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Forum Review

Regulation of Heme Oxygenase-1 by Redox Signals Involving Nitric Oxide

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ABSTRACT

Heme oxygenase-1 (HO-1) is an inducible stress protein the expression of which can be markedly augmented in eukaryotes by a wide range of substances that cause a transient change in the cellular redox state. The importance of this protein in physiology and disease is underlined by the versatility of HO-1 inducers and the functional role attributed to HO-1 products (carbon monoxide and bilirubin) in conditions that are associated with moderate or severe cellular stress. An intriguing aspect is the recent evidence showing that nitric oxide, a ubiquitous signaling molecule, finely modulates the activation of HO-1 expression. As the effects of oxidative stress on the regulation of the HO-1 gene have been well established and characterized, this review will focus on the biological relevance of redox signals involving nitric oxide and reactive nitrogen species that lead to up-regulation of the HO-1 pathway, with particular emphasis on vascular tissues and the cardiovascular system.
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INTRODUCTION

THE BIOLOGICAL SIGNIFICANCE OF HEME DEGRADATION by heme oxygenase enzymes in mammalian tissue is currently enticing the interest of many investigators. Both constitutive (HO-2) and inducible isoforms (HO-1) of heme oxygenase catalyze the oxidation of heme to carbon monoxide (CO), iron, and biliverdin, the latter being converted to bilirubin by biliverdin reductase (56). The common conception that products of heme degradation are merely eliminated from tissues as toxic wastes has been disputed by recent evidence demonstrating that endogenously generated CO and bilirubin act as important effector molecules in the cardiovascular system (49). Indeed, studies have revealed that increased production of CO following induction of the *ho-1* gene in vascular tissue significantly affects the regulation of vessel tone *in vitro* (72, 88) and blood pressure *in vivo* (65). It has also been shown that up-regulation of HO-1 and consequent overproduction of intracellular bilirubin are associated with protection against peroxynitrite ($ONOO^-$)-mediated apoptosis (26), suppression of oxidant-induced microvascular leukocyte adhesion (34), and amelioration of postischemic myocardial function (17). An analysis of the dynamics of HO-1 expression and bilirubin production after stimulation with hemin revealed that cells display high resistance to oxidant damage only when actively producing the bile pigment, strongly implicating the HO-1 pathway in cytoprotection against oxidative stress (16, 27). As oxidant-mediated cell injury appears to contribute significantly to the pathogenesis of vascular disease, HO-1 is widely regarded as a key player in the restoration of vascular function under conditions of increased generation of reactive oxygen species (ROS). Notably, the hypothesis that the HO-1 system may act in a similar fashion to counteract the excessive production of nitric oxide (NO) and reactive nitrogen species (RNS) has recently been postulated (24). The high inducibility of HO-1 in eukaryotes in response to NO and NO-related species makes this stress protein a likely candidate to participate in NO detoxification. This review will discuss our current understanding of the chemical reactivity of NO with intracellular components and its redox signaling properties in relation to HO-1 regulation.

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REDOX ACTIVITIES ELICITED BY NO

NO and RNS

The signaling molecule NO, which is generated in mammals by a family of constitutive (nNOS and eNOS) and inducible (iNOS) NO synthase (NOS) enzymes, plays an essential regulatory role in a variety of physiological and pathophysiological processes that take place within the cardiovascular, nervous, and immune systems (62). The distinctive biological activities evoked by NO can be achieved by virtue of its nature as free radical and, consequently, by the reactivity of the NO group with a wide range of structural and functional targets comprising the intracellular milieu. It is then not surprising that such a ubiquitous gaseous molecule can effectively interact with specific nucleophiles, preferentially metal centers (77), to modulate disparate and important functions, including smooth muscle relaxation, neurotransmission, and immunoregulation. Contrary to its well recognized beneficial effects when generated in small quantities within vascular tissues and other cell types, overproduction of NO has been established as a potent cytotoxic weapon in host defense against infection, inflammation, and cancer (67). Considerable amounts of NO can originate from activated iNOS when appropriately induced by cytokines, endotoxins, oxygen free radicals, or other stressful stimuli, and this response appears in many circumstances to be critical for predetermining the maintenance or loss of cellular function (67). In fact, an augmented expression of iNOS and the consequent increase in NO levels have been shown to cause either deleterious or beneficial effects to the organism (67), and data on iNOS-deficient animals have confirmed the dual role of this inducible protein in physiology and disease (54).

The difficulty in delineating a mechanistic involvement of iNOS as pro- or antiinflammatory agent and the controversy arising on whether excessive NO elicits cytoprotective or cytotoxic actions are better appreciated by recognizing the complexity of NO chemistry when applied to biological systems. As minutely detailed by Stamler and colleagues, the reactivity of the NO groups is dictated by the oxidation state of the nitrogen atom, which enables the molecule to exist in different redox-activated forms (81). In contrast to NO, which contains one unpaired electron in the outer orbital, nitrosonium cation (NO^+) and nitroxyl anion (NO^-) are charged molecules being, respectively, the one-electron oxidation and reduction products of NO (Eq. 1).

$$NO+ \overset{-e^-}{\longleftarrow} NO \overset{+e^-}{\longrightarrow} NO^- \quad (1)$$

The rigorous investigation on the potential involvement of NO redox-related species as signaling factors in eukaryotes as well as prokaryotic cells is justified by the persuasive evidence that NO^+ can be transferred reversibly between cysteine residues (transnitrosation) (77) and that NO^- can be formed by hemoglobin (28), neuronal NOS (75), and *S*-nitrosothiols (RSNO) (4). Thus, a broad range of chemical reactions can be predicted to arise once NO and other RNS are generated in the vicinity of proteins or biomolecules; some of these reactions have been already documented *in vitro* and *in vivo*. For example, NO at high concentrations in aqueous solutions can be quickly air-oxidized to nitrogen dioxide (NO_2), which subsequently disproportionates to nitrite (NO_2^-) and nitrate (NO_3^-) (Eqs. 2 and 3); notably, the concentration of oxygen and, in the case of biological fluids, the local pO_2 are crucial for determining the fate of NO and its reactivity with cellular components.

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

$$2NO(aq) + H_2O_2 \rightarrow NO_3^-(aq) + NO_2^-(aq) + 2H^+ \quad (3)$$

In pathophysiological conditions, NO reacts rapidly with superoxide anion ($O_2^{\bullet -}$) leading to the formation of $ONOO^-$ (Eq. 4), a potent oxidant species that has been shown to cause nitration of proteins, as well as lipid peroxidation and cytotoxicity (5, 51).

$$NO + O_2^{\bullet -} \rightarrow ONOO^- \quad (4)$$

$$ONOO^- + H^+ \rightleftarrows ONOOH$$

$$ONOOH \rightarrow OH^{\bullet} + NO_2^{\bullet} \quad (5)$$

$$NO + NO_2 \rightleftarrows N_2O_3 \quad (6)$$

Other RNS effectively formed when NO reacts with molecular oxygen or $O_2^{\bullet -}$ are nitrogen dioxide radical ($NO_2^{\bullet}$) (Eq. 5) and dinitrogen trioxide (N_2O_3) (Eq. 6), which can also cause nitration and oxidation of intracellular components. In the context of the chemical modifications that are transduced into specific physiological functions, the interaction of NO-related species with sulfhydryl residues and transition metals such as Fe(II) or Fe(III) heme in proteins assumes great significance. The activation of heme-dependent guanylate cyclase by NO to promote vasorelaxation and the way hemoglobins have evolved to use redox chemistry of NO to facilitate oxygen transport and delivery to tissues, as well as NO consumption or detoxification (29, 33, 41, 82), are remarkable examples of the specificity of certain NO-responsive targets. Similarly, formation of iron-dinitrosyl complexes and modulation of the activity of proteins containing thiols or iron-sulfur centers appear to be critical for cellular signaling and can be viewed as part of NO's versatile, albeit not fully defined, biological role (20, 86).

More recent works have attempted to discriminate between the effects of NO and NO^- on cell toxicity and assess how the interconversion of one species to the other in tissues may determine a defined function or response. In hamster lung fibroblasts, NO^- seems to cause a greater cytotoxicity compared with NO, and the one-electron oxidation reaction of NO^- to NO promoted by ferricyanide results in increased cell survival (89). In another study, it was found that strong oxidants are generated from NO^- to induce site-specific DNA damage in human breast cancer cells, and this effect is exacerbated by hydrogen peroxide (H_2O_2); in the same *in vitro* model NO appears to be ineffective (11). Opposite effects of NO and NO^- on the postischemic myocardium have been reported showing that, although NO is protective, NO^- in-

creases cardiac tissue injury and decreases myocardial hemodynamic functions during ischemia–reperfusion (53). In contrast, both exogenous and endogenously generated NO have been shown to be toxic to neuronal cells, whereas transfer of NO^+ to thiol groups (*S*-nitrosation, see below) of the *N*-methyl-D-aspartate (NMDA) receptor down-regulates its activity and is neuroprotective (51). In addition, interaction of NO^- with a critical cysteine in the NMDA receptor provides cellular protection against excitotoxic insults (44). Once again, these contrasting findings must be interpreted in view of the way cells respond to a given effect mediated by NO or its redox-related forms, their concentrations and location, the presence of thiols, and the composition of the cellular microenvironment. Nevertheless, evidence is mounting on the functional consequences of the signals evoked by RNS in normal conditions and disease states; controlling their levels *in vivo* is essential for cellular homeostasis, and their potential role as modulators of cell survival and death is rationalized by the striking similitude that exists with certain oxidants and ROS in promoting cellular adaptation to a particular type of stress.

RSNO and nitrosative stress

A fundamental aspect of NO biochemistry is the attachment of NO groups to sulfhydryl centers to form *S*-nitrosyl derivatives or RSNO (Eq. 7).

$$RSH + NO^+ \rightarrow RSNO + H^+ \quad (7)$$

This chemical process, known as *S*-nitrosation, has been suggested to represent a refined endogenous tool to stabilize and preserve NO biological activity. Indeed, proteins such as albumin, tissue-type plasminogen activator, and hemoglobin that are nitrosylated at specific cysteine residues can exert, similarly to NO, vasorelaxation and inhibition of platelet aggregation (41, 79, 80). Low-molecular weight RSNO, such as *S*-nitrosoglutathione, and *S*-nitrosocysteine, may also represent a mechanism for storage and transport of NO *in vivo*. In this regard, glutathione becomes an important determinant of the reactivity and fate of NO because this cysteine-containing tripeptide is very abundant (5–10 m*M*) in most tissues and biological fluids. In aqueous solutions, RSNO are quite stable, but they can be rapidly decomposed by several factors, including light, transition metal ions, pH, and the presence of reducing agents (79). RSNO can undergo both homolytic and heterolytic cleavage of the S-N bond; the first reaction leads to the formation of disulfide and NO (Eq. 8), whereas the second one releases NO^+, which can promote transnitrosation of other thiols (Eq. 9).

$$RSNO \rightarrow NO + 1/2RSSR \quad (8)$$

$$RSNO \rightarrow NO^+ RS^- \quad (9)$$

S-Nitrosation is also an important process in modulating the activity and function of several enzymes and proteins, the best example of which is provided by *S*-nitrosohemoglobin. Hemoglobin, whose cysteine 93 (Cys-93) in the β chain is charged with a nitroso group when passing through the lung, delivers NO in arterioles, thereby regulating their diameter in response to the need for flow, as sensed by oxygen tension (41). Although this provocative and controversial model of oxygen/NO transport is a matter of debate in the recognition of a physiological mechanism that could explain a direct involvement of blood in vascular control (59, 82), the general concept that S-nitrosation represents a posttranslational modification sustained by cellular components to transduce NO signals is well supported by previous and recent findings (20, 40, 80). Other functional proteins that are regulated by *S*-nitrosation include the ryanodine receptor (91), caspase-3 (57), and NMDA receptor (40, 51). All these data sustain the notion that redox reactions of thiols involving endogenous NO and RNS must depend upon the activity and regulation of the three NOS isoforms. Thus, it is inferred that the distinct chemistry elicited by RNS is also affected by the amount of NO locally produced or targeting a given tissue, which will ultimately determine the cellular response to nitrosation.

As mentioned above, and in analogy to the accepted evidence that an excessive production of oxidants and ROS leads to oxidative damage, alteration of protein functions may occur when RNS reach a critical threshold. This phenomenon, which is driven by uncontrolled nitrosative reactions, has been termed nitrosative stress (32). The intriguing aspect in the parallelism between the effects mediated by increased RNS and ROS is the ability of cells to respond to these two types of stress; most notably, depending on the severity of the nitrosative/oxidative insult, this response may result in both adaptation and resistance to toxicity. Studies initially performed on bacteria revealed that an oxidative-stress response involving several inducible genes occurs in *Escherichia coli* after exposure to NO; this effect is mediated by the redox-sensitive transcriptional factor SoxR and results in increased resistance of these bacteria to the lethal effect of NO-generating macrophages (69). As the cytoprotective mechanism triggered by SoxR in *E. coli* includes the expression of critical antioxidant defensive proteins such as superoxide dismutase (30), the emerging concept from this elegant study was that analogous systems might operate in mammalian cells. That the antioxidant protein heme oxygenase could "sense" NO and act effectively as a pivotal player in cytoprotection against ROS and RNS insults was instigated by the following original findings: (a) NO and NO-related species induce HO-1 expression and increase heme oxygenase activity in hepatocytes (46), liver (64), human glioblastoma cells (84), and aortic vascular cells (22, 25, 31, 63, 72); (b) cells pretreated with various NO-releasing agents acquire increased resistance to H_2O_2-mediated cytotoxicity at the time heme oxygenase is maximally activated (45, 63); and (c) bilirubin, one of the end products of heme degradation by heme oxygenase, protects against the cytotoxic effects caused by the strong oxidants H_2O_2 and $ONOO^-$ (16, 26, 63). Given that further investigations have revealed that NO-mediated activation of the HO-1 pathway is a stress response that can be extended to a variety of mammalian systems (24), several important issues on the possible signal transduction mechanism(s) that leads to HO-1 induction by NO and RNS remain to be carefully examined. These and other aspects will be analyzed and discussed in the next paragraphs.

HO-1: A REDOX-SENSITIVE INDUCIBLE PROTEIN

Induction of HO-1 by oxidative and nitrosative stress

The first evidence showing that both ultraviolet radiations and oxidizing agents potently induce HO-1 expression in human fibroblasts (43) set the basis for the emerging hypothesis that activation of this protein is a ubiquitous cellular response to oxidative stress. Since then, several reports and comprehensive reviews have appeared in the literature where detailed information can now be found on the molecular regulation of *ho-1* gene expression in response to agents and conditions that are known to promote an increase in endogenous ROS (13, 55, 85). From these and other studies, it has been established that a prerequisite for oxidant-mediated HO-1 expression *in vitro* and *in vivo* is the alteration of the cellular redox state and that in several circumstances, if not all, changes in glutathione levels appear to be the common denominator (see below). Less studied and not fully elucidated are the physiological significance and the mode of regulation of the HO-1 system by NO (24).

The conception that NO and RNS can be directly involved in the modulation of HO-1 expression in eukaryotes is based on the evidence that different NO-releasing agents can markedly increase HO-1 mRNA and protein, as well as heme oxygenase activity, in a variety of tissues (see ref. 24). *S*-Nitroso-*N*-acetylpenicillamine (SNAP), GSNO, sodium nitroprusside (SNP), and 3-morpholinosydnonimine (SIN-1) have been extensively used as "NO-generating systems" in biological studies. Both SNAP and GSNO are nitrosating agents with different half-lives and can release NO (but to a greater extent also NO^+ and NO^-) in physiological buffers (4). SNP is an iron nitrosyl complex in which NO is formally bound as NO^+ to the metal in the ferrous state, whereas SIN-1 releases stoichiometric amounts of NO and $O_2^{\bullet -}$ to promote $ONOO^-$ formation. The fact that in endothelial and smooth muscle cells, as well as isolated aortic tissue, all these NO-releasing agents have been shown to induce HO-1 and augment heme oxygenase activity (22, 25, 63, 72, 92) would suggest that the effect mediated by the NO group is independent of its redox state. However, the following considerations are required at this point: first, the presence of exogenous thiols significantly diminishes SNP-mediated HO-1 expression, suggesting that nitrosation of specific targets (NO^+ transfer) is required for the induction (25); second, thiols are much more effective toward SNP than SNAP in preventing heme oxygenase activation, revealing that, in the case of SNAP, transnitrosation reactions might be part of the induction mechanism (25); third, superoxide dismutase almost completely prevents SIN-1-mediated increase in heme oxygenase activity, indicating that NO originating from sydnonimine does not appear to contribute to the observed effect (25). Moreover, superoxide dismutase has also been reported to attenuate both SNAP- and SNP-mediated increase in heme oxygenase activity (25). Thus, it can be deduced that reaction of NO with $O_2^{\bullet -}$ and the extent of the conversion of NO to NO^+ or NO^- by intracellular components could be critical to determine the modulation of HO-1 gene expression. Not surprisingly, $ONOO^-$ has also been shown to increase endothelial HO-1 protein expression and activity as part of the cellular response to the oxidative/nitrosative threat (25, 26). Whether NO^- can directly promote a similar effect has yet to be investigated; however, preliminary data from our laboratory reveal that Angeli's salt, a spontaneous NO^- generator, promotes HO-1 induction *in vitro*. Figure 1 shows a direct comparison between agents that release or donate NO, NO^+, or NO^- and their intrinsic ability to increase heme oxygenase activity in endothelial cells. Although the induction of HO-1 by NO and NO intermediates seems to be dependent on the concentration of the species used, the maximal increase in heme oxygenase activity can only be achieved by a critical threshold (87) as higher concentrations of NO would ultimately lead to cytotoxicity. In fact, a marked elevation in HO-1 mRNA, which is not reflected by a parallel increase in heme oxygenase activity, can still be observed with concentrations of SNP that are prohibitive or damaging to cells (87). This is in keeping with the concept that the extent of specific redox reactions discriminates between signaling events and oxidative/nitrosative stress inflicted by NO-related species (78). It emerges that the acquired resistance or susceptibility to stress mediated by NO and involving HO-1 induction should not be assessed on the basis of gene and protein expression only (7). If a biological role for HO-1 in protecting cells against various forms of stress is postulated, then up-regulation of enzymatic activity and, consequently, HO-1 products will be the crucial elements in preserving cell function and survival (24). An increase in HO-1 transcript has been reported also in cells treated with NO gas or NONOates (which release NO in a more physiological fashion), but the NO-related species that promote this induction and the intracellular targets mediating this response remain to be identified (9, 31).

Similarly to the effects reported about NO-releasing agents, modulation of HO-1 expression appears to be affected by increased endogenous NO derived from stimulated iNOS.

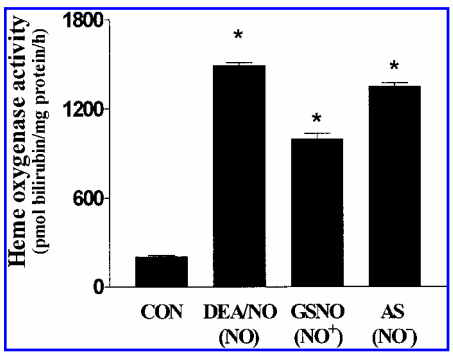

FIG. 1. Effect of NO and its redox-activated forms on endothelial heme oxygenase activity. Bovine aortic endothelial cells were exposed for 6 h to various agents (0.5 m*M* final concentration) that possess the ability either to release NO [diethylamine-NO (DEA/NO)], donate NO^+ (GSNO), or liberate NO^- [Angeli's salt (AS)] at physiological pH. Heme oxygenase activity was measured as previously described (63). As shown, all three agents significantly increased heme oxygenase activity at the end of the treatment. Columns represent the means ± SEM of five independent experiments (*$p < 0.05$).

In rat glial cells, treatment with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) results in a rapid increase in both iNOS expression and nitrite levels followed by enhancement of HO-1 protein (47). In the same study, the presence of NOS inhibitors suppressed both nitrite accumulation and HO-1 protein expression. Modulation of HO-1 mRNA expression by iNOS-derived NO following stimulation with LPS has also been reported in mesangial and Kupffer cells (18, 39); in these *in vitro* systems, NOS inhibitors prevented LPS-mediated HO-1 induction. Moreover, the early increase in iNOS protein levels observed in endothelial cells exposed to low oxygen tension seems to precede the stimulation of HO-1 expression and activity, an effect that appears to be finely regulated by redox reactions involving glutathione (66). Collectively, these findings reveal that endogenously generated NO can trigger the expression of HO-1, which by virtue of its protective action may lead to adaptation and resistance of cells to subsequent nitrosative and oxidative stress insults (7, 24, 63). The exact molecular mechanism(s) by which both exogenous and endogenously formed NO (or NO-related species) modulate the induction of the *ho-1* gene remains obscure. In studies performed using rat hepatocytes, Kim and colleagues advanced the hypothesis that NO displaces the heme prosthetic group from cytochrome P450 proteins, leading to an increase in intracellular heme pool, which would ultimately promote HO-1 induction and heme oxygenase activation (46, 92). This is in contrast to the data reported in endothelial cells showing that nitrosation of intracellular free heme by treatment with SNAP inhibits heme oxygenase activity, thereby preventing heme degradation (42). Based on the evidence that NO is known to activate guanylate cyclase, some authors reported an increase in HO-1 transcript and protein levels after exposure of cells to relatively high concentrations of cyclic GMP analogues (37, 38). In contrast, others have consistently found that activation of the HO-1 pathway is a cyclic GMP-independent process (7, 12, 31, 46, 63). Recent evidence suggests that a translation-independent stabilization of HO-1 mRNA occurs upon exposure of human fibroblasts to NONOates (9). However, in rat smooth muscle cells, these NO-releasing agents have been shown to increase HO-1 gene expression by enhancing both gene transcription and mRNA stability (31). Thus, a direct interaction of NO groups with selective chemical sites localized in transcription proteins that can be activated through nitrosative reactions could effectively contribute to the pronounced overexpression of HO-1. Although transcription factors implicated in *ho-1* gene induction that are sensitive to both ROS and RNS remain to be fully characterized, important information to this end can be drawn from the way bacterial cells regulate the expression of certain genes in response to oxidative and nitrosative stress.

Regulation of gene expression by oxidative and nitrosative stress

As mentioned above, signaling mechanisms adopted by regulatory proteins to control gene expression in response to alterations in the intracellular redox status are very common in prokaryotes. The most elegant example is provided by the transcription factor OxyR, an activator of antioxidant genes that is responsive to oxidative stress in both *E. coli* and *Salmonella typhimurium* (14, 19). It has been shown that oxidized, but not reduced, OxyR activates transcription *in vitro* and that, upon treatment with oxidants, the conversion between the two forms of OxyR is rapid and reversible (83). The expression of these protective genes after exposure to low doses of H_2O_2 renders the bacteria more resistant to oxidant damage (14). Studies have confirmed that, prior to the binding to specific promoters, OxyR must undergo a conformational change in which the molecular mechanism appears to involve either the oxidation of Cys-199 to the corresponding sulfenic acid (48) or the formation of a reversible disulfide bond (93). Interestingly, *S*-nitrosation of OxyR following treatment of *E. coli* with high concentrations of RSNO under anaerobic conditions has been recently proposed as an alternative mechanism of transcriptional regulation (32). This mechanism appears to: (a) be independent of oxidant-mediated stress; (b) involve the depletion of intracellular glutathione and accumulation of endogenous RSNO; and (c) lead to resistance to nitrosative stress. These events are associated with rapid decomposition of RSNO when cells are returned to aerobic conditions, suggesting that endogenous RSNO are being metabolized. The existence of inducible systems capable of counteracting excessive *S*-nitrosation by consuming NO and RSNO is sustained by strong evidence showing that *E. coli* and yeast flavohemoglobins possess both NO oxygenase and reductase activities (33, 52, 60). These activities, which convert RSNO to nitrate and NO^-, respectively, become a refined tool to detoxify cells from the excessive production of NO. In view of the high inducibility of HO-1 and other cytoprotective systems in eukaryotic cells following exposure to NO and RNS (24), future work needs to be addressed to the molecular regulation of *ho-1* gene expression in response to nitrosative stress. Specifically, the following need to be identified: (a) the transcriptional regulator(s) that is sensitive to redox reactions involving NO; (b) the chemical modification(s) within the target protein that is responsible for NO-mediated activation of transcription; and (c) the biological significance of this response. Notably, redox-sensitive transcription factors that recognize specific binding sites within the promoter and distal enhancer regions of the *ho-1* gene include Fos/Jun [activator protein-1 (AP-1)], nuclear factor-κB (NF-κB) and (more recently identified) Nrf2 proteins (2, 3, 13). Of major interest is the notion that both AP-1 and NF-κB contain cysteine residues whose interaction with oxidant or nitrosant species might be crucial for determining the binding activity to DNA (1). Data in the literature show that NO can either activate or inhibit these transcription factors, and that in many circumstances the activation depends on the reversibility of the posttranslational modification elicited by the various RNS (68, 70, 90). Studies performed on smooth muscle cells revealed that increase in HO-1 transcript by the NO donor, spermine NONOate, is associated with enhanced AP-1 DNA binding activity (31). In contrast, recent work using HeLa cells suggests that mitogen-activated protein kinase ERK and p38 pathways, but not the AP-1 transcription factor, are involved in NO-mediated induction of HO-1 (12); in this case, the mechanism of activation would be unrelated to cyclic GMP and also appears to be independent of redox signaling events. Although NF-κB has also been shown to be inhibited by *S*-nitrosation (58), a direct

link between regulation of NF-κB activity by NO and *ho-1* gene induction remains to be investigated. It needs also to be established whether NO interacts directly with other transcription factors that bind to specific elements in the promoter region of the *ho-1* gene. In this regard, investigations should be conducted on eukaryotic transcription factors that are known to contain cysteines in their DNA binding domain or other crucial regulatory sites. A schematic diagram showing how NO and its redox activated forms may interact with cellular components to modulate gene expression (including *ho-1*) in response to both oxidative and nitrosative stress is represented in Fig. 2.

Glutathione and RSNO: intracellular modulators of HO-1 expression

Glutathione, which exists in either a reduced (GSH) or oxidized (GSSG) form, plays an essential role in maintaining the intracellular environment in a tightly controlled redox state; GSH is the major low-molecular-weight thiol compound, as well as one of the most effective antioxidants and reducing agents in plants and animals. Depletion of glutathione has been shown to occur in conditions of moderate or severe oxidative stress and has been associated with increased susceptibility to cell damage (76). It is then not surprising that, in response to oxidation of glutathione and alteration of the thiol redox state, certain antioxidant genes (including *ho-1*) are activated (76). Data in the literature support evidence for a direct link between a decrease in glutathione levels by oxidant stress and rapid up-regulation of HO-1 mRNA and protein in a variety of cells and tissues, including Chinese hamster ovary cells (73), rat brain (23), human fibroblasts (50), mouse liver (71), endothelial cells (66), rat cardiomyocytes (35), and murine macrophages (10). The strong correlation that exists between changes in thiol redox state and activation of the heme oxygenase pathway is validated by findings showing suppression of oxidant-mediated HO-1 induction by the glutathione precursor, *N*-acetylcysteine (8, 10, 71). In addition to oxidants, increased production of NO and RSNO can also lead to changes in intracellular glutathione. Exposure of smooth muscle cells to NO donors results in a marked decrease in the GSH:GSSG ratio, which is accompanied by stimulation of glutathione synthesis and augmentation of total glutathione (61). Depletion of total glutathione and formation of glutathione disulfide was also found after treatment of murine lymphocytes with *S*-nitrosocysteine and DETA-NO (6). In murine macrophages, stimulation of iNOS by exposure to LPS plus IFN-γ decreases total glutathione, and this effect is prevented by pretreatment of cells with NOS inhibitors (36). Of major interest is that an important role for glutathione metabolism and RSNO formation in the regulation of HO-1 expression by NO has been established (24, 25, 66). Specifically, elevation of intracellular glutathione prior to exposure of endothelial cells to NO donors almost completely abolishes activation of the heme oxygenase pathway; this suggests that thiols can antagonize the effect of NO (or NO-related species) on HO-1 induction (25, 66). In a model of *in vitro* hypoxia (pO_2 = 2 mm Hg) using cultured endothelial cells, the expression of HO-1 and the consequent elevation in heme oxygenase activity are associated with a transient decrease in the GSH:GSSG ratio and formation of endogenous RSNO due to an early induction of the iNOS gene (66). Thus, in conditions of low oxygen tension, both oxidative and nitrosative reactions may participate in the observed stimulation of HO-1 (27, 74). In addition, the rapid formation of GSSG in the early stages of hypoxia was found to be a reversible phenomenon and iNOS inhibitors significantly attenuated hypoxia-mediated HO-1 activation (66). These data are indicative of the signal transduction properties of endogenously generated ROS and RNS in HO-1 activation and are in line with the general principle that nitrosation and oxidation are not mutually exclusive events, but

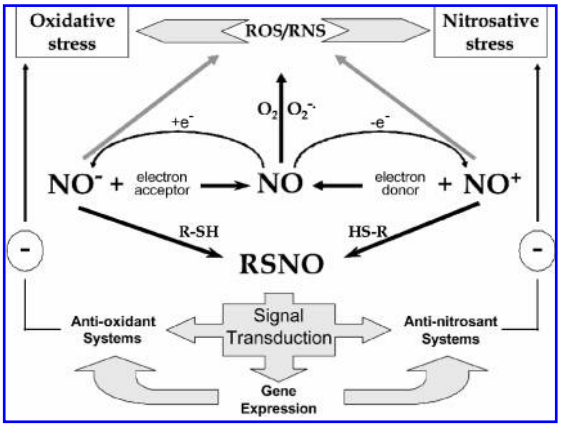

FIG. 2. Regulation of gene expression by redox signals involving NO. A possible mechanism by which cells sense and counteract nitrosative and oxidative stress is represented in this schematic diagram. The chemical versatility of the NO group allows its interaction with regulatory biological components and its conversion to NO^+ and NO^-, the one-electron oxidation and reduction products of NO, respectively. These NO redox species can react either directly with sulfhydryl residues (−SH) as in the case of NO^- or be donated to thiol groups by means of trans-nitrosations, which involve NO^+ transfer. The final result is the formation of RSNO critically located in the regulatory sites of functional and structural proteins. This signal transduction mechanism will modulate selectively also the activity of several transcriptional factors, ultimately controlling gene expression. In the case of excessive production of NO and/or superoxide anion ($O_2^{-\bullet}$), both ROS and RNS can be generated leading to induction of distinct antioxidant and antinitrosant systems (see text for details).

are rather part of a dynamic equilibrium that dictates the reversibility of chemical modifications sustained by intracellular regulatory targets (78). At present, it is not known whether oxidation and nitrosation modulate *ho-1* gene expression via distinct molecular mechanisms or elicit the cellular stress response in a synergistic or additive fashion. It needs to be emphasized that profound changes in GSSG and RSNO levels are important markers for assessing the cellular stress response to oxidation and nitrosation, but are not necessarily indicators of cell death and survival; in this respect, irreversible oxidation of thiols to sulfinic ($S\text{-}O_2H$) and sulfonic ($S\text{-}O_3H$) acids appears to be a better predictor (78). Although data in the literature are very limited (15), it is possible that excessive formation of sulfinic and sulfonic acids represents the point of "no return" for cells in their response to effectively counteract and survive oxidative and nitrosative stress insults. The development of new methodologies that are able to characterize and quantify the amount of these thiol-oxidized species in relation to induction of the HO-1 pathway by various forms of stress will enable scientists to better define the regulation and biological functions of this redox-sensitive inducible protein.

CONCLUSIONS

The biological significance of HO-1 up-regulation by NO and NO-related species remains to be fully elucidated. However, the striking parallelism with oxidant-mediated HO-1 induction in biological systems points to a functional role for this redox-sensitive inducible protein in counteracting nitrosative stress. This hypothesis is sustained by circumstantial evidence showing that products of HO-1 protect cells against the reactivity of RNS and could participate directly in the restoration of cellular homeostasis through modulation of NOS function (24). The fact that HO-1 does not contain cysteines may be indicative of its evolutionary role as a protein insensitive to inactivation by both oxidation and nitrosation (21). Future studies on the redox-dependent molecular mechanisms of HO-1 regulation will enable scientists to characterize structural and functional targets of NO that are essential in promoting the cellular adaptation to stressful conditions.

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ABBREVIATIONS

AP-1, activator protein-1; CO, carbon monoxide; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; GSSG, oxidized glutathione; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; NO^+, nitrosonium cation; NO^-, nitroxyl anion; $O_2^{\bullet -}$, superoxide anion; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSNO, *S*-nitrosothiols; SIN-1, 3-morpholinosydnonimine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

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