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Role of Nitric Oxide and Hydrogen Sulfide in Ischemic Stroke and the Emergent Epigenetic Underpinnings

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Abstract

Nitric oxide (NO) and hydrogen sulfide (H_2S) are the key gasotransmitters with an imperious role in the maintenance of cerebrovascular homeostasis. A decline in their levels contributes to endothelial dysfunction that portends ischemic stroke (IS) or cerebral ischemia/reperfusion (CI/R). Nevertheless, their exorbitant production during CI/R is associated with exacerbation of cerebrovascular injury in the post-stroke epoch. NO-producing nitric oxide synthases are implicated in IS pathology and their activity is regulated, inter alia, by various post-translational modifications and chromatin-based mechanisms. These account for heterogeneous alterations in NO production in a disease setting like IS. Interestingly, NO per se has been posited as an endogenous epigenetic modulator. Further, there is compelling evidence for an ingenious crosstalk between NO and H_2S in effecting the canonical (direct) and non-canonical (off-target collateral) functions. In this regard, NO-mediated S-nitrosylation and H_2S-mediated S-sulfhydration of specific reactive thiols in an expanding array of target proteins are the principal modalities mediating the all-pervasive influence of NO and H_2S on cell fate in an ischemic brain. An integrated stress response subsuming unfolded protein response and autophagy to cellular stressors like endoplasmic reticulum stress, in part, is entrenched in such signaling modalities that substantiate the role of NO and H_2S in priming the cells for stress response. The precis presented here provides a comprehension on the multifarious actions of NO and H_2S and their epigenetic underpinnings, their crosstalk in maintenance of cerebrovascular homeostasis, and their "Janus bifrons" effect in IS milieu together with plausible therapeutic implications.

Keywords Nitric oxide · Hydrogen sulfide · Ischemic stroke · Epigenetic · S-nitrosylation · S-sulfhydration

Introduction

Endothelial dysfunction (ED), characterized by reduced synthesis and bioavailability of endothelium-derived nitric oxide (eNO), is inextricably linked to cerebrovascular dysfunction, co-segregates with stroke-related phenotypes, and is reckoned an independent predictor of ischemic stroke (IS) or cerebral ischemia/reperfusion (CI/R) [1]. NO regulates a distinct array of activities related to brain microcirculation, cerebral vascular resistance, and neurotransmission. It modulates long-term synaptic transmission, promotes synaptogenesis and synaptic remodeling, and is virtually indispensable for preserving cerebral blood flow (CBF), reducing infarct volume and ameliorating neuronal injury in CI/R paradigm [2–4]. Yet another gaseous neurotransmitter that is imperative for physiological control of vascular function is hydrogen sulfide (H_2S), the stunted production of which contributes to ED [5]. It has a substantial role in neuromodulation and transmission, redox homeostasis, free radical neutralization, etc. [6, 7]. Its presence in brain at higher concentrations as compared to peripheral tissues (50–160 μmol/L in rat brain compared to that in peripheral blood: 0–46 μmol/L) designates it as an endogenous neural regulatory factor [7]. This alludes to its ability to rescue primary cortical neurons, astrocytes, and microglia from glutamate-induced oxidative stress (oxytosis) and excitotoxicity-induced cell death [8–10]. The commonality of NO and H_2S in contributing to the maintenance of endothelial function by promoting vasorelaxation and preventing vascular smooth muscle cell (VSMC) proliferation, platelet aggregation, and leukocyte adhesion earned them the descriptive moniker "cytoprotective endogenous modulators" of endothelial function [11–13]. Such a functional overlap is suggestive of common molecular targets and connotes a crosstalk between NO and H_2S in effecting their canonical and non-canonical functions [14–16]. Nevertheless, these gaseous neurotransmitters exhibit a "Janus bifrons" disposition wherein an

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exorbitant generation of NO and H_2S in pathological settings typified by CI/R purportedly associates with worsened I/R-induced vascular and cerebral injury [17–21].

As a crucial addendum to their expanding list of functions, recent studies have indicated that NO is a bona fide epigenetic modulator and in part presides over DNA and histone demethylation that in turn impinges on the transcriptional activity of target genes [22]. Also, genes encoding nitric oxide synthases (NOS) per se are regulated by chromatin-based mechanisms executed by a litany of epigenetic "readers" like methyl-CpG-binding protein 2 (MeCP2), "writers" (DNA methyl transferases (DNMTs), histone acetyl/methyl transferases (HA/MTs)), and "erasers" (DNA and histone demethylases, histone deacetylases) [23]. With an increasing appreciation of the involvement of epigenetic entities in mitochondrial metabolism and oxidative stress in IS, an epigenetic modulation of nitroxidative, nitrosative, and nitrative stress response (increased generation of reactive nitroxidative, nitrosative, and nitrative species, respectively, or protein adducts containing nitrogen oxide functional groups) is apposite in an ischemic brain. [22–24]. As regards H_2S, direct evidence suggestive of a causal relationship with epigenetic modifications has been sparse. The precis presented here provides an update on the multifarious actions of NO and H_2S and their epigenetic underpinnings; their crosstalk in maintenance of cerebrovascular homeostasis and their "Janus bifrons" effect in IS milieu together with plausible therapeutic ramifications.

Nitric Oxide Synthases in Ischemic Brain

NO is synthesized constitutively by endothelial nitric oxide synthase (eNOS). It mediates functional hyperemia that typically couples CBF with neuronal metabolic activity. Plummeting CBF functional thresholds in the core and penumbral regions of ischemic brain is congruent with alterations in functional hyperemia manifested during IS [25]. These promote astrocyte-endothelial uncoupling in somatosensory cortex resulting in altered sensory representations, motor, and cognitive functions [26]. Exorbitant generation of NO by other NOS isoforms, i.e., neuronal NOS (nNOS) and inducible NOS (iNOS) that differ from eNOS in rates of electron flow between oxygenase and reductase domains and hence NO production, occurs during CI/R [27]. In line with this, restoration of CBF and perfusion during early phase of post-ischemic reoxygenation is mediated by nNOS inhibition that lessens microvascular nitrative stress and an early progressive microvascular injury [28]. While nNOS activity majorly accounts for total NO production (90–95%) over eNOS (5–10%) in brain as assessed from wild-type brain homogenates, the preferential enzymatic source of NO production is reversed temporally during transient focal ischemia in cerebral vasculature [29]. Predilection for eNOS-derived NO production in post-ischemic phase over nNOS as demonstrated in nNOS (-/-) and eNOS (-/-) mice could be a compensatory maneuver to facilitate vasodilation and mitigate CI/R-induced damage [29, 30]. In line with this, studies in transgenic mice have demonstrated a decreased infarct size following knockout of nNOS and reduced neovascularization and penumbral territory in eNOS knockout models of focal CI [30, 31]. Factors contributing to the precedence of eNOS over nNOS in the post-ischemic phase include, inter alia, distinctiveness in the mode of Ca^{2+}-dependent activation of NOS isoforms (nNOS and eNOS) in situ and the occurrence of a coterie of nNOS regulatory proteins like PIN. Also, the availability of substrates (L-arginine) and cofactors (tetrahydrobiopterin) in neurons is increasingly limited compared to endothelial cells (ECs) in ischemic brain that could contribute to preferential superoxide anion ($O_2^{\cdot-}$) production. Robust NO generation in glial cells is attributed to cytokine-mediated activation of iNOS that potentiates excitotoxicity and jeopardizes neighboring neurons. Studies have indicated that glial-derived NO inflicts delayed cerebral damage following CI/R which could be contained by administration of selective iNOS inhibitor [32]. In addition, augmented iNOS expression is observed in endothelium and infiltrating neutrophils in frank infarcts caused by sustained focal ischemia [33]. Taken together, the nuanced variation in NO levels resulting from alterations in the activity of NOS isoforms aligns distinctively with the progression of CI/R-induced cerebrovascular injury. Understanding the molecular underpinnings of such trajectories, as discussed in the following sections, is essential for formulation of suitable drug targets with temporal precision to alleviate conditions like CI/R.

Modalities of Regulation of NOS Genes

NO elicits a cornucopia of diverse cellular outcomes and these signaling effects increasingly rely upon the extent of its synthesis (dose and time-dependent), cellular setting, *milieu interier*, redox status, and local oxygen concentration. The latter substantiates the observation that ED stymies the usefulness of normobaric hyperoxia in treating IS patients [34]. In addition to these extraneous factors, NOS expression and activity are regulated by panoply of discrete, site-specific, reversible, and regulatory post-translational modifications (PTMs), the prominent of which are phosphorylation of distinct serine (Ser)/threonine (Thr)/tyrosine (Tyr), deacetylation of lysine (Lys), S-nitrosylation of cysteine (Cys), and S-glutathionylation of Cys residues, respectively. The implications of such NOS-related PTMs in IS etiology are discussed as under.

Extra-Nuclear Mode of NOS Regulation

The PTMs of NOS are indispensable for regulated production of NO, their subcellular localization, and protein-protein interactions. While the list of such PTMs is exhaustive (as reviewed in detail in [35]), we confine our discussion to PTM modules of NOS regulation in relation with ischemic brain that typically experiences insurmountable affronts to brain endothelium. Binding of Ca^{2+}-calmodulin to nNOS following N-methyl-D-aspartate receptor (NMDAR) activation by an excitotoxic stimulus like glutamate elevations triggers a phosphorylation cascade bringing about phosphotidyl inositol-3-kinase (PI3K)-Akt mediated nNOS activation by Ser^{1412} phosphorylation and excessive NO production [36] (Fig. 1). As regards eNOS, decreased Ser^{1177} phosphorylation and an increased monomerization (as eNOS functions as an obligate homodimer) correlate with a marked deterioration in its function despite its increased expression in middle cerebral

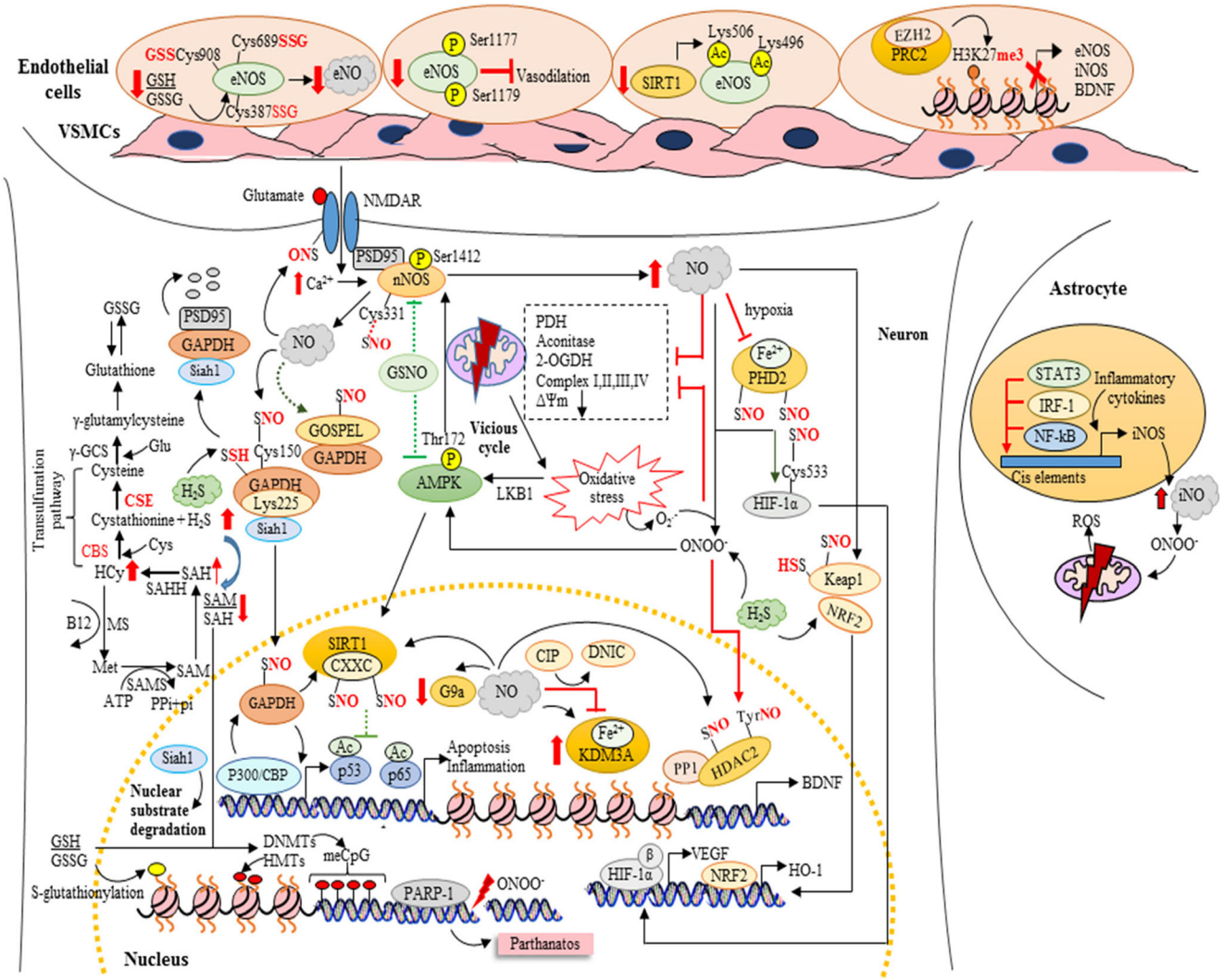

Fig. 1 Diverse aspects of the effects of NO and H_2S on the functional capacities of neurovascular unit components under physiological conditions and during cerebral ischemia/reperfusion (CI/R). The reactive thiol/sulfhydryl (-SH) residues in the target (nuclear, cytosolic, and membrane) proteins as indicated in the figure are susceptible to NO-mediated S-nitrosylation and H_2S-mediated S-sulfhydration/persulfidation forming protein adducts with -SNO (S-nitrosyl) and -SSH (S-sulfhydryl) moieties that affect their functionality and subcellular localization. These posttranslational modifications (PTMs) are accentuated or dysregulated during CI/R that elicit varied cellular sequelae. S-nitrosylated proteins like GAPDH are further capable of transnitrosylation involving transfer of -SNO residues to receptor proteins like sirtuin-1 (SIRT1) that can modulate varied cellular responses like inflammation and apoptosis. NO-producing nitric oxide synthases (neuronal/inducible/endothelial NOS) are also subject to various PTMs that affect their function depending on cellular source. NO also functions as an epigenetic modulator by sequestration of labile iron (Fe) pools and affecting the functionality of various epigenetic enzymes. H_2S can exert epigenetic effects albeit in an indirect manner through altered glutathione (GSH)/oxidized glutathione (GSSG) and S-adenosyl methionine (SAM)/S-adenosyl homocysteine (SAH) ratios that impinge on target gene expression by affecting histone S-glutathionylation and functioning of DNA/histone methyltransferases (DNMTs/HMTs) with respect to DNA/histone methylation (see text for details)

artery (MCA) core and periphery following CI/R [37]. This is linked to infarct expansion, decreased CBF, microvascular abnormalities, and ED that significantly impair tissue repair and functional recovery following IS. In addition, Ser^{1179} phosphorylation is also crucial as it correlates with an increased vascular reactivity and less severe stroke [38]. Owing to this PTM, there is reduced eNOS dependence on Ca^{2+}-calmodulin and an increased electron flux from reductase domain to oxygenase domain that augurs well for constitutive generation of NO. Corrective action in an ischemic brain to this end is enforced by rho kinase inhibitors like fasudil and hydroxyfasudil that promote Akt-dependent eNOS Ser^{1179} phosphorylation, and restore microvessel blood flow in ischemic cortex in an endothelium-dependent fashion that eventually reduces infarct size [39]. Another arrow in the quiver is a derivative of a phytophenolic compound, viz., mitochondria-targeted esculetin that enhances AMP-kinase (AMPK)-mediated eNOS phosphorylation and ensures EC survival following exposure to oxidative and atherosclerotic stressors like angiotensin-II in ApoE-/- mice. In this case, mitochondrial biogenesis is also promoted that relates with an increased activation of SIRT3, a member of class III histone deacetylases (HDACs), viz., sirtuins (that can also deacetylate non-histone proteins) [40].

Apart from phosphorylation, eNOS is also subject to Lys deacetylation by sirtuins. A putative example in the cerebral milieu is SIRT1-mediated eNOS deacetylation of Lys^{496} and Lys^{506} residues in the calmodulin-binding domain. This promotes endothelium-dependent vasorelaxation that can effectively combat cerebral hypoperfusion injury by correcting CBF [41, 42] (Fig. 1). However, inhibition of SIRT1 signaling pathway has been reported to protect against oxidative stress-induced acute endothelial injury through modulation of MAP kinases (JNK, p38MAPK, and ERK) [43]. Further, negative regulation of eNOS by HDAC1-mediated deacetylation has been reported in bovine aortic ECs (BAECs) under basal conditions and upon agonist (endothelin-1) stimulation resulting in reduced NO production [44]. Interestingly, eNOS protein expression and phosphorylation of critical Thr^{497}, Ser^{635}, and Ser^{1179} residues remain unaffected by overexpression or knockdown of HDAC1 [44]. This sparing effect could in fact be suggestive of HDAC1 being embroiled in a complex regulatory mechanism involving acetylation/deacetylation of eNOS-associated proteins and cofactor availability in intracellular compartments thereby sensitizing it to activators, cofactors, or subcellular localization. Alternatively, it could also be a strategy to desensitize eNOS to inactivators. The more apposite PTM regulating eNOS expression under conditions of elevated intracellular Ca^{2+} (that also typifies ischemic brain) in BAECs induced by agents like ionomycin seems to be Thr^{497} phosphorylation that reduces the functionality of eNOS [45]. Under such conditions, an increased Thr^{497} phosphorylation impacts NO production negatively allowing for a speculation that HDAC1-modulated eNOS expression is dispensed with. Needless to say, it would be tenable to validate this modality in cerebral context to ascertain the parallelism (if any) in eNOS regulatory mechanisms during such Ca^{2+} transients.

Pan-HDAC inhibition is increasingly viewed as a communal modality for mitigating CI/R-induced injury [46]. At this juncture, the experimental finding that HDAC1 functions as a molecular switch between neuronal survival and death may be dwelt upon to configure the relationship between HDAC1, eNOS activity, and NO production in IS. This toggling behavior of HDAC1 is dependent upon its interacting partners like HDAC3 (neurotoxic), HDRP (truncated form of HDAC9 being neuroprotective), or class IIa HDACs as shown in mouse models of Huntington's disease and tauopathic neurodegeneration [47]. The neurotoxic effect of HDAC1-HDAC3 interaction has been shown to be abrogated by activation of PI3K-Akt signaling, which indeed is the principal mode of eNOS signaling and also is the reperfusion injury salvage kinase (RISK) pathway [47]. In addition to its collusion with HDAC1, HDAC3 seems to subvert the beneficial effects of aspirin (that is also administered in IS patients) by suppressing aspirin-mediated Lys acetylation and enzyme activity of eNOS, its binding to calmodulin and eNO production [48]. Contrastingly, HDAC1-HDRP interaction is neuroprotective with an epigenetic premise. HDRP interacts with and recruits HDAC1 to c-jun promoter, reduces histone H3 acetylation, and inhibits c-JNK [49]. This seemingly overlaps with the molecular basis of atorvastatin-mediated neuroprotection of CA1 hippocampal neurons involving inhibition of JNK3 and c-jun phosphorylation with concomitant Akt phosphorylation and nNOS activation [50]. While HDAC1-HDAC3 neurotoxic rapport is supported by increased HDAC3 levels in IS brain, the conflicting tenor of the neuroprotective HDAC1-HDRP interaction as against neurotoxic HDAC9 activity needs to be resolved in ischemic milieu [51]. This alludes to amelioration of brain microvessel ED by HDAC9 inhibition as the latter is highly expressed in ischemic cerebral hemisphere and mediates effects such as inflammatory response, cellular apoptosis, and EC permeability dysfunction [51, 52]. HDAC9 also reduces the expression of tight junction proteins and suppresses autophagy. These effects articulate with the identification of HDAC9 as an epigenetic factor whose genetic variants confer an increased susceptibility to large vessel IS [53].

Yet, another distinct redox-based PTM governing NOS activity in neurons and endothelial cells is S-nitrosylation that involves covalent adduction of NO-derived nitrosyl groups to critical Cys-thiol groups residing in signature -SNO motifs that represent consensus groups of amino acids with nucleophilic residues [54–58]. Given the plethora of cellular targets and facile nature of S-nitrosylation, it is reckoned as a ubiquitous effector of NO signaling [57]. Akin to ubiquitous phosphorylation, S-nitrosylation has profound influence on protein

activity, its localization and aggregation, and protein-protein interactions [57, 58]. In line with this, eNOS can undergo reversible and transient S-nitrosylation in resting BAECs and rapid denitrosylation following agonist (VEGF)-dependent receptor-mediated activation [59]. S-nitrosylation by exogenous NO results in collapse of zinc-tetrathiolate cluster at the dimeric interface resulting in eNOS monomerization and reduced eNOS activity that could only be rescued by thioredoxin and thioredoxin-reductase system [60]. This collapse could plausibly contribute to eNOS uncoupling and consequential $O_2^{\cdot-}$ production following disruption of nitroso-redox balance due to oxidative and nitrosative stress in ischemic brain. Further, S-nitrosylation of eNOS requires membrane localization that constitutes a vital determinant of NO-dependent signaling in vascular wall as demonstrated in the arteries of wild-type mice and acylation-deficient transgenic mice [61]. On the flip side, nNOS is extensively S-nitrosylated in resting neurons and NMDAR-dependent denitrosylation of nNOS Cys^{331} by thioredoxin system occurs in the early phase of I/R that augments nNOS activity and hence NO production [62] (Fig. 1).

eNOS can also be reversibly inactivated during oxidative stress through S-glutathionylation involving a thiol–disulfide exchange with oxidized glutathione (GSSG) or reaction of oxidant-induced protein thiyl radicals with reduced glutathione (GSH) [63]. S-glutathionylation of critical cysteine $Cys^{689, 908}$ residues in the reductase domain of eNOS uncouples it (due to weakening of FAD and FMN domains), dampens eNO production, and promotes $O_2^{\cdot-}$ production resulting in impaired vasorelaxation [64] (Fig. 1). The GSSG/GSH ratio that serves as an index of oxidative stress in the cell also contributes to eNOS regulation. This is arbitrated by glutaredoxin, a GSH-dependent enzyme and a protein partner of eNOS that S-glutathionylates Cys^{382} on the surface of oxygenase domain with a net effect of reducing NO production without causing eNOS uncoupling in ECs [65]. The steering factor here is GSSG/GSH ratio, wherein a ratio of > 0.2 that is typical of tissues reeling under oxidative stress burden promotes eNOS S-glutathionylation while the same is reversed following a decrease in ratio below 0.1.

During CI/R, there is a decline in the levels of a crucial signaling molecule of NO metabolome, viz., S-nitrosoglutathione (GSNO), that is an endogenous S-nitrosothiol and a source of bioavailable NO [66]. GSNO keeps nNOS activity and cytotoxic peroxynitrite ($ONOO^-$) formation at bay by nNOS Cys^{332} nitrosylation in resting neurons (Fig. 1). It is superior to 7-nitroindazole (that causes suicidal nNOS inhibition) in conferring neuroprotection during CI/R as it enhances NO bioavailability, preserves CBF, and inhibits blood-brain barrier (BBB) disruption [66]. Exogenous GSNO supplementation invokes a neurorepair process subsuming activation of hypoxia inducible factor-1α (HIF-1α)/vascular endothelial growth factor (VEGF) pathway and enhanced platelet endothelial cell-adhesion molecule-1 levels resulting in reduced infarct volume and decreased neuronal loss [67]. It can also intercept a vicious cycle encompassing AMPK-mediated nNOS activation through Ser^{1412} phosphorylation and the downstream unbridled $ONOO^-$ production resulting in neuroprotection and preserved neurological function [67] (Fig. 1). Taken together, the loss or accentuation of NOS molecular functionality ensuing from inhibitory or stimulatory PTMs respectively can provide an explanation for the nuanced variation in stable and semistable pools of NO (inclusive of nitrite) in varied cellular compartments, biological milieu, and pathological scenarios typified by CI/R.

The Third Wave of NOS Regulation: Epigenetic Regulation of *NOS* Genes

DNA methylation and histone PTMs add another layer to regulation of *eNOS* gene activity that constitutes an epigenetic modality [22, 23, 68, 69]. In line with this, the cell-specific expression of eNOS is attributed to the dense methylation of core promoter CpG dinucleotides in non-expressing vascular cells and hypomethylation of *cis*-acting elements in healthy ECs [70]. Methylated DNA precludes the synergistic action of EC-enriched *trans*-acting factors like Sp1, Sp2, and Ets on *eNOS* promoter activity. This is corroborated by restoration of eNOS expression in HeLa cells and VSMCs following treatment with DNA methyltransferase inhibitor 5-azacytidine [70]. Regulation of eNOS activity at genic level is entrenched in an endothelium specific "histone code" that is formulated by the combinatorial association of various histone PTMs regulating the gene expression [71]. Accordingly, bulk acetylation of histones H3 and H4 on Lys (K) residues at eNOS proximal promoter underlies its activation in ECs. In conjunction with this, its transcriptional activation is also earmarked by selective enrichment of H3K4me2, me3 and H3K9, and H4K12 acetylation marks in the nucleosomes of core promoter in the ECs [68, 69, 71]. All of these are virtually lacking in non-ECs that explains the retarded eNOS repression in non-ECs by a pan-HDAC inhibitor, trichostatin [72]. An acetylation/deacetylation module also is implicated in eNOS regulation as evidenced by an interaction of HDAC1 with Sp1 binding site lying proximal to *eNOS* promoter that precludes its transcription in non-endothelial cells [72]. HDAC1 is recruited to a DNA-binding complex by MeCP2 which invokes transcriptional repression by recruiting co-repressor complexes comprising HDAC and H3K9 methyltransferases at the methylated sites [73]. Paradoxically, trichostatin downregulates *eNOS* gene expression by intercepting HDAC1-Sp1 interaction after an initial overwhelming activation. This is attributed to a putative eNOS mRNA destabilizing factor that binds to 3′UTR of eNOS

mRNA [74]. Further, under conditions of hypoxia (acute and chronic), there is decreased eNOS mRNA and protein expression. This is attributed to histone eviction during acute hypoxia resulting in absolute loss of *eNOS* activating histone PTMs [75]. During chronic hypoxia, there is restoration of histone octamers but not the histone PTMs. Reduced eNOS expression could also be ascribed to expression of an anti-sense eNOS gene, sONE, that overlaps with the 3′ end of eNOS mRNA and regulates it in a manner distinct from anti-sense gene ANRIL [76]. As hypoxia is an integral feature of CI, it would be interesting to explore the validity of these modalities of eNOS regulation in CI/R paradigm.

As regards *iNOS* gene that harbors classic *cis*-elements crucial for cytokine inducibility in its promoter, viz., NF-κB (as *iNOS* is NF-κB responsive gene), interferon-regulatory factor-1 (IRF-1), and STAT3, there is transcriptional hyporesponsiveness in cultured human ECs [77, 78]. This is attributed to heavy methylation of cytosines flanking γ-interferon-activated sites in *iNOS* promoter that interferes with NF-κB p50 binding, resulting in reduced interleukin-1β (IL-1β) inducibility and hence iNOS production [79]. Recalcitrance to cytokine inducibility of iNOS in human ECs results from reduced recruitment of RNA polymerase II at densely methylated iNOS promoter combined with differential recruitment of MeCP2, the purveyor of corepressor complexes [77, 78]. This lends credence to the proposition that iNOS-derived NO in ischemic brain is majorly elaborated from the infiltrating macrophages and microglia, the cell types in which cytokine-inducible iNOS is highly responsive. Concerning *iNOS* regulation, constitutive silencing of human *iNOS* is achieved by *trans*-acting NF-κB repressing factor that binds to a *cis*-acting negative responsive element in *iNOS* promoter as shown in HeLa cells [77]. There is also enrichment of transcriptionally repressive H3K9me2, me3 marks with a concomitant decrease in H3K9ac in human *iNOS* proximal promoter [78]. Treatment of mesangial cells with DNA methylation inhibitors or anti-sense knockdown of DNMT-3b expression and activity seemed to augment cytokine (IL1β)-modulated endogenous NO production, *iNOS* expression, and promoter activity [79]. Directed reactivation of *iNOS* can also be achieved by catalytically active thymidine DNA glycosylase, an enzyme involved in methylcytosine demethylation at promoter and CpG islands that restores responsiveness to lipopolysaccharide LPS and IFN stimuli [80]. Summing up, an erratic activation of iNOS in neurons and cerebrovascular ECs by a plausible dysregulation of the above discussed epigenetic pathways in an ischemic brain could be conjectured.

The regulation of *eNOS*, brain-derived neurotrophic factor (*BDNF*), and *iNOS* by a H3K27me3 methyl transferase, viz., enhancer of zeste homolog 2 (EZH2), could add another wrinkle to the understanding of epigenetic control of EC functional capacity during CI/R [81, 82]. EZH2, a subunit of polycomb repressor complex (PRC2), increasingly associates with *eNOS* and *BDNF* gene promoters during hypoxia-ischemia, making repressive H3K27me3 marks more abundant at these sites (within 1 kb of transcription start sites) resulting in chromatin compaction and silenced gene expression [81]. The ensuing decline in eNOS and BDNF levels during hypoxia could stem from counterpoising elevated gene (*eNOS* and *BDNF*) expression in ECs by PRC2 at genomic regions of poised genes by deposition of H3K27me3 marks. Nevertheless, this regulation modality could be offset by a transient rise in jumonji domain-containing H3K27me3 demethylase-JMJD3 during hypoxia-ischemia [81]. A commensurate rise in H3K27 acetylation or EZH2 inhibition could thereby kick-start endothelial gene expression. Counterintuitively, EZH2 inhibition by S-adenosyl homocysteine (SAH) invokes EC activation (a pathophysiological process) through NF-κB-mediated expression of pro-inflammatory phenotype [83]. SAH is generated from the methyl donor S-adenosyl-methionine (SAM) in the methionine cycle and SAM/SAH ratio is regarded as a precise indicator of methylation potential (Fig. 1). A decline in this ratio signifies hyperhomocystenemia (as would occur during CI/R), the ramifications of which include alterations in global and locus-specific DNA methylation, attendant gene expression, and vascular homeostasis as demonstrated in atherosclerosis [84, 85]. Akin to *eNOS*, EZH2 also enriches *iNOS* DNA with H3K27me3 that can repress iNOS inducibility in human ECs [82] (Fig. 1). Taken together, the reparative angiogenic potential of ECs can be harnessed by EZH2 inhibition and deposition of transcriptionally permissive H3K36me3/H3K27 acetylation marks that promote vascular regeneration or morphogenesis productive for ischemic tissue perfusion.

Nitric Oxide-Mediated Actions

The propensity of NO to react with free radicals and interact with transition metals to form metal-nitrosyl complexes stems from an inherent need to stabilize its unpaired electron. Its ability to react with the iron atom in iron-sulfur containing proteins and non-heme iron proteins defines most of its signaling repertoire [14, 22]. By reacting with the redox active pool of non-heme cellular iron called the "chelatable iron pool" (CIP) and two anions (typically thiols, "R-," like glutathione or Cys), it forms dinitrosyl-iron complex (DNIC). Quantitatively, DNICs are overwhelmingly dominant species (45–90 μM) (among NO-derived cellular adducts) compared to measurable chelatable iron and correlate with upregulated iNOS expression [86]. Under conditions of limiting oxygen concentrations, DNIC generation remains unchanged albeit reduction in NO synthesis. Thereby, DNIC formation and disappearance seem to be independent of oxygen concentration and the existence of NO generating source [86].

An upshot of DNIC formation is iron sequestration that in turn impinges on the functionality of three distinct classes of

epigenetic enzymes. These are iron, oxygen, and 2-oxoglutarate (2-OG)-dependent DNA demethylases, viz., ten-eleven translocases (TETs) that mediate active DNA demethylation, jumonji domain-containing histone demethylases (JHDMs), and HIF-prolyl hydroxylases (PHDs) [87]. Recursive action of TET 1, 2, and 3 generates a demethylated cytosine with 5-hydroxymethylcytosine (5-hmC) being an early intermediate and the most prominent among the congeners of active DNA demethylation in the brain [88, 89]. 5-hmC is associated with transcriptionally active chromatin and inhibition of TETs by NO-mediated iron sequestration correlates with skewed 5-mC/5-hmC balance that potentially alters the expression of target genes. Alongside, NO regulates the expression and activity of a JHDM, viz., KDM3A (that demethylates H3K9) by (i) direct binding to the iron atom that is coordinated by the 2-histidine-1-carboxylate facial triad in the active center of KDM3A that precludes O_2 binding, (ii) sequestering cellular iron, a cofactor for KDM3A, resulting in KDM3A inhibition, and (iii) upregulating KDM3A and tandemly downregulating H3K9 methyl transferase, viz., G9a as shown in human breast carcinoma cultures [87] (Fig. 1). Therein, an attendant global decrease in 5hmC and H3K4ac levels with a reciprocal rise in mono-, di-, and tri-methylated H3K4 and H3K9me2 levels is observed. It is posited that the enzyme (for instance KDM3A)-Fe(II)-2OG-substrate complex exhibits an increased affinity for NO rather than oxygen. Therein, it would be interesting to validate this modality in IS setting and also codify the inhibitory effect of NO over a range of physiological oxygen tensions that would plausibly have a bearing on the application of hyperbaric and normobaric hyperoxia in IS treatment. It is interesting to note that an increase in H3K9me2 levels majorly results from dampened KDM3A activity rather than an increased G9a activity [87] (Fig. 1). Additionally, cellular exposure to NO can also elevate the expression of H3K9MTs like SETDB1 and Suv39H1. In addition to KDM3A, NO has been reported to increase the expression of a host of other KDMs in breast carcinoma cultures which could supposedly compensate inhibition of JHDMs [90].

It is well established that the activity of TETs, JHDMs, and PHDs is regulated by the availability of Krebs cycle intermediates like 2-OG (activating), succinate, and fumarate (inhibitory) [91, 92]. Succinate accumulation majorly by reverse carboxylation and other auxiliary pathways is a sentinel feature of dysregulated oxidative metabolism during CI/R [93]. Further, excessive NO induces metabolic perturbations, mainly by stifling six of eight Krebs cycle enzymes by S-nitrosylation and causing energetic failure [94–96]. Notably, the critical thiol moieties of aconitase, 2-OG-dehydrogenase, and electron transport chain (ETC) complex I protein NADH dehydrogenase form SNO-adducts that are inhibitory. Pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH), succinate dehydrogenase flavoprotein A (SDH), and malate dehydrogenase (MDH) are also S-nitrosylated that in turn influence the steady-state concentrations of key Krebs cycle substrates and hence the functionality of epigenetic enzymes as discussed above [94–96] (Fig. 1). Mitochondrial proteins are also subject to S-nitrosylation by GSNO in mitochondria with an oxidized environment, which in turn is regulated by respiratory substrates like malate/glutamate in a thiol-dependent manner [97]. Low GSH, NADH, NADPH, and high GSSG, NAD^+, and $NADP^+$ characterize oxidized mitochondrial environment, a feature complicit in IS etiology. Glutamate-evoked excitotoxic injury typified by CI/R also induces mitochondrial Ca^{2+} overload that could affect the mitochondrial NO generation by mitochondrial NOS isoform (considered to be a broken cell artifact) and downstream sequelae like S-nitrosylation of target mitochondrial proteins, lowering of mitochondrial membrane potential, mitochondrial permeability transition, etc. [94]. Transient suspension of Krebs cycle and fatty acid oxidation (FAO) by S-nitrosylation of Krebs cycle and FAO enzyme complements could act as a reprieve for ischemic brain from the damaging effect of robust ROS production during reperfusion. Also, S-nitrosylation could prevent irreversible inhibition of the crucial enzymes and also inhibition by S-glutathionylation. Based on the above tenets and the influence of NO on the Krebs cycle enzyme complement, a link between NO-induced metabolic snafu and epigenetic alterations in CI/R paradigm can be anticipated.

Apart from the canonical soluble guanylyl cyclase/cyclic-GMP/protein kinase G signaling pathway, NO can effectively transduce signals to varied cellular compartments through protein S-nitrosylation [56, 57, 95]. In a two-pronged approach, S-nitrosylation can either impose (i) an extra-nuclear influence on the activation, stability, and nuclear targeting of transcription factors (TFs) by S-nitrosylating their regulatory binding partners as exemplified by HIF-1α and p53 [98, 99] or (ii) directly S-nitrosylate critical redox-sensitive Cys residues in the DNA-binding or allosteric sites of TFs as in the case of NF-κB (s-nitrosylation of conserved Cys in p50 and Rel homology domain of p65) to invoke alterations in gene expression [100, 101]. Also, S-nitrosylation- mediated alteration of epigenetic enzyme functionality could explicate the role of NO as an epigenetic modulator [22, 23, 102]. In line with this, the S-nitrosylated forms of epigenetic enzymes like PHD2, SIRT1, HDAC2, and *trans*-acting TFs have regulatory effects on cellular events as hypoxic response, apoptosis, and survival signaling, respectively, that determine IS outcome as can be comprehended from the next few sections [98–100].

S-Nitrosylation and Hypoxia

NO-mediated HIF-1α stabilization occurs during ischemia by S-nitrosylation of HIF-1α Cys^{533} that precludes an interaction

between its oxygen-dependent domain (ODD) and E3 ubiquitin ligase von Hippel-Lindau (vHL) factor [98]. Consequently, HIF-1α can translocate into the nucleus and activate hypoxia-responsive genes by dimerizing with HIF-1β, binding to transcriptional coactivators like cAMP response element binding protein (CREB)-binding protein (CBP)/p300 and hypoxia response elements in target gene promoters (Fig. 1). Another modality involves PHDs that modify the prolyl residues in the ODD of HIF-1α in an oxygen-dependent manner, thereby marking them for vHL-mediated polyubiquitination and subsequent proteasomal degradation. However during hypoxia, PHD2 inactivation by S-nitrosylation and reaction of NO with Fe(II) at the active site of the enzyme could induce HIF-1α-VEGF axis for adaptive neovascularization during reperfusion (Fig. 1) [103]. Akin to succinate, iNOS-derived NO can induce pseudo-hypoxic stabilization of HIF-1α and NF-κB by repressing PHDs [92]. Based on this and the vivid role of PHD inhibition in conferring cell survival compensation by endogenous adaptive hypoxic response during early phase of CI, the contribution of NO towards invocation of hypoxic response in ischemic brain can be substantiated [104, 105]. Also, as hypoxia is associated with global alteration of histone PTMs, it would be interesting to explore the modulatory role of NO in invoking or sorting of such histone PTMs in ischemic brain [106].

S-Nitrosylation and Synaptic Plasticity

S-nitrosylation is instrumental in propagating the ubiquitous influence of NO on cellular signaling mechanisms and the derivative cellular events [54–58, 107]. For instance, neurotrophins and other stimuli that induce an intracellular Ca^{2+} rise and resultant nNOS-derived NO accumulation in a PLC-Trk-IP3/ Ca^{2+}-calmodulin-dependent manner can affect S-nitrosylation of nuclear factors that bind to *cis*-regulatory elements [102, 108, 109]. Accordingly, in developing cortical neurons, BDNF and synaptic activity induce NO signaling-dependent expression of CREB target genes (*c-fos*, *nNOS*, and *vegf*) as demonstrated in pharmacologically manipulated neurons and nNOS knockout mice [108, 109]. This is arbitrated by nNOS-derived NO-mediated S-nitrosylation of HDAC2 causing its release from the chromatin [102, 110]. CREB-CBP then binds to CRE-containing gene promoters with coincident histone acetylation. While CREB has been implicated in many neuronal processes, including axon and dendrite growth, survival and plasticity, HDAC2 negatively regulates synaptic plasticity [111]. H3 and H4 acetylation is mediated by histone acetyl transferase (HAT) activity of CBP that promotes the assembly of transcriptional initiation complexes. This event indeed is indispensable for neuronal resistance against ischemic injury and preservation of synaptic plasticity [112]. HDAC2 blockade by NO-mediated S-nitrosylation is seemingly essential for therapeutic response to NO donors as nNOS knockout mice exhibit cognitive deficits as exemplified by contextual fear conditioning [113]. This, in part, can be rescued by increased H3 and H4 acetylation following administration of HDAC inhibitor, viz., sodium butyrate. Another putative feature is the convergence of the canonical RAS-MEK-CREB Ser^{133} phosphorylation and NO-HDAC2 signaling axes in promoting stimulus-inducible CREB binding to DNA. This affirms that distinct PTMs act in unison for transcriptional activation of target genes [95, 110].

In neurons replete with HDAC2, S-nitrosylation of two specific (regulatory) Cys $residues^{262,274}$ could plausibly exert a calibrated influence on cognitive improvement as NO disrupts the interaction between HDAC2 and the repressive complexes on the target genes like *BDNF* [109, 110]. Herein, a therapeutic strategy can be convened that involves disruption of the integrity of HDAC-containing repressive complexes without obliterating the protein function per se. A pliable strategy would be HDAC2 downregulation or eNOS restitution. Moving in this direction, HDAC2 suppression by direct inhibition, knockdown, or knockout in the peri-infarct cortex of rodents was shown to enhance cell survival in the peri-infarct area. This could promote neuroplasticity of surviving neurons and abated neuroinflammation, the consolidated effect of which was recovery of motor function [113]. Temporally, HDAC2 is overexpressed between 5 and 7 days post-stroke (which coincides with nNOS upregulation) and is associated with stroke-induced functional impairment in wild-type and HDAC2 conditional knockout mice [114]. On the flip side, the applicability of HDAC inhibitors as neuroprotective agents has a predicament of lack of specificity and adverse side effects in clinical setting [115]. Therein, eNOS restitution could prove to be a more viable recourse to achieve reduced HDAC2 influence. Further, as Ca^{2+} levels and NO modulate activity-dependent recruitment of CREB, characterizing the repressive complexes to the resolution of cell types and target gene promoters could take precedence in designing small molecule inhibitors (mimicking NO). This can plausibly avert stroke-related cognitive decline and neurodegeneration. In continuity, another interesting contender would be MeCP2 that is increasingly abundant in mature neurons and invariably occupies the methylated DNA globally [116]. It is subject to activity-dependent phosphorylation that promotes *BDNF* gene expression and dendritic growth that is contingent on elevated Ca^{2+} concentrations (that typifies ischemic brain) and calmodulin-dependent kinases [117]. It would be interesting to explore the nexus (if any) between MeCP2 and NO-dependent modulation of synaptic plasticity and long-term potentiation which are of absolute importance in post-stroke recovery.

S-Nitrosylation in Ischemic Stroke

While ambient S-nitrosylation is essential for diverse functions such as transcriptional activity, synaptic plasticity, and neuronal survival, pathological release of NO triggers aberrant S-nitrosylation [118, 119]. An aberrant S-nitrosylation of putative targets by NO and $ONOO^{\cdot-}$ is widespread during CI/R which has varied regulatory influences on gene transcriptional modules. The former event mostly is the handiwork of NO elaborated by nNOS and iNOS. Of note, the reliance of brain S-nitrosocysteine proteome on eNOS could be minimal during secondary neuronal injury owing to substantial contribution of other NOS isoforms. Exemplary instances of S-nitrosylation modulating IS pathogenesis are provided by GAPDH and matrix metalloproteinase 9 (MMP9) as discussed below.

GAPDH

Compartmentalization of NO bioactivity can be appreciated by S-nitrosylation of de novo GAPDH (unbound to glycolytic substrates) at critical Cys^{150} by nNOS/iNOS-derived NO [120] (Fig. 1). This triggers its binding to seven in absentia (Siah)1 homolog complex (an E3 ubiquitin ligase) at Lys^{225} and piggybacking into the nucleus. Acetylation of GAPDH $Lys^{117,227,251}$ by p300/CBP-associated factor (PCAF) also facilitates nuclear translocation. Once there, Siah1 mediates proteasomal degradation of nuclear substrates that spurs a cell death cascade. GAPDH accumulates in penumbral apoptotic neurons following MCAO [121]. Correspondingly, an increased GAPDH-Siah1 interaction is demonstrable in cerebellar granular neurons (CGNs) following glutamate-induced excitotoxicity. SNO-GAPDH can transnitrosylate (transfer of nitroso groups between thiols) nuclear substrates such as SIRT1, HDAC2, DNA-PK, and PARP-1 which disseminate signals of neuronal activity to apoptotic or metabolic enzyme transcriptional repertoire through p300/CBP-mediated histone acetylation [99, 102, 111, 120–122]. SNO-GAPDH-mediated SIRT1 transnitrosylation on $Cys^{387,390}$ residues in Zn^{2+} -binding CXXC motif inhibits its activity and abrogates PGC-1α deacetylation that precludes HNF-4 coactivation, gluconeogenic gene induction, and mitochondrial biogenesis. SIRT1 S-nitrosylation correlates with abundant p53 and p65 acetylation and activation of p53 and NF-κB target genes inciting pro-inflammatory and apoptotic processes [99, 118]. In a similar vein, transnitrosation of SIRT1 by exogenous GSNO was shown to release Zn^{2+} from conserved sirtuin Zn–tetrathiolate cluster, resulting in loss of α-helical structure and SIRT1 inhibition [123]. SIRT1 is also subject to GSNO-mediated S-glutathionylation that correlates with acetylation of SIRT1 substrates and apoptotic induction [124].

Nuclear signaling of NO in response to pro-apoptotic cell stressors summons neuronal apoptosis. In keeping with this, p53 modulates Siah1-GADPH axis, as Siah1 is a p53 transcriptional target and p53 upregulates GAPDH in cerebellar neurons [118, 120–123]. Nuclear GAPDH is acetylated on Lys^{160} by p300/CBP resulting in stimulation of acetylation and catalytic activity of p300/CBP which in turn activates downstream targets like p53 thereby inducing apoptosis [122] (Fig. 1). Additionally, accumulated iNOS in microglia partakes in neuronal apoptosis by enhancing GAPDH-Siah1 interaction during CI. Reciprocally, SNO-GAPDH can regulate iNOS-dependent NO production in a negative feedback loop by precluding heme insertion into iNOS and its activation in glial cells [125]. Also, iNOS induced S-nitrosylation of p65 and IKK (inhibitor of nuclear factor kappa-B kinase) dampens NF-κB activity as demonstrated in respiratory epithelial cells and macrophages and hence iNOS expression, which could be perceived as a negative feedback mechanism [100, 101].

A preemptive mechanism becomes operative during excitotoxicity that adaptively counteracts nitrosative stress. This involves disruption of NO-GAPDH-Siah cascade by GOSPEL, a cytosolic protein which when S-nitrosylated at Cys^{47} outcompetes Siah1 in binding GAPDH, thereby preventing the latter's nuclear translocation [126] (Fig. 1). GOSPEL overexpression is neuroprotective and averts glutamate-induced excitotoxicity while its depletion portends neuronal death. In an alternative mode, a nucleolar protein, viz., B23/nucleophosmin, binds GAPDH-Siah1 in a ternary complex and is transnitrosylated by SNO-GAPDH at Cys^{275} only to dislodge Siah1 from GAPDH [127]. Apart from impeding proteasomal degradation of other nuclear substrates, this reaction inhibits the self-directed ligase activity of Siah1 thereby downregulating Siah-mediated neuronal death signaling. Consistent with this, GOSPEL and B23 overexpression in cerebral cortex curtails neurotoxicity resulting from excessive NMDAR action. In this direction, deprenyl derivatives that mimic GOSPEL in binding Rossman fold in GAPDH could offer promise in exerting neuroprotective action. A diverging theme emerges at this junction when stimulated with neurotrophins like BDNF and nerve growth factor. They activate nNOS and hence NO production, causing SNO-GAPDH-Siah to bind, transnitrosylate, and degrade a H3K9 methyltransferase, viz., suppressor of variegation 3-9 homolog 1 (Suv39H1). A coexistent modification of SNO-GAPDH is methylation that promotes the same [102, 108]. An imminent reduction in H3K9 trimethylation promotes histone acetylation and transcriptional activation of neurotrophin-dependent genes related to neuronal plasticity and neurite growth (*c-fos*) in a CREB-dependent fashion. This modality seems to complement and consolidate activity-dependent CREB-mediated H3K9 acetylation following S-nitrosylation and dissociation of HDAC2 from chromatin in embryonic cortical neurons that

regulates dendritic growth and branching [108–110]. Taken together, the above-discussed signaling modules can be availed therapeutically by designing agents that enhance the stability and integrity of ternary complexes that can either sequester Siah1 or selectively degrade Suv39H1 to inhibit neuronal apoptosis and epigenetically induce dendritic outgrowth respectively following CI/R.

Matrix Metalloproteinase 9

MMP9 is another target that is selectively S-nitrosylated by NO and is acutely activated during CI/R. Colocalization of MMP9 with nNOS and iNOS in migrating cells testifies the role of NO in MMP activation [118, 128]. This principally involves S-nitrosylation of "cysteine switch" at the active site of enzymic proform thereby exposing the catalytic Zn^{2+} to substrate. This together with ROS-mediated oxidation of MMPs to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid derivatives results in unbridled activation of MMP9 which triggers anoikis, an apoptotic form of cell death [118, 128].

S-Nitrosylation and Neurogenesis

From a redox standpoint, NO as an epigenetic modulator can modulate neurogenesis and neuronal death through S-nitrosylation of myocyte-enhancement factor 2 (MEF2) family of TFs at a critical Cys inhibitory switch that prevents DNA-binding [129]. Accordingly, the formation of SNO-MEF2A abrogates neurogenesis by impinging in part on nuclear receptor-tailess signaling cascade that is essential for neurogenesis in adult hippocampus. In a similar vein, SNO-MEF2C formation promotes death of cerebrocortical neurons during an excitotoxic insult in part by abrogating the anti-apoptotic Bcl-xl pathway. Mechanistically, disrupting nNOS-PSD-95 coupling (that negatively controls regenerative repair) in neurons promotes a litany of regenerative features, viz., neuronal differentiation of NSCs, migration of newborn cells into the injured area, neurite growth, and dendritic spine formation of mature neurons in ischemic brain of rats [130].

Crosstalk Between S-Nitrosylation and Other PTMs-Pleiotropic Effects

Given the expanse of S-nitrosylation in cells with around 3000 S-nitrosylated proteins, its holistic influence on the signaling mechanisms is reliant on its crosstalk with other PTMs like phosphorylation and ubiquitylation that constitutes a central operational modality [95, 119]. This can be exemplified by inhibition of phosphatase and tensin homolog (PTEN) by S-nitrosylation of Cys^{83} following Akt activation, resulting in PTEN getting marked for degradation by NEDD4-1 via the ubiquitin-proteasome system (UPS) [131, 132]. PTEN dephosphorylates phosphatidyl inositol-3,4,5-triphosphate (PIP3) and is a counter-regulatory enzyme of the pro-survival PI3K-Akt pathway in neurons [131]. It plays a critical role in the functional coupling between JNK and PI3K-Akt pathways in ischemic brain injury [132]. S-nitrosylation in conjunction with NO-mediated enhanced ubiquitination regulates both the lipid phosphatase activity and protein stability of PTEN in the hippocampal CA1 region of rats subjected to transient global CI [133]. This is distinct from H_2O_2-dependent PTEN oxidation that dampens its activity and does not affect the integrity. In addition, Akt per se is subject to S-nitrosylation that reduces it activity and impedes PI3K-Akt signaling [132]. By doing so, it can circumvent or nearly obviate the need for the inhibitory effect of PTEN on PI3K-Akt pathway. It is important to note that the magnitude of NO production determines the substrate for S-nitrosylation. Low levels of NO prefer PTEN S-nitrosylation while exorbitant quantities prefer Akt as substrate [131]. Therein, it would be appealing to formulate a tenable therapeutic target by unequivocally deciphering as to which arm of S-nitrosylation is activated during excitotoxicity (as PTEN has been reported to be S-nitrosylated in CI/R) in ischemic brain with temporal precision in view of the dichotomous outcome of neuroprotection or neurotoxicity. Apart from this, it would be interesting to explore the effect of epigenetic silencing of PTEN through promoter methylation as demonstrated in some cancers and the plausibility of any influence on S-nitrosylation in effecting Akt derepression [134].

Another pertinent example of the proposed crosstalk in the neuronal milieu is provided by the anti-phasic relationship between S-nitrosylation and S-palmitoylation that articulates the synaptic clustering of post-synaptic density protein-95 (PSD95) (scaffolding the glutamatergic receptors) with NMDAR and nNOS in the event of glutamate excitotoxicity [135]. A dynamic reciprocity operates wherein S-palmitoylation of $Cys^{3,5}$ of PSD95 that favors synaptic localization of PSD-95 is dampened by nNOS activation following NMDAR activation. S-nitrosylation of NMDAR on Cys^{399} of NR2 subunit reduces the permeability of Ca^{2+} and hence its influx in a bid to constrain the damage elicited by elevated intracellular Ca^{2+} during NMDAR-mediated glutamate-dependent excitotoxicity [136]. S-nitrosylation of $Cys^{744,798}$ of NR2 subunit is also implicated in stroke and hypoxia which could plausibly have an additional suppressive effect on S-palmitoylation [136, 137]. Further, interactions of NO with other histone PTMs like ISGylation and SUMOylation have been postulated to impinge on signaling events and need to be explored in ischemic brain [95]. Recently, S-nitrosylation has also been shown to collaborate with yet another PTM, viz., farnesylation and modulate synaptic transmission by controlling neurotransmitter

release [138]. Taken together, S-nitrosylation constitutes a tractable therapeutic option that engenders improved astrocyte functioning and neuronal survival.

Hydrogen Sulfide

The production of H_2S in the vasculature from L-cysteine is catalyzed by two pyridoxal 5′-phosphate-dependent enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), and by the combined action of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST). In the brain, H_2S generation is majorly driven by CBS and 3-MST. In an IS setting, most of the H_2S-synthesizing activity is ascribed to CBS in the cerebral cortex [7, 19]. CBS is increasingly expressed in astrocytes and microglia, while 3-MST is the predominant H_2S-synthesizing enzyme in neurons [139–141]. CBS inhibition or knockout significantly affects H_2S production in the brain [6, 7, 139]. In line with this, CBS expression and H_2S synthesis were shown to decline in an ischemic brain that impelled microglial polarization to pro-inflammatory phenotype [142]. This effect could be counteracted by CBS overexpression or exogenous H_2S supplementation that promoted microglial polarization to anti-inflammatory phenotype in an AMPK-dependent manner. As regards 3-MST, it has been posited to less likely contribute to endogenous H_2S production as it is downregulated in cortex and striatum following permanent MCAO and also in astroglia in an ischemic brain [143]. The multifaceted contribution of H_2S to the maintenance of cerebrovascular homeostasis is well appreciated [5–9]. Its anti-apoptotic, anti-inflammatory, and anti-oxidant role in mitigating secondary neuronal injury from CI/R is pivotal and has been reviewed in detail in [7, 8]. An alluring feature of the anti-oxidative action of H_2S is restoration of favorable ratio of GSSG/GSH through increased activation of γ-glutamyl cysteine synthase (GCS) that catalyzes the conversion of Cys to γ-glutamylcysteine, the precursor for GSH [7–9] (Fig. 1). In a concerted action, there is also enhanced cystine (precursor for cysteine) transport through cystine/glutamate antiporter (that is otherwise diminished during IS) thereby robustly generating GSH. Homocysteine (Hcy) can be regarded as a pivot for H_2S production by virtue of its ability to act as a branch point for methionine passage either to cysteine through transsulfuration pathway via pyridoxal phosphate-dependent CBS and CSE to cystathionine and cysteine or remethylation that is contingent on betaine, folate, or vitamin B_{12} (cobalamin) availability (Fig. 1). Hcy accumulation and the availability of methylation donors like SAM also seem to impact the predominance of either of the pathways (remethylation to methionine or transsulfuration) and redox status of cell by impinging on GSH/GSSG ratios [139]. It is understood that oxidative stress effectively skews the GSH/GSSG and SAM/SAH ratios. GSH metabolism can induce epigenetic alterations through substrate/cofactor availability (SAM/SAH, GSH/GSSG ratios). Given the ability of GSH to function as a histone modifier (as it induces histone H3 S-glutathionylation), it would be appropriate to consider H_2S as an epigenetic modulator albeit in an indirect manner [144]. In relation with oxidative stress, H_2S is also mitoprotective as it averts energy failure and upregulates superoxide dismutase (SOD) expression with a reciprocal downregulation of cytochrome oxidase [9]. Paradoxically, both inhibition of H_2S production and therapeutic H_2S donation seem to substantially influence the disease progression in various animal models of inflammation, reperfusion injury, and circulatory shock [145].

H_2S Donors in Ischemic Brain

H_2S donors like 5-(4-methoxyphenyl)-1,2-dithiole-3-thione anethole dithiolethione (ADT) and sodium hydrosulfide (NaHS) have been shown to prevent ischemic neuronal death and BBB disruption following MCAO [10]. This occurs by intercepting pro-inflammatory NF-κB axis, attenuating MMP9 and NADPH oxidase isoform 4 (NOX4)-derived ROS production, and preserving the expression of tight junction proteins in ischemic brain. Administration of NaHS prior to I/R in acute stroke model has been shown to confer neuroprotective effects on the peri-infarct region during the evolution of I/R injury [146]. Treatment with H_2S also has been shown to reduce brain injuries and post-ischemic cerebral edema in a dose-dependent manner possibly by blocking apoptosis [147]. In yet another study, NaHS has been shown to improve cerebral energy metabolism by restoring cerebral blood flow and decreasing cerebral vascular resistance, thrombogenesis in animals subjected to acute cerebral anoxia [148]. Further, a synthetic NMDAR antagonist, viz., S-memantine eliciting H_2S release was shown to decrease infarct volume and an improved survival and neurological function in mice subjected to global CI/R [149]. It was also shown to reduce glutamate-induced intracellular calcium accumulation in primary cortical neurons. In the case of hyperhomocystenemia that portends ED and hence IS, NaHS lessens Hcy-induced NMDAR-mediated mitochondrial toxicity in an epigenetic manner by reducing oxidative stress (NOX4, ROS, and NO), restoring ATP production, and attenuating Hcy-induced mitophagy (lowering LC3-I/II, CSE, Atg3/7). By restoring eNOS, CD31, VE-cadherin, and endothelin-1 levels, it seemingly reinstates endothelial function in brain ECs exposed to Hcy [150]. In the brain endothelium, NAHS dampens oxidative stress and improves H_2S and CSE levels in a SIRT6-dependent mechanism [151]. Further, H_2S mitigates oxidative stress-induced cell injury by upregulating SIRT3 expression through increased activator protein 1-binding interaction with SIRT3 promoter. It

thereby reverses H_2O_2-induced reduction in SOD1 and IDH2 activity [152]. Na_2S/NaHS can also abrogate pro-inflammatory phenotype by reducing the chromatin openness at IL-6 and TNF-α genes [153]. Further, its anti-apoptotic action involves attenuation of poly (ADP-ribose) polymerase (PARP-1)-AIF pathway [154]. Another putative application involves co-administration of H_2S with tissue plasminogen activator (tPA) to counteract tPA-induced hemorrhagic transformation by disrupting tPA-induced Akt-VEGF-MMP cascade [155]. Combining the administration of NaHS with mild hypothermia for resuscitation following I/R injury has been shown to avert hippocampal apoptosis by shifting the NMDAR subunit NR2A/NR2B balance towards synaptic neuron stimulation and PI3K/Akt signaling [156]. In a hypoxic milieu, H_2S can counteract hypoxic accumulation of HIF-1α and the downstream VEGF expression through eukaryotic translation initiation factor-2α (eIF-2α) Ser^{51} phosphorylation that contravenes eIF-2α ternary complex formation and hence HIF-1α translation initiation in ECs [157]. Another school of thought supports modulation of intracellular oxygen homeostasis by H_2S through HIF-1α destabilization in a vHL and mitochondria-dependent manner [158]. Contrastingly, H_2S augments VEGF and angiopoietin-1 expression by promoting Akt and ERK phosphorylation that induces angiogenesis in peri-infarct region and ameliorates functional outcomes in a rat MCAO model [159]. Subsequently, H_2S enhances the activity of NMDA receptor by generating bound sulfane sulfur (sulfurhydrate or sulfurate) in the cysteine residues of the receptors [160]. CBS-derived H_2S facilitates long-term potentiation (LTP) at active synapses and hence linked to associative learning [161]. Further, H_2S-mediated inhibition of VSMC proliferation has been reported to have an epigenetic basis that subsumes ATP-dependent chromatin-remodeling SWI/SNF complex. H_2S has been shown to inhibit the central catalytic ATPase of this complex, viz., Brahma-1 that interferes with the transcriptional activation of genes related to cell proliferation and differentiation [162]. It would be interesting to explore the possible operation of such a mechanism in CI/R paradigm.

Is H_2S Donation a Pyrrhic Victory in Ischemic Stroke?

Despite all these telltale signs of neuroprotection afforded by H_2S, contradictions have been raised. In a rat stroke model, an increase in H_2S level in the lesioned cortex along with an increase in H_2S-synthesizing activity was observed and inhibition of H_2S synthesis seemed to reduce MCAO-induced infarct volume dose-dependently [19]. Administration of H_2S inhibitors like amino-oxy acetic acid (a CSL inhibitor) at low concentrations (0.025 mmol/kg) in a rat model of tFCI seemed to reduce infarct volume, brain edema, and neurological deficits. These effects were inconspicuous at higher concentrations and exacerbated ischemic injury [163]. During ischemia, ATP depletion associates with reduced GSH pool that correspondingly elevates Cys levels. This correlates with elevated H_2S levels synthesized through transsulfuration pathway during early ischemia [20] (Fig. 2). In this regard, pre-administration of Cys or NaHS/Na_2S enhanced infarct volume, an effect counteracted by CBS inhibition that unequivocally reduces H_2S production [164]. CBS is a rapid responder to ischemic insults as evinced from upregulated CBS expression in the cortical and striatal regions post-MCAO. A truncated form of CBS is evident in cortical and striatal regions together with primary astrocytes. Truncated CBS signifies CBS cleavage with no endogenous H_2S generation and is an avid producer of ROS due to the similarity in its reduction potential with hemithiolates like cytochrome p450 and NOS [164]. Taken together, H_2S impinges on neuronal survival and BBB integrity that seems to be reliant on *milieu interier* during stroke, H_2S concentrations, and agents that induce ischemic injury. Interestingly, a potent "zipped" inhibitor of CBS, viz., 6S dampens H_2S production significantly and reduces infarct volume in a rat tMCAO model [165]. Given its ability to facilitate synaptic remodeling and LTP which is of obvious significance pursuant to stroke, there is an immediate need to clarify the equivocal effects of H_2S on neurological outcomes.

Crosstalk Between H_2S and NO in Neurovascular Unit—It Takes Two to Tango

H_2S and NO engage in a metabolic crosstalk that elicits varied effects on angiogenesis and vasodilation which can be capitalized for the development of effective therapeutic agents [11]. There are umpteen examples suggesting a crosstalk between NO and H_2S, some of which pertinent to ischemic brain are discussed below. H_2S-mediated attenuation of oxidative stress and maintenance of mitochondrial homeostasis involve potentiation of VEGF/Akt/eNOS/NO/cGMP pathway [11, 12]. The beneficial effects of NO signaling seem to get a "bigger bang for the buck" by H_2S-mediated endothelium-dependent vasorelaxing/ hyperpolarizing action [15, 166]. In relation with this, a PTM similar to S-nitrosylation, viz., H_2S-derived S-sulfhydration or persulfidation [forming hydropersulfide moieties (-SSH) by interaction with thiol groups in active Cys residues of target proteins] underlies the modulatory impact of H_2S (CSE/CBS/3-MST-dependent) on NOS [167, 168]. As regards sulfhydration, in lieu of the above mechanism, a series of reactive sulfur species (persulfides, polysulfides, and thiosulfate) generated by sulfide oxidation pathways (primarily mechanisms for disposing of excess sulfide) are also posited to modify target proteins [169]. It is thereby proposed that sulfide oxidation pathways

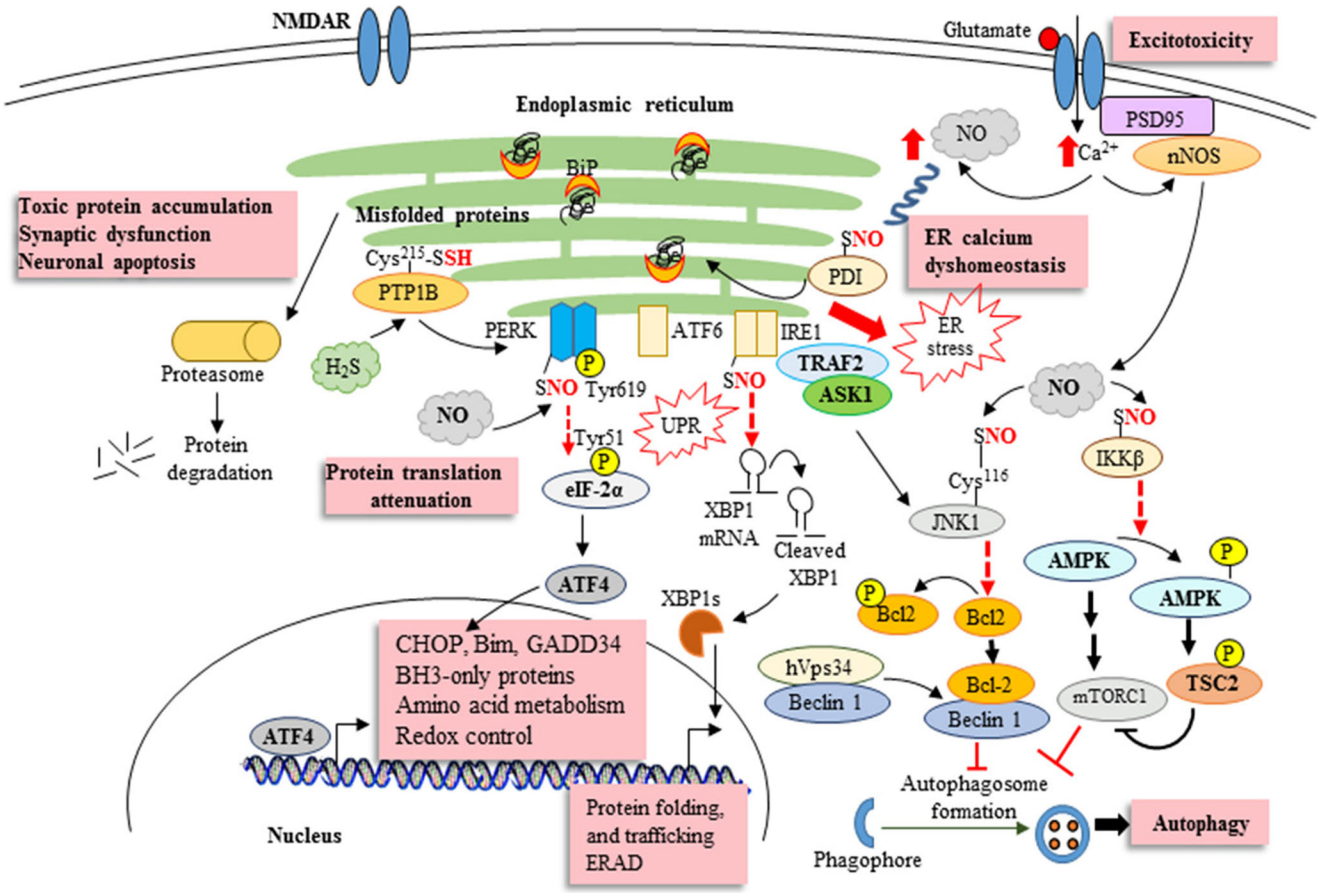

Fig. 2 Contribution of NO and H_2S to modulation of integrated cell stress response with particular reference to endoplasmic reticulum stress (ERS) pathway and autophagy induction. S-nitrosylation of protein disulfide isomerase (PDI) results in improper folding of proteins that triggers unfolded protein response (UPR). Accumulation of unfolded proteins stifles the ER chaperone binding immunoglobulin protein (BiP) equilibrium causing its dissociation (and binding to misfolded proteins) from the three classical ERS sensors, viz., protein kinase-like ER kinase (PERK), inositol-requiring enzyme (IRE1), and activating transcription factor 6 (ATF6) and their subsequent oligomerization, activation, and unplugging of UPR. The activity of ERS sensors like PERK and IRE1 is modulated by NO-mediated S-nitrosylation and H_2S-mediated S-sulfhydration. PERK is activated by S-nitrosylation and S-sulfhydration which in turn activates phosphorylation of eIF2α resulting in transient suspension of cap-dependent mRNA translation. The expression of cap-independent mRNAs like activating transcription factor 4 (ATF4) proceeds that in turn promotes the expression of genes related to anti-oxidant response, amino acid biosynthesis, and transport essential for cell survival. ATF4 also promotes expression of pro-apoptotic Bim, growth arrest and DNA damage-inducible protein 34 (GADD34) and BH3 only proteins. Delayed expression of C/EBP homologous protein (CHOP) ensues to mediate ER stress-induced apoptosis. S-nitrosylation of IRE1 inhibits its ribonuclease activity that suspends the unconventional X-Box protein 1 (XBP1) mRNA splicing to produce active transcription factor (XBP1s) resulting in inhibition of target gene expression responsible for protein folding, trafficking, and ER-associated protein degradation program (ERAD). S-sulfhydration of PTP1B also induces PERK phosphorylation and triggers ERS. Active IRE1 is implicated in ER stress-induced autophagy induction, by way of sequentially recruiting TNF receptor-associated factor 2 (TRAF2) and apoptotic regulatory kinase 1 (ASK2) that activates c-Jun N-terminal kinase 1 (JNK1). Activated JNK1 relieves the autophagy-inhibitory effect of anti-apoptotic Bcl-2 by causing it to dissociate from Beclin 1 and promoting autophagosome formation. Nevertheless, NO functions as an autophagic suppressor by way of S-nitrosylation of JNK1 and IKKβ components of JNK1-Bcl-2-Beclin 1 and IKKβ-AMPK-tuberous sclerosis complex 2 (TSC2)-mTORC1 (mammalian target of rapamycin complex 1) axes thereby precluding autophagosome formation

mediate sulfide signaling and that target specificity is ensured by sulfurtransferases [169]. With respect to eNOS, by sulfhydrating it on Cys^{443}, H_2S potentiates eNOS activity by promoting its phosphorylation and dimerization and inhibiting S-nitrosylation of the same residue [170]. This modality is CSE-dependent as eNOS from CSE knockout (CSE-/-) mice exists in monomerized form with compromised NO production. This is in stark contrast to the effect of NO on eNOS that promotes eNOS monomerization. H_2S (CSE-backed) augments eNOS Ser^{1177} phosphorylation by accentuating PI3K-Akt pathways and delays cGMP degradation by exerting tonic inhibitory effect on cGMP-specific phosphodiesterase, PDE5 [11]. This response is evoked by eNO stimulating factors like VEGF and calcium mobilizing hormones like acetylcholine [11, 166].

Activation of PI3K–Akt pathway in ECs exposed to H_2S donors occurs by sulfhydrative inhibition of PTEN (Cys^{124}) and protein tyrosine phosphatase (PTP1B) (Cys^{215}) that

results in increased PIP3 levels and Akt (phosphorylated on Ser^{473})-dependent activation of eNOS through Ser^{1177} phosphorylation [11, 170, 171]. Interestingly, sulfhydration of Cys^{71} and Cys^{124} residues dissuades concomitant S-nitrosylation [168]. It can thereby be construed that S-sulfhydration can occur on the same sites as S-nitrosylation presumably indicating that the two processes are mutually exclusive. The mutual dependence of NO and H_2S in regulating angiogenesis and endothelium-dependent vasorelaxation is implicit in suspended NO-stimulated cGMP accumulation, angiogenesis, and acetylcholine-induced vasorelaxation following silencing of H_2S-synthesizing CSE [11, 13]. Reciprocally, substrate bioavailability and expression of CSE can be upregulated by pharmacological NO donors resulting in vasodilation [11, 13, 15]. H_2S can also prime ECs for angiogenesis that alludes to its pivotal role in wound healing through increased phosphorylation of Akt, ERK, and p38 [172]. Further, the combined presence of H_2S and NO irreversibly inhibits iNOS and nNOS at modest H_2S concentrations that would dampen NO production under conditions of excessive formation of H_2S/NO [173]. Nevertheless, as CBS is the predominant H_2S-producing enzyme in the brain, the pertinence of the above discussed findings needs to be evaluated in ischemic brain.

Further, both NO and H_2S can rein in excessive ROS production by inhibiting electron transport chain complexes I, IV, and cytochrome C [8, 11, 168]. Additionally, S-nitrosylation of keap1 enhances Nrf2-mediated anti-oxidant gene expression by facilitating the dissociation of Keap1 from Nrf2 [174]. H_2S can also activate Nrf2 through keap1 S-sulfhydration on Cys^{151} that can disarm ROS and diabetes-accelerated atherosclerosis through hemoxygenase-1 (HO-1) expression [175] (Fig. 1). In addition, recursive interactions between NO and H_2S generate a panoply of chemical species like thionitrous acid (HSNO), nitrosothiol, sulfinyl nitrite ($HSNO_2$), and nitroxyl (HNO) that have substantial impact on physiological functions as oxygen delivery, vasorelaxation, cardioprotection, etc. [176]. At therapeutic concentrations, H_2S can abrogate pro-apoptotic and oxidative effects of $ONOO^-$ by formation of $HSNO_2$. This could impact PARP-1 activation as $ONOO^-$-induced DNA damage triggers PARP-1, an excessive activation of which is deleterious as it induces neuronal specific cell death "parthanatos" [177] (Fig. 1).

S-nitrosylation and S-sulfhydration also present a contrasting picture of the "molecules in cohesion" in terms of elaboration of cellular sequelae like synaptic remodeling, dendritic spine retraction, and attendant cognitive deficits. A pertinent example involves H_2S-induced GAPDH sulfhydration resulting in its binding to Siah and this duo binds to PSD95 in dendrites resulting in ubiquitin-mediated degradation of the latter thereby contributing to dendritic spine loss and cognitive deficits [178] (Fig. 1). This modality relies on CBS functionality and forms the basis for IL-1β-induced memory impairment (as IL-1β is involved in learning and memory and promotes LTP) in various neurological diseases.

A Case in Point: Mediation of Endoplasmic Reticulum Stress Response by S-Nitrosylation and S-Sulfhydration

Apart from regulating vascular tone and inflammation, the PTMs, viz., S-nitrosylation and S-sulfhydration mediate a consolidated cellular response such as endoplasmic reticulum stress response (ERSR) [118, 179, 180]. Excess Ca^{2+} influx resulting from glutamate-induced NMDAR overactivation primes endoplasmic reticulum (ER) dyshomeostasis that subsumes dysregulated protein folding within ER [181, 182]. The latter effect could in part be ascribed to dysregulated S-nitrosylation of protein disulfide isomerase (PDI). PDI is the sentinel molecule for optimal oxidative protein folding and S-nitrosylated PDI (SNO-PDI) forges a functional relationship between nitrosative stress-induced protein misfolding in ER and neurodegeneration [183]. Upregulated expression of PDI has been reported to reduce DNA-fragmented cells in CA1 region of hippocampus in rats with brain ischemia thereby conferring resistance to ischemic injury [184]. Nevertheless, S-nitrosylation of ER chaperones, viz., PDI and glucose-related protein (GRP) causally relate with accumulation of ubiquitinated protein aggregates in cultured astrocytes following oxygen and glucose deprivation [185]. Upregulated PDI during CI/R is subject to iNOS-derived NO-mediated S-nitrosylation that compromises its oxidation, isomerase, and chaperone activity [186]. This intercepts proper protein folding in ER that triggers ER stress, unfolded protein response (UPR), and apoptotic induction. UPR is characterized by chaperone induction, increased degradation of misfolded proteins, and suspension of protein translation (Fig. 2). Cell viability is compromised as SNO-PDI is inept in rescuing neurons from proteotoxicity, proteasome dysfunction, and prolonged UPR. It is interesting to note that SNO-PDI is stable in ER due to its vicinal thiols that react with NO to form double -SNO and in part due to the oxidizing environment of ER [183]. A compromised anti-oxidant action seems to be an epiphenomenon of PDI S-nitrosylation, as SOD1 ubiquitination and degradation in the cytosol and nucleus of astrocytes occur due to an ineffective SNO-PDI and SOD1 binding [185]. The resultant mutant SOD1 forms monomers or multimers with reduced thiols in lieu of a functional homodimer with an evolutionarily conserved disulfide bond. These forms could be in part be faultily localized to ER that portends worsened oxidative stress and neuronal death. In vitro studies have shown that mutant SOD1 can also launch a positive feedback loop as it augments iNOS production which in turn

accentuates SNO-PDI formation and mutant SOD1 aggregation [186]. These unwarranted sequelae could be abrogated by treatment with Nω-nitro-L-arginine (l-NNA), a broad-spectrum NOS inhibitor [186].

In addition, S-nitrosylation also targets the salient components of UPR pathway that initiate and control ERSR such as inositol-requiring enzyme 1 (IRE1) and double-stranded RNA-activated protein kinase-like ER kinase (PERK), where in it evokes contrasting effects [187]. S-nitrosylation of IRE1 inhibits its ribonuclease activity and that of PERK activates it resulting in activation of its downstream effector eukaryotic translation initiation factor (eIF2α) and inhibition of protein translation (Fig. 2). In a similar vein, H_2S activates the PERK arm of UPR albeit in an indirect manner. Underlying PERK activation is sulfhydrative inhibition of PTP1B that targets the regulatory phospho-Tyr^{615} of PERK [180]. Sulfhydrated PTP1B is subject to reduction by thioredoxin that overrides GSH or Dithiothreitol. This activation is incumbent on CSE activity as suppression of CSE dampens H_2S production and decreases the phosphorylation of Tyr^{615} of PERK that relieves the inhibition on protein synthesis [180]. This is convergent with ERS-induced activation of CSE that leads to preferential H_2S production. A recent study indicated that H_2S-mediated increase in eIF2α is realized by sulfhydration of Cys^{127} of PTP1c [188]. On the contrary, S-nitrosylation is posited to prevent oxidative stress-induced permanent inactivation of PTPs as evinced from protection of PTP1B from subsequent H_2O_2-induced irreversible oxidation by S-nitrosylation of Cys^{215} [189]. Augmenting cellular NO by pretreating cells with a NO donor or by activating ectopically expressed NOS seemed to inhibit ROS-induced irreversible oxidation of endogenous PTP1B [189]. Taken together, S-nitrosylation and sulfhydration modulate the activity of ER machinery and could be construed as precocious determinants of ERSR as they seem to mediate an integrated stress response by inducing metabolic alterations and priming the cells for stress response.

The continuum of cellular stress response invoked by major cellular stressors like ER stress, hypoxia, mitochondrial damage, and nutrient and energy deprivation spans across quality control of protein turnover to degradation of complex macromolecular structures and apoptotic cell death [190, 191]. ER stress is one of the major cellular stressors for autophagic induction. Given the shared molecular machinery and common stressors for NO production and autophagic induction, it is tenable to assume that NO production is a communal feature of an integrated response to cellular stress. Increased NO production by NOS overexpression or NO donation has been shown to suppress autophagy in primary cortical neurons, HeLa and HEK cells, while decreased endogenous NO production promotes autophagic flux [192]. This is affected by S-nitrosylation of the components of two canonical autophagic signaling pathways i.e., JNK1-Bcl-2-Beclin1 and IKKβ-AMPK-mammalian target of rapamycin complex 1-upstream autophagy regulator (mTORC1) axes (Fig. 2). S-nitrosylation of JNK1 precludes the formation of core autophagophore for clearance of large protein aggregates. This occurs by reduced phosphorylation of Bcl-2 that in turn increasingly interacts with Beclin 1 whose complex formation with hVps34 is disrupted. Alternatively, S-nitrosylation of Bcl-2 has been reported to stabilize its interaction with Beclin 1 resulting in inhibition of the latter and hence autophagy in transformed lung epithelial cells [193]. Further, S-nitrosylation of IKKβ decreases AMPK phosphorylation resulting in activation of autophagic inhibitor, mTORC1 (Fig. 2). Nevertheless, a moot question arises as to what provokes autophagic induction as proautophagic effects of NOS inhibition are independent of both Bcl-2/Beclin 1 function and mTORC1 activity [192].

nNOS-derived NO-induced S-nitrosylation of PTEN also has been reported to invoke PTEN-mTOR signaling axis that limits excessive autophagy in nasopharyngeal cancer cells [194]. Further, iNOS and NO which constitute well-defined downstream effectors of IRF-1 arbitrate the inhibitory effect of IRF-1 on autophagy by way of mTOR activation [195]. An interesting speculation about H_2S contribution in this regard can be provided by the regulatory aspect of IRF-1 by CSE-backed S-sulfhydration that inhibits autophagy in lipopolysaccharide-stimulated macrophages. A recent study has shown that exogenous H_2S restores cardioprotection from pre-conditioning by upregulation of autophagy via activation of AMPK/mTOR pathway in the aged hearts and cardiomyocytes [196]. Similarly, treatment of hepatocellular carcinoma cell lines with NaHS seemed to promote autophagy as evidenced from an increased expression of autophagy-related proteins LC3-II and Atg5 and upstream inhibition of PI3K-Akt-mTOR pathway [197]. In CI/R setting, a recent study demonstrated that NaHS confers neuroprotection in the peri-infarct region by suppression of over-activated autophagic response [198]. This said, not much research has been initiated into parsing the coordinated action of NO and H_2S and the putative epigenetic underpinnings of these mechanisms in an ischemic milieu. Summing up, given the breadth of the pleiotropic effects of NO and H_2S in modulating ERSR and its downstream sequelae, it would be interesting to seek their unrealized influence on the epigenetic network in ischemic brain for establishing durable drug targets.

Conclusions and Future Directions—Wearing a Questioning Hat

Cellular localization and compartmentalization of NO production define the protein repertoire subjected to regulation by S-nitrosylation. A more refined account of the trajectory of NOS activity in CI/R paradigm will be required

as majority of the observations concerning NO production were made ex vivo in brain homogenates and correlated with in vivo NO production. In addition, a major caveat in unfolding its varied sequelae in different cellular contexts by assessment of the degree of modification of the target proteins is compartmentalized modification of substrate proteins. Nevertheless, determining the stoichiometry of different PTMs co-occurring with S-nitrosylation on protein subsets would circumscribe the theoretical expanse of the signaling protein repertoire. Further, the emerging minutiae concerning the interaction between NO and H_2S in determining the cellular fates allow us to refurbish our considerations of drug administration, wherein a combination treatment would offer considerable promise in containing the disease and also in its alleviation in the post-stroke epoch. Efforts need to be expended in this area as ischemic brain is the locus for grossly altered expression of various genes. Given the profound impact of NO and H_2S signaling on maintenance of brain-endothelial homeostasis, it would be highly appealing to study the coercive nature of highly elevated NO and H_2S levels in the backdrop of metabolic trajectories and ischemic brain transcriptome with an underlying epigenetic basis. Given the multifarious actions of these "molecules in cohesion," it would be appealing to study the epigenetic basis of these recursive interactions in steering various transcriptional responses in CI/R paradigm. Another emerging aspect that merits emphasis is the effect of NO on mRNA methylation that seems to have a dichotomous outcome of either increasing or decreasing translation. Though the concept is still nascent, the phenotypic sequelae of NO-mediated changes in mRNA translation are worth being explored. Finally, the regulation of stress responsive pathways like ERSR and autophagy by S-nitrosylation and S-sulfhydration may reek of redundancy in terms of molecular targets of these PTMs, which offers an exciting opportunity for identification of a single common target for attenuation of overactive and cell-damaging disease (CI/R) responsive pathways. A clearer appreciation of the complicity of NO and H_2S in CI/R possibly by backward validation of epigenetic disease signatures needs to be attained which could open the turnstiles for development of formidable drug targets.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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