSNO-mediated neuroprotection might suggest therapeutic approaches for ischemic brain injury (Yin et al., 2013).

S-glutathionylation of caspase-3 by GSNO, or SNAP plus GSH, has been described by several studies (Klatt et al., 2000; Mitchell and Marletta, 2005). Recent observations claim that caspase-3 experiences *S*-glutathionylation

on several thiol residues and that this process is intracellularly regulated by Grx, a mechanism relevant for the regulation of TNF-α-dependent endothelial cell death (Pan and Berk, 2006). Clearly, caspase-3 constitutes an interesting model to study both types of PTMs at the cellular level, particularly with respect to regulation of cell death. However, definitive evidence establishing implications at whole organism level is still missing.

Myosin

NO is synthesized in skeletal muscle and its production increases during contractile activity. Although myosin is the most abundant protein in muscle, it is not known whether myosin is a target for NO or its derivatives. Nogueira et al. (2009) have shown that exercise increases *S*-nitrosation in muscle proteins, and, among contractile proteins, myosin is the principal target of exogenous *S*-nitrosothiols in both skinned skeletal muscle fibres and differentiated myotubes. Myosin contains 42 Cys residues, and different populations have been described based on their reactivity to sulfhydryl-specific reagents and the resulting changes in ATPase activity (Buttkus, 1971; Schaub et al., 1975). The reaction of isolated myosin with GSNO results in *S*-nitrosation at multiple cysteines and produces two populations of protein-bound SNOs with different stabilities. The less stable population inhibits the physiological Mg^{2+}-ATPase activity without affecting the affinity of myosin for actin. However, myosin is neither inhibited nor *S*-nitrosated by the NO-donor diethylamine NONOate, indicating a requirement for transnitrosation between lmw. SNOs and myosin cysteine thiols rather than a direct reaction of myosin with NO or its auto-oxidation products. Interestingly, alkylation by N-ethylmaleimide of the most reactive thiols in myosin does not inhibit formation of a stable population of protein-SNOs, suggesting that these sites are located in regions of the protein less accessible than those affecting activity. This early study established thus a link between exercise and *S*-nitrosation of contractile proteins that may be important in pathophysiology of skeletal muscle (Buttkus, 1971).

High levels of GSNO-dependent *S*-nitrosation of proteins from the cytoskeleton and the contractile machinery were recently identified under oxidative stress conditions (Belcastro et al., 2017). In particular, the detailed pattern of *S*-nitrosated proteins indicates that most of *S*-nitrosylated proteins are of primary relevance for the performance of SMC functions being often altered in vascular diseases, such as cell communication, cytoskeletal organization, morphogenesis, contraction and movement. Interestingly, the process involves actin and cytoskeleton dynamics at several key regulatory proteins, including myosin light polypeptide 6 (MYL6). The role of *S*-nitrosation in regulating these proteins is largely unknown, particularly during oxidative stress. Each of them has been shown to play a role in modulating smooth muscle contraction. *In vitro S*-nitrosation of skeletal muscle myosin, for example, increases the force of the actomyosin interaction while decreasing its velocity, indicating a relaxed state (Evangelista et al., 2010). MYL6 regulates light chain of myosin and does not bind calcium, but is anyway involved in muscle contraction and development of skeletal muscle tissue. Other proteins *S*-nitrosated by GSNO under oxidative stress conditions include calponin-2 (CNN2) and transgelin (TAGLN). Together these proteins, implicated in Ca^{2+}-dependent contractility and NO signaling, constitute a potential interactome, and discovering their behavior as *S*-nitrosated proteins may further help our understanding of processes such as contraction-relaxation signaling in SMCs, or their phenotype switching during atherosclerosis progression.

S-glutathionylation of myosin has an important impact on the protein structure, as documented by the lower fluorescence quantum yield of *S*-glutathionylated protein and its increased susceptibility to proteolytic cleavage. Myosin ATPase activity is also sensitive to modulation by *S*-glutathionylation, depending on GSSG redox balance. Thus, similar to the phosphorylation/dephosphorylation cycle, *S*-glutathionylation may represent a mechanism by which glutathione modulates sarcomere functions depending on the tissue redox state, and myosin may constitute the muscle redox-sensor (Passarelli et al., 2008; Pastore and Piemonte, 2013).

α-Actin

Actin filaments are important components of the cell cytoskeleton, where they are often involved in the process of exocytosis. Actin of rabbit skeletal muscle has five cysteine residues at different positions, but only Cy*S*-374 in the C-terminal region is exposed and important for polymerization (Aspenström et al., 1993). The same residue is a decisive site for *S*-nitrosation (Dalle-Donne et al., 2000). Recently it has been demonstrated that NO-induced *S*-nitrosation is an effective inhibitor of actin polymerisation (Lu et al., 2009). Actin was rapidly *S*-nitrosated *in vitro* by exogenous NO-generating donors (e.g. GSNO), and *in vivo* in the spinal cord of a mouse model of inflammatory pain. NO showed an inhibitory action on inhibitory synaptic transmission in the spinal

dorsal horn, implicating *S*-nitrosation in pain transmission via disinhibition of inhibitory neurons (Lu et al., 2009, 2011). In rat aortic SMCs stressed *in vitro* with a free radical generator (2,2′-azobis(2-amidinopropane dihydrochloride, AAPH), actin was *S*-nitrosated following exposure to GSNO (Belcastro et al., 2017), but the real impact of this modification on SMCs functions remains to be elucidated.

α-Actin is also particularly sensitive to binding of glutathione, as shown in isolated cardiac and skeletal myofibrils under conditions of oxidative stress (Passarelli et al., 2010). *S*-glutathionylation of cardiac α-actin occurs non-enzymatically, via spontaneous oxidation of a Cys residue to a cysteinyl-sulfenic acid intermediary (Dalle-Donne et al., 2003; Johansson and Lundberg, 2007). It has been shown that cardiac α-actin polymerizes slower than the native protein, when *S*-glutathionylated *in vitro*. Thus, like cytoskeletal β-actin (Dalle-Donne et al., 2003; Fiaschi et al., 2006) and skeletal muscle actin (Dalle-Donne et al., 2003), even α-actin could constitute a direct target for oxidative modification in human heart, and its *S*-glutathionylation may represent a mechanism by which glutathione can modulate sarcomere function, depending on the redox state of the tissue.

The balance between nitrosation and glutathionylation of thiols: general considerations

How proteins become *S*-glutathionylated and *S*-nitrosated *in vivo* is not yet well established, despite continuing attempts to clarify this issue. The effects of *S*-nitrosation and *S*-glutathionylation on protein activity may be similar, and both modifications are reversible, but the resulting products, PSNOs and PSSGs, do possess different biological properties. The list of proteins undergoing these changes is long and not definitive; a summary of the main protein targets modified by *S*-nitrosation and/or *S*-glutathionylation is however reported in Table 2.

The relative importance of *S*-nitrosation and *S*-glutathionylation likely depends not only on the environment surrounding the protein -SH groups, but also on the chemical nature of the intervening lmw. RSNO, which can affect the accessibility of the protein thionylate to either the nitrogen or the sulfur of the RSNO. This was shown *in vitro* for CPK, which is predominantly either *S*-glutathionylated or *S*-nitrosated, depending on the different species of RSNOs it is exposed to (Konorev et al., 2000). CPK is reversibly inhibited by incubation with *S*-nitrosothiols (Giustarini et al., 2005). Loss of enzyme activity is associated with the depletion of 5,5′-dithiobi*S*-(2-nitrobenzoic acid)-accessible thiol groups, and is not due to nitric oxide release from RSNOs. Full enzymatic activity and protein thiol content are restored by incubation of the *S*-nitrosothiol-modified protein with GSH. SNAP, which contains a more sterically hindered *S*-nitroso group than GSNO, predominantly modifies the CPK protein thiols via a transnitrosation reaction. In contrast, GSNO modifies CPK thiols predominantly by *S*-thionylation, while bovine serum albumin is nitrosated by both SNAP and GSNO. Therefore, using CPK as a model, the degree of *S*-thionylation and *S*-nitrosation appears to depend both on the protein structure and the chemical nature of the *S*-nitrosothiol, and these differences are likely due to both steric factors and pKa of the protein thiols (Konorev et al., 2000). Data suggest that *S*-glutathionylation can be a quantitatively relevant process likely running in parallel to *S*-nitrosation in some proteins, and that denitrosation is a largely faster process than deglutathionylation. In addition, it may be hypothesized that stable intracellular protein RSNOs pools exist, likely located either in hydrophobic protein pockets inaccessible to GSH and other reducing agents, or in other specific cellular microenvironments (Zhang and Hogg, 2004).

Concluding remarks

In this review we have discussed the biologically relevant role of NO as a conveyor of signals, as well as the function of the endogenous nitrosating agent, GSNO, in protein activation and/or inactivation through *S*-nitrosation, transnitrosation, disulfide bond formation, and/or *S*-glutathionylation. In a general perspective, these processes represent just one aspect of a much wider array of reactions and processes related with the redox homeostasis of the cell, involving reactive species of oxygen and nitrogen but also, as increasingly recognized in recent years, of sulfur itself. By now, such reactive species (ROS, RNS and RSS) are considered as players in a complex redox signaling network that interacts with protein thiol targets, acting as redox switches to control protein structure and function. Such a complex network has intriguingly been named ‘reactive species interactome’ (RSI), a novel concept in cell biology liable to provide critical insights for the development of prevention and treatment strategies, in what could be called today ‘redox medicine’ (Cortese-Krott et al., 2017). The RSI perspective is somehow complementary to (and integrated with) the redox code, and within that, to

the redox proteome, another concept recently introduced to describe how protein redox-sensitive Cys residues participate in a wider network of redox switches, linked to the NAD and NADP systems and, through those, to the general metabolism of the cell (Jones and Sies, 2015).

Much work is still needed to more precisely define how the different modifications may be balanced, the detailed mechanisms underlying their relationships, and their functional implications in physiological settings. The recent development of new methodologies (proteomics, subcellular localization studies) has moved knowledge some steps further, but significant analytical improvements are still needed in order to gain deeper insight into the physiological relevance of *S*-nitrosation and *S*-glutathionylation. The low, negligible levels of protein *S*-nitrosation/*S*-glutathionylation generally detectable in homeostatic conditions suggest that these PTM may often lack true pathophysiological roles, playing as mere bystanders. On the other hand, the combined effects of altered *S*-nitrosation/denitrosation and *S*-glutathionylation/deglutathionylation on different or the same proteins could represent a coordinated adaptive response aimed to promote cell survival during short-term oxidative/nitrosative stress. In the cardiovascular system, evidence is accumulating of the (patho)physiological relevance of these PTMs in ensuring protection of Cys residues from oxidation. It will be important then to determine exactly which proteins and Cys residues, which would otherwise undergo oxidation, can be *S*-nitrosated and/or *S*-glutathionylated. *S*-Nitrosation of some proteins can be beneficial, whereas it can be detrimental in others, while *S*-glutathionylation of the same proteins could still have other effects. In the future, it will be necessary to understand how these processes can combine many individual protein modifications into a global metabolic map, in order to fully understand the importance of reversible oxidative/nitrosative protein modifications. As advances in both the methodology and technology accelerate the study of *S*-nitrosated and *S*-glutathionylated proteins, the critical role they play in CVD is beginning to emerge. In perspective, the full understanding of these modifications may offer indications for the development of new drugs, and they may be as well exploited as biomarkers or predictors in cardiovascular pathology.

Acknowledgments: The authors are indebted to Prof. H. Sies for having inspired and encouraged the compilation of this work. Financial supports by the Université de Lorraine and the Région Lorraine, Programme VINCI 2014 – Université Franco Italienne (Project no. C2-56) and the Italian Cystic Fibrosis Research Foundation (Verona, Italy; Project FFC #18/2014) are gratefully acknowledged. Additional funds were provided by University of Pisa, Italy (P.R.A. 2015 funds).

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