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Nitrosothiol signaling and protein nitrosation in cell death

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Abstract

Nitric oxide, a reactive free radical, is an important signaling molecule that can lead to a plethora of cellular effects affecting homeostasis. A well-established mechanism by which NO manifests its effect on cellular functions is the post-translational chemical modification of cysteine thiols in substrate proteins by a process known as *S*-nitrosation. Studies that investigate regulation of cellular functions through NO have increasingly established *S*-nitrosation as the primary modulatory mechanism in their respective systems. There has been a substantial increase in the number of reports citing various candidate proteins undergoing *S*-nitrosation, which affects cell-death and -survival pathways in a number of tissues including heart, lung, brain and blood. With an exponentially growing list of proteins being identified as substrates for *S*-nitrosation, it is important to assimilate this information in different cell/tissue systems in order to gain an overall view of protein regulation of both individual proteins and a class of protein substrates. This will allow for broad mapping of proteins as a function of their *S*-nitrosation, and help delineate their global effects on pathophysiological responses including cell death and survival. This information will not only provide a much better understanding of overall functional relevance of NO in the context of various disease states, it will also facilitate the generation of novel therapeutics to combat specific diseases that are driven by NO-mediated *S*-nitrosation.

Keywords

Nitrosation; S-nitrosylation; Apoptosis; Cell death; Survival; Cancer

1. Introduction

Nitric oxide (NO) is a relatively short-lived inorganic free radical, which functions as both an effector and second-messenger [1]. Since its first description as endothelium-derived relaxing factor (EDRF) in 1987, NO has been shown to be important in a broad range of physiological and pathological processes [2–4]. In mammals, various NADPH-dependent enzymes called nitric oxide synthases (NOS), together with their co-factors such as tetrahydrobiopterine (BH₄), oxygen and protoporphyrin IX, modify the terminal guanidino

nitrogen group of L-arginine, resulting in the formation of L-citrulline and the generation of NO (Fig. 1). Three isoforms of NOS have been characterized thus far - NOS1 (neuronal or nNOS), NOS2 (inducible or iNOS) and NOS3 (endothelial or eNOS) [5–7]. NOS1 and NOS3 are calcium-dependent enzymes that are constitutively expressed at low levels in neuronal and endothelial cells respectively, and are collectively referred to as constitutive NOS (cNOS). The more ubiquitous and stably expressed inducible NOS (iNOS) is calcium-independent and is induced primarily in response to proinflammatory cytokines and gram-negative endotoxins. The expression of these isoforms varies in different tissues, and are dysregulated in several pathological situations including cancers [8]. Several studies have demonstrated the importance of NOS-mediated signaling in several tumor models, and the inhibition of NOS activity is sufficient to inhibit tumor growth and progression in several tumor subtypes [9–11]. As compared to cNOS, iNOS produces much higher physiological levels of NO [12]. It merits mentioning, however, that inducibility is more a function of stimulus and applies to all forms of NOS. Therefore, the traditional definitions of ‘constitutive’ versus ‘inducible’ may be deemed inaccurate under certain circumstances.

NO has several physiological roles, including regulation of endothelial cell contraction, angiogenesis, and mediating both leukocyte and thrombocytes functionality during injury and infection [13–17]. These effects are mediated due mainly to reactivity of NO with various reactive oxygen species (ROS), resulting in the generation of reactive nitrogen-oxygen species (RNOS) such as dinitrogen trioxide and peroxynitrite, leading to nitrosative stress inside the cell [18–21] (Fig. 2). These effects are countered by antioxidant enzymes such as glutathione and ascorbate, which inhibit reactivity of RNOS. In addition to signaling through intermediaries, NO can directly modify biological molecules via nitration of peptides and lead to aberrant signaling responses. For example, nitrotyrosines formed by nitration of tyrosine residues can prolong the activity of NO by acting as reservoirs that slowly release NO, or by allowing for these modified proteins to act either as second messengers [22,23].

Nitric oxide, either directly or indirectly through various NOS, has a well established role in mediating physiological cell death through host defense mechanisms in response to pro-inflammatory activators such as interferon- γ and TNF- α [24]. Both mesangial cells and macrophages produce NO as a result of pro-inflammatory stimulation, which can directly induce apoptosis in not only pathogens invading the body, but also on epithelial and endothelial cells. In addition, there have been several reports of such direct effects of NO production in driving apoptotic death of various types of tumor cells including that of the blood, lymph and breast [25–28].

In recent years, reversible coupling of a nitroso group to the reactive sulfur atom of protein thiols in cysteine residues, now widely described by the term ‘S-nitrosation’ (there is some debate about the terminology, discussed below), has been demonstrated as being one of the most significant effects of NO signaling. Proteins mediating several important cellular functions have been shown to serve as substrates for S-nitrosation [29,30]. Such modifications may have pleiotropic effects on cells in both physiological and pathological conditions, and influence several aspects of cellular function ranging from immune cell

response, endothelial cell regulation, cardiac functionality and neurodegeneration. Some of these aspects will be discussed in this review.

As mentioned above, the use of the term 'S-nitrosation' in scientific literature has evolved over the past several years. At least three chemical reactions may be mediated by intracellular NO - (a) protein nitration, which refers to the addition of a Nitro-NO₂ group to the side-chains of proteins that is mediated by intermediates such as peroxynitrite [31], (b) protein nitrosylation, or addition of a NO group to metal centers such as that of the heme group of guanylyl cyclase, and (c) protein nitrosation, which refers to direct addition of nitroso group to organic moieties, mainly thiols. However, a significant number of researchers used *S*-nitrosylation (instead of *S*-nitrosation) to describe the reversible coupling of nitroso groups to reactive cysteine thiols, leading to the formation of *S*-nitrosothiol (SNO) moieties in proteins [30,32–35], and this terminology has since been widely accepted and adopted. For example, Simon et al. state that "The covalent attachment of the NO group to sulfhydryl residues in proteins is defined as *S*-nitrosylation. General NO attachment to nucleophilic centers is referred to as nitrosation" [36]. In fact, almost all the studies cited in this review also use *S*-nitrosylation to describe the modification of protein thiols by NO. Therefore, what we refer to as 'S-nitrosation' in this review (the process of nitrosation of protein thiols per the aforementioned biochemical reactions) is referred to as *S*-nitrosylation by several other groups. We submit to the reader that by definition, nitrosylation refers to the addition of a nitrosyl group to metal centers of proteins, whereas addition of NO to sulfhydryl groups is termed nitrosation.

1.1. Nitrosothiols - implications for protein S-nitrosation

In *S*-nitrosated proteins (RSNOs), the coupled NO moiety may be derived from free NO radicals in the system, one or more NOS species, metal-NO complexes or other RSNOs. For example, NO reactivity has been shown to rely heavily on the availability of oxygen and its intermediaries. This dominant metal-independent mechanism under biological conditions involves NO reacting with superoxide to generate peroxynitrite ion, which can mediate *S*-nitrosation of proteins indirectly through the formation of nitrogen dioxide. This occurs via the reaction of nitrogen dioxide with thiol resulting in one-electron oxidation to thiyl radical, which in turn rapidly reacts with free NO, producing nitrosothiols [37]. It is to be noted that in biological systems, metal-independent mechanisms involving nitrogen dioxide-mediated nitrosation (depicted in Fig. 2) are unlikely to occur due to relative preference of nitrogen dioxide for cellular targets such as glutathione [38]. In some cases, proteins may serve as substrates for transnitrosation, which involves the transfer of the SNO group from an *S*-nitrosated protein to the substrate protein. Several substrate proteins including hemoglobin (AE1 nitrosylase), caspase-3 (XIAP nitrosylase) and thioredoxin-1 (procaspase-3 nitrosylase) undergo such modification [39]. Relatively stable *S*-nitrosated molecules, such as *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylpenicillinamine (SNAP) may also directly or indirectly (SNAP is not a NO donor but can still nitrosate substrates) transnitrosate substrate cysteines [40]. The interaction between NO and oxygen also leads to the formation of intermediaries such as dinitrogen tetroxide and dinitrogen trioxide. They are capable of transferring nitrosonium ions to substrate proteins, leading to the formation of

RSNOs (although this does not occur physiologically since nitrosonium ions do not exist in aqueous solutions due to its instantaneous reaction with water).

In metal-containing proteins such as hemoglobin, nitrophorin, cytochrome P450 and albumin, NO has been shown to form stable metal-nitrosyl intermediary with the free metal ion (typically iron or copper). Such metal-nitrosyl complexes *S*-nitrosate the cysteine thiol groups of the parent metal-containing proteins, and represent the major mechanism of nitrosothiol formation inside the cell, catalyzed either by transition metal pools of chelatable iron or through cytochrome-*c* [41–43]. On the other hand, “reductase” enzymes, such as GSNO reductase, alcohol dehydrogenase III, carbonyl reductase and thioredoxin-1, may catalyze the removal of SNO groups from substrate proteins, leading to protein denitrosation [39].

Of the several factors that determine substrate specificity, the protein thiol electrostatic microenvironment is probably the most important in determining the specific cysteine thiols that may be modified by addition of a nitroso group [39]. A number of studies now show that proteins undergoing *S*-nitrosation demonstrate the presence of polar residues immediately following acidic or basic residues immediately preceding their reactive cysteines [39]. Thus, the acid-base motif surrounding a cysteine may serve as a consensus sequence for determination of cysteine susceptibility to either addition or removal of a nitroso group. In addition, local hydrophobicity also enhances *S*-nitrosation mainly due to enhanced solubility of NO and its intermediaries in such environments. Likewise, the presence of metal ions such as copper or calcium may also help catalyze the addition of nitroso groups to cysteine residues. Finally, the proximity of redox centers, that have relative abundance of oxygen and ROS, and the presence of NO-generating systems, such as NOS, have an obvious effect on the degree of *S*-nitrosation of substrate proteins [30,44]. Although various molecules that play an important role in catalysis of this process have been identified, specific enzymes that exclusively mediate *S*-nitrosation have not yet been fully characterized [45].

Not only does *S*-nitrosation directly affect function of the substrate protein, it also impacts its stability. Most proteins undergo recycling through the ubiquitin-proteasomal pathway, and this mechanism is routinely used by the cell for post-translational termination of the effects of these proteins [46,47]. *S*-nitrosation of several proteins, including iron regulatory protein 2 (IRP2), X-linked IAP (XIAP), Bcl-2, cellular FLICE inhibitory protein (c-FLIP), the nuclear factor- κ B (NF- κ B) family and several other can directly impact (either positively or negatively) its ubiquitination and subsequent degradation through the proteasomal pathway in various conditions including cancer, neuromuscular diseases and immune function [48–50]. Several proteins within the proteasomal machinery itself including several of the ubiquitin ligases are susceptible to *S*-nitrosation-mediated regulation, suggesting multifarious direct and indirect effects of *S*-nitrosation on protein function within the cell [48].

In recent years, specific NO-releasing chemosensitizing agents have been developed that drive tumor cell death through modulation of *S*-nitrosation of specific substrate proteins. NO-releasing agents such as NO-NSAIDs (non-steroidal anti-inflammatory drugs)

including m-NO-aspirin, NONO-aspirin and NO-naproxen and the diazeniumdiolate class of nitric oxide prodrugs have demonstrated potent anti-cancer activity in a number of tumor models [51–53]. Substrate proteins such as NF- κ B, caspase-3 and β catenin are targeted for S-nitrosation, leading to increased apoptotic death in tumor cells [52].

1.2. Protein S-nitrosation and cell death

Homeostasis in tissues is maintained by a constant balance between cell survival and cell death signals, which are predominantly initiated and maintained by relatively distinct protein families in specific cellular compartments. During both physiological and pathological situations, imbalances of these signaling responses result in either tissue growth or tissue deterioration. An increase in survival signals and a decrease in death signals will promote cell and tissue growth, but sustained growth signals often lead to pathological conditions such as cancer. Conversely, increased death signals, decreased survival signals will lead to tissue destruction. Both processes occur constantly in order to maintain cellular homeostasis and to discard unhealthy tissue. Physiological cell death typically occurs through a process known as apoptosis, characterized by the shrinking of cells, followed by membrane blebbing and DNA condensation and fragmentation [54]. Apoptosis is a complex phenomenon that involves a myriad of proteins, with a family of cysteine proteases known as caspases playing a central role [54]. Apoptosis may be initiated either intracellularly (the intrinsic pathway), typically through RNOS produced due to cellular stress, or extracellularly (the extrinsic pathway), by binding of soluble and insoluble factors to “death receptors” on the cell surface [55] (Fig. 3). Although these pathways may be triggered at distinct sites, the signaling mechanisms eventually converge, leading to activation of effector caspases and subsequent cell death. Activation of the intrinsic pathway proceeds by the permeabilization of the outer membrane of cellular mitochondria, leading to influx of members of the Bcl-2 family from the cytosol and release of mitochondrial cytochrome-*c*. This release triggers the formation of the apoptosome, which facilitates the enzymatic activation of caspase-9 [56,57], which subsequently activates executor caspases leading to apoptosis. The extrinsic pathway involves binding of death-inducing ligands such as Fas ligand (FasL) and tumor necrosis factor- α (TNF- α) to their respective receptors, resulting in the recruitment and activation of caspase-8 to the death-inducing signaling complex (DISC) by the Fas-associated death-domain (FADD)/TNFR1-associated death domain protein (TRADD) [58–61]. Activated caspase-8 leads to apoptosis directly through effector caspase-3. This can also occur indirectly through cross talk with caspase-9 via Bid protein belonging to the Bcl-2-family of proteins. Similarly, TNF-related apoptosis-inducing ligand (TRAIL) also induces cell death by engaging the extrinsic pathway through death receptors, leading to the formation of the DISC, but TRAIL can augment this effect by engaging the intrinsic pathways of apoptosis as well [62].

Apoptosis may also be precipitated due to loss of contact between a cell and its extracellular matrix environment, which is a process called anoikis [63,64]. Cells may also undergo death by a process known as autophagy, whereby cells may trigger the turnover of several organelles and proteins in a general fashion, or selectively discard specific organelles, such as mitochondria or endoplasmic reticulum. With autophagy, several intracellular organelles

and parts of the cytoplasm are sequestered within multi-membraned vacuoles called autophagosomes, which are subsequently degraded by the lysosome [65,66].

Several of the regulatory proteins previously mentioned are shared by apoptosis, autophagy and anoikis [66]. All of these intracellular processes have been shown to be sensitive to changes in intracellular NO levels, and are directly modulated in both physiological and pathological conditions through the process of *S*-nitrosation [67,68].

Most studies that delve into the mechanisms of action of NO in the context of cell death or cell survival do so by modulation of NO levels either using molecular inhibitors (siRNA against specific NOS species) or by using pharmacological inhibitors (including arginine-based inhibitors, nitronyl nitroxides, aminoguanidine) and donors (including organic nitrates, diazeniumdiolates, *S*-nitrosothiols, NO-hybrid drugs) of NO [69–72]. However, the cellular response to NO modulation depends on at least two important parameters - the type of cell systems being studied (and the differences may be based upon both the cell type or pathology and the intracellular proteins involved) or on the specific type of donor or inhibitor being used. Both factors may have significant bearing on both the specificity of the substrate proteins that may be affected as well as the overall response that might be observed. For example, as summarized below, our group has shown previously that using the NO donor dipropyleneetriamine (DPTA) NONOate in lung cancer cells caused *S*-nitrosation-mediated stabilization of the anti-apoptotic protein Bcl-2, leading to cell survival. On the other hand, the NO donor diethylenetriamine (DETA) NONOate bound and inhibited the transcriptional repression of death receptor DR5 by Yin Yang 1, leading to increased apoptosis in prostate cancer cells. Interestingly, DETA NONOate has been shown to exert both neuroprotective effects in cortical neurons, and promotes arteriogenesis [73,74]. There are several such examples in addition to what has been summarized below, and for the many instances demonstrating a protective role for NO in terms of overcoming apoptosis and promoting survival, there are also reports that clearly reveal a pro-death effect of increasing intracellular NO. Although this has brought about its share of controversy, there remains no doubt that NO plays a crucial role in mediating both cell death and cell survival pathways, and overall homeostasis.

In this review, we will specifically focus on literature that provides demonstrable evidence of protein *S*-nitrosation in response to NO, and its resulting effects on cellular homeostasis. It is to be noted that while NO-mediated *S*-nitrosation may drive cell survival in some cases, it also directs cells to undergo cell death. As alluded to earlier, these seemingly contradictory effects are due to the differences in target proteins that are being *S*-nitrosated, the cellular model and the specific type of modulator being used. We will discuss some of the key proteins (Table 1) related to cell death that serve as substrates for *S*-nitrosation in the section below.

1.2.1. Caspases—Caspases are key regulators of apoptotic cell death, and are triggered both in the extrinsic and intrinsic pathways [102]. Initiation of cell death occurs by the enzymatic activation of initiator caspase-8 and -9 generated by cleavage of their proenzyme forms. The mature enzymes trigger intracellular cascades leading to activation of effector caspases such as caspase-3, -6 and -7, and eventually cell death. Since these proteins are

ubiquitously expressed in various tissue types and their activation is irreversible (and therefore a marker for apoptosis). Resting cells have been found to constitutively *S*-nitrosate caspase-3 and -9, which are subsequently denitrosated following treatment with death ligands such as FasL and TNF- α [103]. For example, Maejima et al. demonstrated that NO plays a protective role against doxycycline-induced apoptosis in cardiomyocytes. Specifically, treatment with NO donors protected cardiomyocytes from undergoing doxycycline-induced apoptosis due to *S*-nitrosation of caspase-3, leading to suppression of its activity and an inhibition of cell death [75]. Multiple groups have demonstrated that *S*-nitrosation of procaspase-9 prevents its cleavage and subsequent activation, thereby protecting cells from caspase-dependent apoptosis in human colon adenocarcinoma cells [77]. Likewise, Kim et al. demonstrated that nitric oxide protects rat hepatocytes from TNF- α -induced apoptosis by increased *S*-nitrosation of Caspase 8, preventing the activation of the proapoptotic protein Bid, and release of cytochrome-*c* from the mitochondria [76]. Thus, *S*-nitrosation seems to have a repressive function on caspases in general, leading to an overall cytoprotective effect in tissue.

1.2.2. c-FLIP—Cellular FLICE-like inhibitory protein (c-FLIP) is an important regulator of the extrinsic death-receptor mediated pathway of apoptosis. It was first identified as an anti-apoptotic protein that was overexpressed in several tumors, and allowed escape of tumor cells from *in vivo* immune surveillance [104–106]. At high levels, c-FLIP forms inactive heterodimers with procaspase-8 at the DISC, thereby preventing enzymatic activation of caspases and rendering cancer cells resistant to death. Down-regulation of c-FLIP is sufficient to sensitize cancer cells to FasL-mediated apoptosis. Degradation of c-FLIP is mediated through the ubiquitin-proteasomal pathway of protein degradation, where select ϵ -NH₂ groups of lysine residues in the substrate proteins are ubiquitinated, initiating its proteasomal degradation [107,108]. However, studies demonstrate a pro-apoptotic role for c-FLIP at extremely low physiological levels, where it seems to facilitate the proteolytic cleavage of procaspase-8, suggesting a dichotomous role for c-FLIP [109]. Chanvorachote et al. demonstrated that, upon treatment with NO donors in the presence of FasL, c-FLIP is *S*-nitrosated at Cys-254 and Cys-259 in its caspase-like domain, leading to increased c-FLIP levels. The resistance to FasL-mediated inhibition of c-FLIP resulted from *S*-nitrosation interfering with the ubiquitination and proteasomal degradation of c-FLIP, thereby stabilizing the protein and causing sustained anti-apoptotic effects [79].

1.2.3. Bcl-2—B-cell lymphoma-2 (Bcl-2) is an important apoptosis-regulatory protein of the intrinsic mitochondrial death pathway [56]. Bcl-2 is an anti-apoptotic protein that inhibits death in several ways, including the formation of heterodimers with pro-apoptotic proteins such as Bax, regulation of the mitochondrial transmembrane potential, and inhibition of cytochrome *c* release [110,111]. Bcl-2 is known to be overexpressed in almost all malignant cells [112–114]. An overexpression of Bcl-2 leads to increased resistance to apoptotic cell death induced by various carcinogens. Since NO is also found to be overexpressed in several cancers, our group investigated the potential cross talk between NO and Bcl-2 in normal and cancer lung epithelial cells. We reported that *S*-nitrosation of Bcl-2 at Cys-158 and Cys-229 was increased in cells exposed to NO [78]. Furthermore, increased Bcl-2 *S*-nitrosation led to its stabilization by preventing its ubiquitination and subsequent

proteasomal degradation in a manner similar to c-FLIP. Mutation of its reactive cysteines led to degradation of Bcl-2 and increased susceptibility of cells to apoptosis [115–117].

1.2.4. XIAP and parkin—X-linked inhibitor of apoptosis (XIAP) belongs to the family of ‘inhibitor of apoptosis proteins’ (IAPs) that is highly conserved in vertebrates [118]. They regulate cell survival through the binding via baculoviral IAP repeat (BIR) motifs and inhibition of executioner caspases such as caspase-3. Not only is XIAP found to be upregulated in a number of tumors, it is also found to play a neuroprotective role in the progression of neurodegenerative disorders that are sensitive to nitrosative stress, such as Parkinson's disease. An important mechanism for neuroprotection in Parkinson's disease involves upregulation of the protective protein parkin, which aids in the degradation of unwanted toxins that contribute to the progression of the disease [119]. Studies from the Dawson laboratory at Johns Hopkins demonstrated that both parkin and XIAP are *S*-nitrosated under periods of neurological nitrosative stress. It was reported that *S*-nitrosation of parkin leads to inhibition of its E3 ligase activity that is critical for the degradation and disposal of toxic substances in neurons through the ubiquitin-proteasomal pathway. This eventually leads to the accumulation of these substances and progression of disease [82]. A similar result was found by the Lipton group, who demonstrated that although *S*-nitrosation of parkin initially increases its activity, sustained *S*-nitrosation inhibits its E3 ligase activity, leading to increased protein aggregation resembling Lewy bodies [83]. *S*-nitrosation of XIAP on its BIP domain, on the other hand, leads to an inability to inhibit caspase-3, allowing for caspase-mediated cell death in neuronal cells [80]. Nakamura et al. also found a similar result in neuronal cells, wherein *S*-nitrosation of the RING domain of XIAP led to decreased E3 ligase activity and increased apoptosis [81]. Thus, nitrosative stress has multiple effects on neuronal cells, and these effects are mediated through *S*-nitrosation.

1.2.5. Fas—Fas is a transmembrane receptor in the TNF superfamily that binds death ligands and induces apoptosis through the extrinsic pathway of apoptosis, both in normal and cancer cells [120]. Although several proteins such as caspases and c-FLIP directly influence Fas-mediated death through intracellular pathways, post-translational modifications such as palmitoylation and *S*-glutathionylation can also influence Fas function [121]. While not well understood, antitumor agents such as resveratrol and cisplatin can influence the clustering of Fas in lipid rafts, which is a crucial step in determining Fas-mediated apoptosis. Leon-Bollotte et al. demonstrated that iNOS can have a direct impact on the translocation of Fas to lipid rafts, leading to apoptosis in colon cancer cells [84]. The NO-mediated translocation was stimulated due to the *S*-nitrosation of both Cys-199 and Cys-304 in Fas. Domain cysteine mutants showed that mutation of Cys-304 but not Cys-199 led to a decrease in NO-mediated translocation of Fas to rafts, affected the formation of the DISC, and led to a decrease in FasL-induced apoptosis. In addition, direct *S*-nitrosation of the transcriptional repressor Yin Yang 1 (YY1) led to inhibition of its DNA-binding and released its downstream effector Fas from repression, causing upregulation of Fas expression and increased Fas-induced apoptosis [85]. These data suggest that *S*-nitrosation plays an important role in mediating the pro-apoptotic functions of Fas, by facilitating the cell surface initiation signals in response to death inducing ligands such as FasL. A similar finding was established by Gabran et al., who demonstrated that NO inhibits the binding

activity of Yin-Yang1 at the silencer region of the Fas promoter allowing for increased Fas gene expression in human ovarian carcinoma cells, and a consequent increase in levels of the death receptor Fas [122].

1.2.6. TRAIL receptor DR4 and DR5—In addition to the FasL and TNF- α that induce cell death through the extrinsic pathway, cells also undergo apoptosis through the binding of TRAIL (TNF-related apoptosis inducing ligand) to its receptors on the cell surface. Of the four TRAIL receptors discovered thus far, only TRAIL-R1 (DR4/Apo2) and TRAIL-R2 (DR5/Killer) are capable of inducing death upon binding their respective ligand [123,124]. Interestingly, cancer cells are found to be extremely vulnerable to TRAIL-induced apoptosis as compared to normal tissue. This suggests that these receptors might serve as markers for several types of cancers and may be targeted to achieve specificity with respect to drug delivery. However, the effect of second messengers such as nitric oxide on TRAIL receptor functionality was not addressed. Tang et al. demonstrated that the anti-cancer drug nitrosylcobalamin (NO-Cbl), a vitamin B12 analogue that generates NO and signals through the extrinsic pathway, was required to bind the DR4 receptor to exert its anti-proliferative activity [86]. The binding of NO-Cbl to DR4 receptor led to an increase in *S*-nitrosation of DR4, which was critical to transfer the pro-apoptotic stimulus generated from NO-Cbl binding to caspase-8 in the extrinsic pathway. Substitution of the critical Cys-336 residue on DR4 required for *S*-nitrosation rendered cells expressing the mutant receptor resistant to NO-Cbl action. Thus, *S*-nitrosation of DR4 seems to play an important role in mediating its pro-apoptotic role in cancer death.

In a similar manner, Huerta-Yepe et al. demonstrated that treatment of TRAIL-resistant prostate cancer cells with the NO donor DETANONOate led to the upregulation of the DR5 receptor, and sensitized the cells to TRAIL-induced apoptosis [125]. The increase in DR5 levels was due to inhibition of the transcriptional repressor YY1 and NF- κ B, which negatively regulates DR5 transcription. The NO-dependent increase in DR5 levels led to increased sensitivity of the tumor cells to TRAIL, thereby leading to increased apoptotic cell death.

1.2.7. GAPDH—Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) is an important enzyme that catalyzes the glycolysis reaction in the cell to produce both energy and carbon molecules [126]. However, recent studies have shown that GAPDH has non-metabolic functions as well, including functioning as a transcriptional regulator, and an initiator of apoptosis. The Snyder group has elucidated the role of NO and GAPDH in mediating several aspects of the apoptotic response. They first demonstrated that upon apoptotic stimuli, neuronal cells produce iNOS, which leads to the *S*-nitrosation of GAPDH at Cys-150 [87]. GAPDH then binds the E3 ubiquitin ligase Siah1 in the cytosol, from where the complex is translocated to the nucleus. Such SNO-GAPDH-dependent recruitment of Siah1 stabilizes its expression in the nucleus, leading to enhanced Siah1-mediated degradation of nuclear proteins, leading to cell death. SNO-GAPDH transnitrosylates other nuclear proteins such as sirtuin 1 (SIRT1) and histone deacetylase (HDAC2) in a Siah1-dependent manner, leading to an overall repression of gene transcription. Alternatively, a protein known as GOSPEL (GAPDH's competitor Of Siah1 Protein Enhances Life)

competes with Siah1 for the binding site, retaining it in the cytosol and preventing its apoptotic effects in neuronal cells. Interestingly, GOSPEL may also be *S*-nitrosated at Cys-47, which enhances binding with GAPDH and protects neurons from NMDA-induced neurotoxicity [88].

1.2.8. Thioredoxin—Thioredoxin (Trx) and thioredoxin reductase are two important components of the Trx system that regulate redox function inside the cell, and mediate a variety of functions related to cell proliferation and apoptosis [127]. In normal cells, one of the important functions of Trx is the redox-dependent binding, inhibition and degradation of ASK1 (Apoptosis signal-regulating kinase 1), a member of the MAPK family. Trx when oxidized releases ASK1, which then forms homodimers and binds TNFR2, leading to apoptotic cell death through both JNK and p38 MAPK. Multiple reports have shown that Trx is stably *S*-nitrosated at Cys-69, which is critically important for its repressive effect on ASK1 in endothelial cells, thus leading to cell survival [89]. In addition, *S*-nitrosation of Trx at Cys-62 and Cys-73 has also been shown to be important for its activity within the thioredoxin system, and mediates the regulation of caspase-3 [90]. The thiol specificity of the individual cysteines within Trx has been shown to be differentially regulated, and depends upon protein conformation, which may be driven by either access to the *S*-NO moiety or by the nucleophilicity of the individual cysteines themselves [128]. Interestingly, the activity of both JNK and ASK1 may be modulated by *S*-nitrosation, which adds another dimension to the complexity of NO-based signaling [101].

1.2.9. Cyclophilin D—The mitochondrial permeability transition pore (mPTP) is a nonspecific pore found in the inner mitochondrial membrane that is composed of several protein subunits, and allows for the selective movement of small molecules (less than 1500 Da) into the mitochondria [129]. The mPTP exhibits increased permeability during ischemia/reperfusion (I/R) injury in the heart, leading to increased cell death of cardiomyocytes from the loss of mitochondrial membrane potential, matrix swelling and ATP depletion. Inhibition of mPTP plays a cytoprotective role in several organs including the heart, brain and kidney. Cyclophilin D (CypD), a mitochondrial matrix protein, is an important regulator of mPTP opening, and a reduction in CypD leads to decreased mPTP activity, resulting in decreased cell death during I/R *in vivo* [130]. Studies by the Nguyen and Murphy groups at Johns Hopkins have demonstrated that CypD undergoes protein *S*-nitrosation at Cys-203, leading to inactivation of the protein. This leads to inhibition of mPTP activity, resulting in an overall reduction in cell death [91]. Thus, the cardioprotective effects of increased NO in the heart may be mediated through the *S*-nitrosation of CypD.

1.2.10. Dynamin—Angiogenesis, or new blood vessel formation, requires activation of survival signals in endothelial cells (EC) that are generally susceptible to death-induced signaling by TNF- α . NO signaling and generation of eNOS have been shown to counterbalance such TNF- α -mediated apoptosis. One such mechanism involves the dynamin family of GTPases that drive clathrin and caveolin vesicle internalization, and regulate the transduction of death signals from the cell surface (such as TNF- α) [131]. Kang-Decker et al. demonstrated that increased NO activity in the endothelial cells led to *S*-nitrosation of dynamin at both Cys-86 and Cys-607, leading to an increase in GTPase activity of dynamin

and decreased susceptibility to TNF-mediated apoptosis [92]. Dynamin domain mutants with alanine substitutions for the cysteines abolished the protection offered by NO in the face of TNF-induced apoptosis. Thus, *S*-nitrosation of dynamin contributes to survival and growth of endothelial cell, allowing them to overcome effects from death-inducing ligands.

1.2.11. Ras—The Rat sarcoma (Ras) proteins are a set of small membrane bound guanosine-nucleotide-binding G proteins that transduce signals from the cell surface to several downstream intracellular signaling cascades [132]. p21^{Ras} and other members of the Ras family act as focal points for a number of intracellular proteins including phosphoinositide 3-Kinase/protein kinase B (PI3K/Akt), extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK). The Ras family regulates several functions including growth, development and differentiation, and several studies have demonstrated that Cys-118 of p21^{Ras} may be *S*-nitrosated, leading to increased nucleotide exchange through Ras and subsequent increase in intracellular signaling [93–96]. This can play a protective role as is the case of cardiomyocytes during ischemic injury, where protective ATP-sensitive potassium channels are triggered by Ras. On the other hand, both p21^{Ras} and N-Ras *S*-nitrosation also leads to an increase in levels of the pro-apoptotic protein Bcl2/adenovirus E1B 19 kDa protein-interacting protein (BNIP3) through ERK in macrophages and T-cells respectively, which leads to increased cell death. In addition to Ras, proteins such as Akt, epidermal growth factor receptor (EGFR), and phosphatases PTB1B (protein tyrosine phosphatase 1B) and PTEN (phosphatase and tensin homolog) are also *S*-nitrosated downstream of Ras, leading to multifarious effects [133].

1.2.12. NF- κ B—Nuclear factor kappa B (NF- κ B) is an important transcription factor that regulates a number of physiological responses, including cell growth, differentiation, inflammation and death [109]. NF- κ B is a dimer consisting of two subunits, p50 (NF- κ B 1) and p65 (RelA), and is sequestered in the cytoplasm by a family of inhibitory proteins known as I- κ B. Activation of NF- κ B occurs when upstream regulatory proteins such as I- κ B kinase (IKK) phosphorylate I- κ B, leading to its ubiquitination and degradation. This allows the NF- κ B dimer to translocate to the nucleus and promote transcription of substrate genes. Studies have demonstrated that all the subunits associated with NF- κ B, including p50, p65, I- κ B and IKK may be *S*-nitrosated at specific cysteine residues, suggesting multiple points of NF- κ B regulation [98]. *S*-nitrosation at Cys-179 is sufficient to significantly impair the capacity of both IKK α and IKK β to phosphorylate I- κ B, thereby preventing its degradation. As a result, NF- κ B is retained in the cytoplasm, which reduces its transcription ability. Importantly, subsequent phosphorylation of IKK by TNF- α treatment could not overcome the inhibitory effect of *S*-nitrosation. Likewise, *S*-nitrosation of both p50 and p65 subunits of NF- κ B at Cys-60 and Cys-38 respectively caused a strong and significant decrease in the transcription of NF- κ B-dependent genes [97]. For example, *S*-nitrosation of p50 and p65 subunits has also shown to regulate various targeted gene products such as ASK1, p21^{Ras} and Jnk1 (discussed below) [98]. Thus, such repressive signals would play an important role in determining the transduction of NF- κ B-dependent signaling pathways that involve production of NO and increased activity of NOS.

1.2.13. Jnk/SAPK—The MAPK family of kinases is composed of several components, including the MAPK isoforms ERK, p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase (Jnk/SAPK). Jnk is strongly activated due to various stresses including radiation, metabolic inhibitors and proinflammatory cytokines such as TNF- α [134]. Activation of Jnk leads to its translocation to the nucleus, where it activates downstream transcription factors and cell cycle regulation proteins including p53, ATF-2, Elk1 and c-Jun, leading to effects such as growth, differentiation, apoptosis and autophagy. Park et al. reported that exposure of macrophages to pro-inflammatory cytokines such as interferon gamma (INF- γ) led to an increase in NO production, leading to suppression of Jnk activity [100]. This suppression was caused due to the S-nitrosation of its CyS-116 residue, leading to impaired activation of c-Jun. Recently, Sarkar et al. demonstrated that NO has a potent inhibitory effect on the formation of autophagosome, primarily due to the S-nitrosation of Jnk and IKK- β [99]. Exposure of neuronal cells to NO led to increased S-nitrosation of Jnk, which decreased the interaction of Bcl-2-dependent formation of the Beclin1-hVps34 complex that is required for the formation of the autophagosome. Thus NO may exert both neurotoxic effects through activation S-nitrosation of XIAP and Parkin (activation of apoptosis - see above) or neuroprotective effects through S-nitrosation of Jnk (inhibition of autophagy).

1.3. Concluding remarks

S-nitrosation has come a long way, from being considered as just an artifact when first reported, to now gaining widespread acceptance as a significant post-translational modification that may be as powerful as protein phosphorylation and ubiquitination. Evidence in the literature has demonstrated time and again that S-nitrosation of cysteines is controlled in a very specific manner and can have multifarious effects depending on the innate function of the substrate proteins (Fig. 4). It has shown to modulate a variety of responses in various organs and tissues including heart, lung, liver, brain and the vascular system, and can modulate responses in a wide variety of situations. S-nitrosation offers an important mode for control of the functionality of its substrate proteins, and effectively translates the effects of NO inside the cell in the context of varied cellular responses including cell growth, differentiation, metabolism and cell death.

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Fig. 1.

Synthesis of nitric oxide. Nitric oxide (NO) is synthesized by several nitric oxide synthases (NOS) that catalyze the conversion of the amino acid L-arginine to form the intermediate N-hydroxy-L-arginine as an intermediate, finally resulting in the release of L-citrulline. This process involves several important factors such as molecular oxygen and NADPH, and involves important cofactors such as flavin mononucleotide (FMN), flavin amino dinucleotide (FAD), tetrahydrobiopterin (BH4), calcium-calmodulin and enzyme-bound heme to catalyze this process.

Fig. 2.

Modes of protein S-nitrosation. Nitric oxide may lead to S-nitrosation through several processes. At low levels, NO can directly react with transition metals of proteins containing heme groups such as hemoglobin, guanylyl-cyclase, cytochrome-C oxidase, etc., which can serve as intermediate reservoirs of SNO that can be transferred to the substrate proteins. At higher concentration of NO (>1 mM, typically generated due to iNOS activity), it can react with either molecular oxygen or superoxide, leading to intermediates such as peroxynitrite, dinitrogen trioxide (N_2O_3) and dinitrogen tetroxide (N_2O_4) that can cause protein S-nitrosation. Alternatively, proteins such as glutathione (GSH) may serve as chaperones by forming S-nitrosoglutathione (GSNO) that can directly transnitrosate substrate proteins.

Fig. 3.

Pathways of apoptosis: In response to nitrosative stress either by activation of death receptors (extrinsic) or through the initiation of genotoxic insults that increase pro-apoptotic molecules such as p53 and Bax, the mitochondrial membrane becomes permeable, releasing cytochrome c. Proteins such as Apaf1 and caspase-9 are activated, resulting in the formation of a death complex or apoptosome. Formation of the apoptosome stimulates the activation of effector caspases such as caspase-3, -6 and -7, leading to apoptotic cell death. This pathway may also be stimulated extrinsically through the formation of truncated Bid (tBid), which transduces these signals to cause increased permeabilization of the mitochondrion. On the other hand, in response to external nitrosative stressor stimulated through binding of death receptors such as Fas/DR4/TNFR to their respective ligands, proteins such as TRAF and FADD/TRADD are recruited to the receptor to form a death-inducing signaling complex. Caspase-8 is recruited to the DISC, leading to its processing and subsequent activation of effector caspases, leading to apoptosis. In addition, Bid is also activated, which transduces these death signals to the intrinsic pathway. Proteins that have a protective effect (pro-survival) upon *S*-nitrosation are highlighted in green, whereas those that have pro-apoptotic effects upon *S*-nitrosation are highlighted in red. Proteins that have been reported to have dual effects are indicated in orange.

Fig. 4.

The balance of life and death. Proteins that regulate cell survival and cell death pathways may be differentially modulated, leading to either cell death (via apoptosis or autophagy) or survival. Thus, a constant balance needs to be maintained to achieve cellular homeostasis.

Table 1

S-nitrosation targets.

Protein	Cellular function	S-NO site	NOS species	Functional Effect	Effect	Ref
Caspase-3	Apoptosis induction	Cys-163	iNOS	Decreased enzymatic activity	Survival	[75]
Caspase-8	Apoptosis; Activation of caspase-3	Cys-287	iNOS	Decreased enzymatic activity and apoptosis	Survival	[76]
Caspase-9	Apoptosis; Activation of caspase-3	Cys-287	iNOS	Decreased enzymatic activity	Survival	[77]
Bcl-2	Cytochrome c inhibition	Cys-158 and Cys-229	iNOS	Inhibition of Bcl-2 degradation	Survival	[78]
c-FLIP	Caspase-8 inhibition	Cys-254 and Cys-259	iNOS	Inhibition of c-FLIP degradation	Survival	[79]
XIAP	Apoptosis inhibition	Multiple cysteines in its BIR domain	nNOS	Increase in caspase-3 activity	Death	[80,81]
Parkin	Ubiquitin E3-ligase activity	Multiple cysteines in its IBR domain	iNOS and nNOS	Accumulation of toxins	Death	[82,83]
Fas	Apoptosis induction	Cys-199 and Cys-304	iNOS	Accumulation of receptors in lipid rafts	Death	[84,85]
TRAIL Receptor DR4	Apoptosis induction	Cys-336	iNOS	Increased sensitivity to cell death	Death	[86]
GAPDH	Glycolysis; Apoptosis	Cys-150	iNOS and nNOS	Increased binding to Siah1 and nuclear translocation	Death	[87]
GOSPEL	Apoptosis inhibition; Siah1 inhibition	Cys-47	nNOS	Decreases GAPDH-induced apoptosis	Survival	[88]
TRX	Redox regulation; Apoptosis	Cys-62, Cys-69 and Cys-73	iNOS	Decreased apoptosis; increase in ROS	Survival	[89,90]
Cyclophilin D	Regulation of mPTP	Cys-203	iNOS	Increased opening of mPTP	Survival	[91]
Dynamin	GTPase	Cys-86 and Cys-607	iNOS	Increased PKG activation and survival	Survival	[92]
p21 ^{Ras}	GTPase	Cys-118	nNOS	Increased cellular GTPase activity	Survival	[93-96]
NF-κB p65	Transcription factor	Cys-38	iNOS	Decreased iNOS transcription	Death	[97]
NF-κB p50	Transcription factor	Cys-62	iNOS	Decreased DNA transcription	Death	[97]
IKK	Regulation of NF-κB	Cys-179	iNOS	Decreased NF-κB phosphorylation	Death	[98,99]
JNK1	Apoptosis induction	Cys-116	iNOS	Decreased activation of c-Jun and increased apoptosis	Death	[100]
Yin Yang 1	Apoptosis inhibition	Not reported	iNOS	Increased activity of Fas	Death	[85]
ASK1	Apoptosis inhibition	Cys-869	iNOS	Inhibits ASK1 activity and binding to MKK-3 and -6	Survival	[101]

Note: This table, adapted from Iyer et al., summarizes some of the proteins involved in cell death that undergo S-nitrosation and have been discussed in the article. Numerous others also have been shown to undergo S-nitrosation, but have not been included as they are beyond the scope of this article in terms of involvement in cell death. Work by Stamler et al. offers an exhaustive list of proteins (some of which are also included here) that have been shown to undergo S-nitrosation. In addition, an excellent database by Zhang et al. provides a summary list of all the proteins that have been shown to be S-nitrosated, the model system that has been reported and the effects of S-nitrosation on cell function.