Free Radical Biology and Medicine (xxxx) xxxx–xxxx

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

How widespread is stable protein S-nitrosylation as an end-effector of protein regulation?

Kathryn Wolhuter, Philip Eaton*

King's College London, Cardiovascular Division, The British Heart Foundation Centre of Excellence, The Rayne Institute, St Thomas' Hospital, London SE1 7EH, UK

ARTICLE INFO

Keywords:
S-nitrosylation
S-nitrosation
Thiol
Nitric oxide
Cysteine
Redox

ABSTRACT

Over the last 25 years protein S-nitrosylation, also known more correctly as S-nitrosation, has been progressively implicated in virtually every nitric oxide-regulated process within the cardiovascular system. The current, widely-held paradigm is that S-nitrosylation plays an equivalent role as phosphorylation, providing a stable and controllable post-translational modification that directly regulates end-effector target proteins to elicit biological responses. However, this concept largely ignores the intrinsic instability of the nitrosothiol bond, which rapidly reacts with typically abundant thiol-containing molecules to generate more stable disulfide bonds. These protein disulfides, formed via a nitrosothiol intermediate redox state, are rationally anticipated to be the predominant end-effector modification that mediates functional alterations when cells encounter nitrosative stimuli. In this review we present evidence and explain our reasoning for arriving at this conclusion that may be controversial to some researchers in the field.

1. Introduction

Nitric oxide (NO) is perhaps best known as the endothelium-derived relaxation factor responsible for vasodilation, but it also plays fundamental roles in a diverse array of disparate biological processes [1]. Much of the biology associated with NO production is via it binding and altering the function of heme proteins [2]. For example NO activates soluble guanylyl cyclase in this way, stimulating synthesis of the second messenger cyclic guanosine monophosphate which binds and activates protein kinase G to induce phosphorylation-dependent signalling [3]. NO and a number of related NO chemical variants can also react with cellular molecules to yield nitroso (i.e. R-NO) derivatives [4], which may alter their physiochemical properties and so perhaps also their biological activities. Thiol (R-SH) containing molecules are especially susceptible to such nitrosation reactions, and the formation of these species in proteins is considered to occur widely. Formation of S-nitrosothiols (P-S-N=O), a process commonly referred to as S-nitrosylation, is widely held to be a pervasive post-translational modification that serves as a regulatory mechanism by altering protein function. Indeed, protein S-nitrosylation is often presented as an evolutionary-conserved signalling mechanism that affects most, if not all, classes of proteins. The putative roles for protein S-nitrosothiols within the cardiovascular systems is vast with over 1000 proposed targets in the heart alone [5]. Many reviews ascribe S-nitrosothiols as end-effectors of NO signalling that contribute to homeostasis during health [6–8], with dysregulation of these processes contributing to disease pathogenesis. For example, hyper- or hypo-S-nitrosylation contributes to a range cardiovascular disease including type 1 and 2 diabetes [9], atherosclerosis [10], cardiac ischemic injury [11], hypertrophy [12] and sepsis [13].

The current consensus is that S-nitrosylation, much like phosphorylation, is a stable post-translational modification that directly mediates signalling by altering the function of end-effector proteins. However, such a widespread role would appear incompatible with the inherent lability of the nitroso bond [14], especially *in vivo* where thiol-containing molecules are abundant and react rapidly with S-nitrosothiols to generate disulfide bonds [15,16]. These transient S-nitrosothiol intermediates can yield intramolecular or intermolecular protein disulfides, as well as disulfides with small molecular weight thiols such as glutathione (GSH) or cysteine. These disulfide post-translational modifications, which are fully anticipated to occur widely when S-nitrosylation reactions occur in cells, have been known for many decades to regulate protein function [17]. Disulfide formation is also known to play a crucial role in redox signalling within the cardiovascular system, however it is less well studied in the setting of nitrosative stress [18].

Although transient S-nitrosothiols are already acknowledged to be one mechanism by which disulfides form [19,20], it is important to reconsider and highlight such studies. This is because the vast majority of work relating to protein S-nitrosylation fails to recognise that in many cases, if not most, a disulfide is likely the predominant post-

* Corresponding author.
E-mail address: philip.eaton@kcl.ac.uk (P. Eaton).

http://dx.doi.org/10.1016/j.freeradbiomed.2017.02.013
Received 2 December 2016; Received in revised form 26 January 2017; Accepted 5 February 2017

translational modification that mediates NO-dependent alterations in function. This does not mean that protein S-nitrosylation does not occur or play crucial roles in biological systems, but instead that the current vision of many that it is a ubiquitous end-effector of protein function is likely to be significantly incorrect. It is notable that the term nitros**yl**ation chemically refers to NO bound to a transition metal, whereas a more correct chemical term for NO bound to a thiol is S-nitrosation. The inclusion of the '**yl**' may be used to infer that it reflects an enzyme-mediated process, perhaps instead of passive chemistry. S-nitrosylation is not only an inappropriate term that can facilitate confusion, but is perhaps deliberately used in an attempt to conflate and assist with establishing parallels with other post-translation regulatory mechanisms such as phospho**ryl**ation. For this reason, the chemically correct term S-nitrosation is predominantly used in this review. Our aim is to critically evaluate the evidence for S-nitrosation as the stable post-translational and universal end-effector modification that it is so widely perceived or advocated to be.

2. Stable protein S-nitrosation as a regulatory end-effector mechanism

Strong parallels have been drawn between protein S-nitrosation and other well-established post-translational modifications, especially phosphorylation [21,22]. Kinases are often co-localized with their substrates, with additional selectivity achieved by consensus motifs in the primary sequence of a target protein that is phosphorylated. Similarly, targets of S-nitrosation can localise with NO synthase (NOS) enzymes [8], and may contain linear amino acid consensus motifs [23], thus mirroring key features of kinase-mediated phosphorylation. Furthermore, denitrosylase enzymes that remove NO from proteins are described to play the corresponding role of phosphatases in phospho-regulated proteins [22]. Protein S-nitrosation as a post-translational regulatory mechanism is additionally supported by the fact that it can occur stoichiometrically [24], and that pharmacological inhibition of the enzyme that mediates denitrosation leads to elevated S-nitrosation [25]. Moreover genetic ablation of a target cysteine not only prevents formation of the S-nitroso modification, but also abrogates the functional alteration that otherwise occurs in the wild type protein [26]. It would therefore seem irrefutable that stable protein S-nitrosation is a ubiquitous end-effector regulatory mechanism. However, when the primary evidence for each of the individual facets that support a role for regulation by stable S-nitrosation is considered in depth, we contend that significant discrepancies become apparent. Indeed, as considered below, we suggest that protein S-nitrosation per se is unlikely to directly underlie functional alterations to the extent it is commonly described to.

2.1. Enzymatic regulation of protein S-nitrosation

A keystone in the conceptual foundations of regulatory S-nitrosation was the identification of so-called 'nitrosylases' and 'denitrosylase' that enzymatically regulate protein nitrosothiol levels by adding or removing the modification respectively. Genetic knock out of neuronal NOS *in vivo* resulted in decreased S-nitrosation of several proteins, illustrating that S-nitrosation is ultimately NOS dependent [27]. NOS enzymes have therefore been described as nitrosylases, playing an analogous role to kinase-mediated phosphorylation, by co-localising with target proteins susceptible to S-nitrosation [28]. NOS-derived reactive nitrogen species can directly transfer to target proteins directly or alternately this can occur indirectly, via a cascade of trans-nitrosation transfer reactions. Direct binding of NOS to a target has been shown experimentally for inducible NOS and cyclooxygenase-2 [29]. Indirect trans-nitrosation by NOS via intermediate scaffold and adaptor proteins was summarized by Hess et al. [8]. They described how the N-methyl-D-aspartate (NMDA) receptor is S-nitrosated by neuronal NOS [30] via postsynaptic density protein 95 (PSD95), which serves as a scaffold. PSD95 is known to interaction with both the neuronal NOS [31] as well as the NMDA receptor [32], supporting its role as a scaffold. However, whether disrupting PSD95 interactions alters S-nitrosation of NMDA receptor remains unclear. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can also transfer NO from itself to another target protein thiol, therefore acting as a trans-nitrosylase [33]. These examples of localized direct or trans-nitrosation-mediated modification of proteins involving organized multicomponent signalling complex are often assumed to reflect a generic, widespread situation. Whilst there are multitudes of studies describing co-localized, multicomponent signalosomes in the context of phospho-regulation, we note that studies providing primary experimental evidence for protein S-nitrosation to be generically organized in this way are relatively rare.

A significant argument against the existence of regulatory nitrosylases is that S-nitrosothiol formation is principally a chemical reaction between sulfur and various derivatives of NO that occurs rapidly and without enzyme catalysis [34]. As NO is unable to directly oxidise thiols at a meaningful biological rate, S-nitrosothiol formation is dependent on oxidation of NO to a reactive forms in the presence of a one-electron acceptor, such as oxygen [35]. Several mechanisms for attack by NOS-derived reactive nitrogen species have been proposed, which are summarized in Fig. 1 [36].

In the presence of oxygen one prominent theory is S-nitrosation via the formation of N_2O_3 ($^+ON…NO_2$) (**Eq.** (1) and (2)).

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO_2 + NO \rightarrow N_2O_3 = {}^+ON…NO_2^- \quad (2)$$

S-nitrosothiols are formed by the electrophilic attack of a deprotonated thiol (PS^-), known as a thiolate, by NO^+. Here formation of N_2O_3 is the rate-limiting step in S-nitrosation as it occurs relatively slowly in aqueous solutions ($6.6 \times 10^6\ M^{-2}\ s^{-1}$); although this increases to $8.8 \times 10^7\ M^{-2}\ s^1$ in the lipid phase or within hydrophobic protein pockets [36]. Kinetic modelling has predicted that physiologically NO concentrations, likely < 1 μM, would result in femtomolar concentrations of N_2O_3 [37]. Therefore, when taking diffusion into account, S-nitrosation is likely to primarily occur when a NOS enzyme is proximal to a target protein thiol, especially those in a hydrophobic environment. The efficiency of this third-order reaction is highly dependent on the concentration of oxygen. If oxygen is depleted, such as during hypoxia, S-nitrosation is unlikely to occur via this NO_2/N_2O_3 pathway. At a low

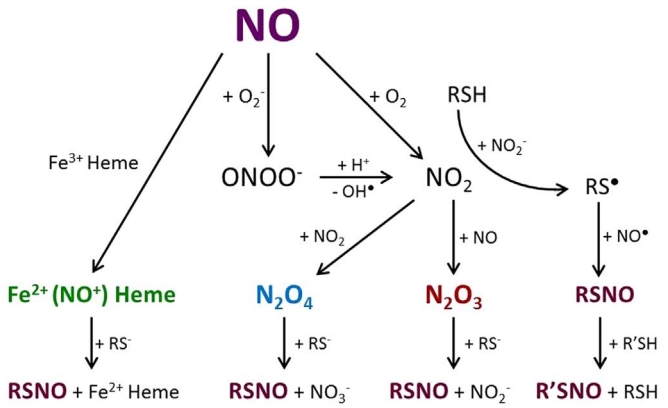

Fig. 1. *Proposed mechanisms of S-nitrosation.* Formation of S-nitrosothiols (RSNO) is predicted to occur through multiple distinct pathways. Nitric oxide (NO) can react with transition metals to form an intermediate followed by direct transfer of NO to target thiols. NO will also react with superoxide to form peroxynitrite ($ONOO^-$) followed by loss of $^{\bullet}OH$ to form NO_2, although perhaps this is unlikely to occur under physiological conditions. It is perhaps more likely that NO_2 is formed by NO reacting with oxygen. NO_2 then forms nitrosating species, such as N_2O_3 and N_2O_4, that directly S-nitrosate target thiols. Intermediates in the NO/NO_2 pathway can convert a thiol to a thiyl radical ($RS^{\bullet}$) which then directly react with $NO^{\bullet}$ forming RSNO. Finally thiols can be trans-nitrosated whereby one S-nitrosothiol transfers its NO group directly to another thiol.