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Invited Review

The Heme Oxygenase Pathway and its Interaction with Nitric Oxide in the Control of Cellular Homeostasis

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Heme oxygenase is the rate limiting enzyme in heme degradation to carbon monoxide (CO), iron and bilirubin. The inducible isoform of the protein, heme oxygenase-1 (HO-1), is susceptible to up-regulation by a diverse variety of conditions and agents in mammalian tissue, leading to the common conception that HO-1 is a stress related enzyme. However, as attempts are made to unravel the mechanisms by which HO-1 is induced and as we discover that CO, iron and bilirubin may be important effector molecules, we are learning to appreciate that heme oxygenases may be central to the regulation of many physiological and pathophysiological processes besides their established function in heme catabolism. One such process may be closely linked to nitric oxide (NO). It has been demonstrated that NO and NO donors are capable of inducing HO-1 protein expression, in a mechanism depending on the *de novo* synthesis of RNA and protein. Thus, it is postulated that NO may serve as a signaling molecule in the modulation of the tissue stress response. This review will highlight the current ideas on the role of CO–heme oxygenase and NO–nitric oxide synthase in cell signaling and discuss how the two systems are interrelated.

Keywords: Heme oxygenase, NO synthase, CO, NO, peroxynitrite, stress response, bilirubin, CO sensing, S-nitrosation, signal transduction

INTRODUCTION

Nitric oxide (NO) is generated intracellularly by the enzyme nitric oxide synthase (NOS) using L-arginine as a substrate.[1] It is generally accepted that NO is a major component in signal transduction pathways controlling smooth muscle tone, platelet aggregation, neurotransmission, host response to infection and a wide array of other biological processes.[2] Another gaseous molecule, carbon monoxide (CO), has been recently attributed with biologically active properties. Similarly to NO, CO has been shown to be implicated in vasodilatation,[3–6] to act as modulator of cGMP

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levels[3,5–11] and to inhibit platelet aggregation.[12,13] Heme oxygenase is the main endogenous source of CO, which is produced during the degradation of heme in a mechanism requiring molecular oxygen and a reducing agent (NADPH).[14,15] The fact that heme oxygenase occurs almost ubiquitously in nature, from mammals to plants and blue-green algae, and its activity can be greatly enhanced by many stimuli of diverse nature, including NO, led us and other investigators to pursue the aim of identifing the inherent characteristics of the products of heme catabolism. Our own research in the field of heme oxygenase has mainly focused on studying the mechanisms by which HO-1 can be induced and the possible role of this stress protein in the pathophysiology of cardiovascular diseases. Recently, detailed reviews on the function of the heme oxygenase–CO–bilirubin and NO synthase–NO pathways in a variety of systems have been prepared by investigators in the field.[2,16–25] The purpose of this review is to analyze with particular emphasis the specific effect of NO and NO related compounds and their ability to induce HO-1; the physiological significance of the HO-1 pathway in the restoration of cellular homeostasis will also be discussed.

THE CO- AND NO-GENERATING PATHWAYS

The Heme Oxygenase System

To date three isoforms of heme oxygenase have been identified: HO-2 or constitutive isoform,[26,27] HO-1 or inducible enzyme[26,28,29] and the recently discovered HO-3.[30] They are all products of distinct genes and while both HO-2 and HO-1 possess heme degrading activity, HO-3 is a poor heme catalyst and displays two heme regulatory motifs that may be involved in heme binding. Immunolocalization techniques for HO-2 have shown that the enzyme is present constitutively in most tissues, including brain and the nervous system, liver, spleen, vasculature and testis;[15,19] the only known modulators of HO-2 gene expression are adrenal glucocorticoids.[31] Recent studies suggest that the HO-2–CO system controls normal physiological functions likely related to neurotransmission,[7,8] NO-independent relaxation of vascular tone[4] and regulation of vascular resistance in sinusoids of rat liver.[32] In addition, Dore and colleagues have recently reported that phosphorylation of HO-2 by protein kinase C results in enhancement of HO-2 activity in brain cultured cells,[33] reinforcing the hypothesis that the heme oxygenase pathway plays a significant role in the nervous system.

HO-1, also known as the stress protein HSP32, is present in normal conditions in the spleen; however, its augmented expression can be observed in a wide range of tissues following stressful stimuli. These include heavy metals, heat shock, GSH-depletion, UVA radiation, endotoxin, ischemia–reperfusion, hypoxia, hyperoxia, NO donors and peroxynitrite, as well as its own substrate heme and various hemoglobins.[10,19,34–42] Indeed, there seems to be no other known enzyme that responds to such a variety of distinct agents and conditions, as does HO-1. Based on these findings, it has been proposed that HO-1 induction is part of the defensive mechanism that cells and tissues are capable of mounting against stress situations. This possibility is sustained by evidence from several experiments. Induction of HO-1 has been reported to be a rapid, protective response to rhabdomyolysis in rat[43] and to confer high resistance to oxidative injury when coupled to increased ferritin production in the endothelium.[44] Otterbein *et al.* demonstrated that hemoglobin administration to rats protects against septic shock,[45] in a mechanism largely dependent on augmented expression of the stress inducible gene HO-1 and not ferritin expression.[46] We have shown that NO-mediated increase of HO-1 expression and heme oxygenase activity in cultured endothelial cells diminishes the susceptibility to oxidant damage by hydrogen peroxide.[37] Furthermore, our results indicate

that HO-1 up-regulation by hemin is associated with decreased apoptosis caused by peroxynitrite in aortic endothelial cells[39] and reduced ischemia–reperfusion injury in an *ex vivo* model of isolated perfused heart[47] and skeletal muscle *in vivo* (M. Vesely and R. Motterlini, manuscript in preparation). Recently, it has been observed that knock-out mice for HO-1 are more sensitive to damage caused by lipopolysaccharide[48] and that HO-1 expression is functionally associated with long-term survival of cardiac xenografis.[49] In addition, HO-1 induction in vascular tissue is protective against chronic cardiac allograft rejection and suppresses the development of transplant arteriosclerosis.[50] In general, the observed protection afforded by HO-1 is attributed to increased metabolism of the pro-oxidant heme and the concomitant production of the powerful antioxidants biliverdin and bilirubin, the vasoactive gas CO and iron, a gene regulator. However, only few studies show a direct causal link between endogenous bilirubin and CO derived from heme oxygenases and control of tissue's functions.[5,6,11,32,33,39,51,52] It has to be noted that, in hamster fibroblasts, HO-1 is reported to ameliorate resistance to oxygen toxicity, but the authors speculate that the beneficial effect can be obtained only within a narrow range of expression.[53]

The third isoform of heme oxygenase, HO-3, shares a 90% amino acid homology with HO-2 and is constitutively expressed in all tissues examined.[30] Although HO-3 awaits further characterization, the presence of two heme regulatory motifs, with a conserved cysteine–proline dipeptide core, suggests an important role for the protein in heme-dependent intracellular functions.

The protein isoforms belonging to the heme oxygenase family seem independent from each other and regulated at molecular and biochemical levels by different factors; at the same time, the existence of some cross-talk among the three enzymes to optimize their function and modulate their activity cannot be excluded *a priori* and this hypothesis represents an attractive field of investigation. In addition, whether heme oxygenase is operating primarily to break down heme or, alternatively, to synthesize specific bioactive molecules from the substrate heme, still remains to be fully elucidated. Depending on the circumstances (physiologic or pathophysiologic) and tissue distribution of heme oxygenases, both perspectives might be true. Relevant to this dualistic function, we recall one of the best examples of heme oxygenase at work in a common condition, a bruise which gradually changes color from black and blue to green, and then yellow. The accumulation of the pro-oxidant heme and hemoglobin from ruptured blood vessels into the damaged tissue is responsible for the dark color. The green color is caused by biliverdin, derived from the oxidation of heme catalyzed by heme oxygenase; the reduction of biliverdin by biliverdin reductase to the bilirubin pigment provides the yellow color. In the light of the possible functions of biliverdin and bilirubin as effective antioxidants and CO as a vasorelaxant, it is reasonable to speculate that the concerted action of the products of heme oxygenase may facilitate and accelerate the healing process.

Heme Oxygenases Versus NO Synthases

The heme oxygenase and NO synthase enzyme families bear strong similarities. Both are characterized by constitutive and inducible isoforms, which produce two important signaling molecules, CO and NO. These two diatomic gases possess similar chemical properties, including molecular weight and diffusibility in water. Due to its unpaired electron, NO is by definition a free radical whereas CO is apparently a stable compound. This structural distinction dictates the reactivity of NO and CO with their target molecules, NO being a highly reactive and short-lived species and CO a mediator with possible long-lasting effects. This becomes evident when considering the interaction of hemoglobin with these two gases. The combination rate of NO with

ferrous hemoglobin is much faster compared to CO;[54,55] in contrast, the rate of NO dissociation from ferrous hemoglobin is slow.[56] Because of the low off rate, the affinity of NO for hemoglobin is high (~ 1500 times that of CO).[57,58] Despite the large body of evidence supporting a major biological role for NO in comparison with CO, a close analysis of the actual amount of these two gaseous molecules measured in the human body under normal conditions reveals that their production is of the same order of magnitude (approximately 850 and 500 μmol/day for NO and CO, respectively).[59–61] This suggests that CO may be as important as NO in the modulation of physiological functions, in view also of the unique characteristic of NO and CO to activate guanylate cyclase and increase intracellular concentrations of the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP) (see below).

The inducible enzymes of heme oxygenase and NO synthase (HO-1 and iNOS, respectively) share common stimulators, such as cytokines, endotoxins and reactive oxygen species.[19] However, the HO-1 and iNOS genes can also be regulated independently from each other (i.e. there are stimuli that selectively affect only the expression of HO-1 and not that of iNOS).[62] Interestingly, it has been described that conditions such as hypoxia down-regulate constitutive NOS protein levels in both human umbilical vein and pulmonary artery endothelial cells[63,64] and transiently increase both iNOS and HO-1 protein expression in the endothelium[65,66] and smooth muscle.[10] These data imply the existence of fine regulatory mechanisms which, under hypoxic conditions, simultaneously control heme oxygenase and NO synthase genes and result in the modulation of vascular functions, such as changes in vessel contractility[67,68] and cell proliferation.[69] Further investigation is required to verify whether this hypothesis can be extended to other 'non-physiological' conditions and to tissues different from the vasculature. Since it has been shown that NO releasing agents stimulate vascular HO-1 expression,[9,38,70] it would be relevant to examine whether iNOS up-regulation always results in HO-1 induction and, if so, whether a temporal relationship underlies this phenomenon.

Taken together, these findings indicate that both the heme oxygenase–CO and NO synthase–NO systems participate in the regulation of tissue functions. It is possible that, according to tissue localization and the physiological or pathophysiological situation being considered, heme oxygenases and NO synthases co-operate to maintain cellular homeostasis. Moreover, under certain conditions, one enzymatic pathway may counter-regulate, compensate or prevail over the other. We have recently found that hemin administration results in a dramatic increase in HO-1 mRNA levels in rat skeletal muscle.[71] Notably, this effect was most prominent in fiber type I (slow twitch) but very weak in fiber type II (fast twitch). Although additional studies are required to establish the exact significance of these data, we postulate that the HO-1/CO pathway may have a role in regulating microcirculatory function in skeletal muscle, especially in conditions such as exercise, which are characterized by HO-1 induction.[72] Since it has been reported that skeletal muscle contains a neuronal-type NO synthase restricted to the sarcolemma of type II fibres,[73] it seems reasonable to speculate that CO and NO may act as signaling molecules following fibre type-specific patterns.

CO and NO in Cellular Signaling

NO and CO exert their signaling activity utilizing diverse mechanisms of transduction. Firstly, both NO and CO are activators of soluble heme-containing guanylate cyclase, in virtue of their ability to bind to ferrous heme.[74,75] The consequent augmented production of cGMP has major influence in the maintenance of vessel tone, brain functions and many other important intracellular processes.[76] However, one subject of controversy is the fact that CO is a poor stimulator of soluble guanylate cyclase in *in vitro* studies

when compared to NO. The enzymatic activity of purified guanylate cyclase is increased 130-fold and 4.4-fold by its interaction with NO and CO, respectively.[75] An additional criticism is based on the findings that the most commonly used inhibitors of heme oxygenase activity, tin protoporphyrin IX (SnPPIX) and zinc protoporphyrin IX (ZnPPIX), have been shown *in vitro* to also inhibit the activity of guanylate cyclase and NO synthase[4,77] (see below). Nevertheless, a fast increasing body of evidence supports a role for CO in the regulation of cGMP levels. In some areas of the brain, for example, the localization of HO-2 more closely resembles that of guanylate cyclase than the localization of neuronal NOS, and cGMP basal production in the olfactory bulb can be depleted by heme oxygenase inhibitors.[7,8] Similarly, HO-2-derived CO appears to control vasomotor and sinusoidal tone by elevating intracellular levels of cGMP.[4,32] It is essential at this point to make an important distinction between physiological and non-physiological conditions. As HO-1 isozyme cannot be detected in most tissues under unstressed conditions,[19] it can be inferred that normal cellular functions regulated by CO occur through the enzyme activity of HO-2. On the other hand, in stress situations characterized by augmented levels of HO-1, modulation of endogenous processes can be attributed to the substantial amount of CO produced by the inducible isoform of heme oxygenase. Indeed, smooth muscle cells exposed to hypoxia show increased HO-1 protein expression and activity, which results in enhanced CO release and intracellular cGMP accumulation.[10] By utilizing a platelet/smooth muscle cell co-incubation system, Wagner and colleagues demonstrated that platelet aggregation is inhibited by CO generated from smooth muscle cells HO-1.[62] This effect was associated with a marked increase in platelet cGMP levels and reversed by treating smooth muscle cells with heme oxygenase inhibitors. In our laboratory, we have recently measured CO released from aortic tissue following stimulation of HO-1 expression. We found that increased vascular CO derived from HO-1 suppresses acute hypertensive responses *in vivo* via a cGMP-dependent mechanism.[5] Similarly, in a model of isolated aortic rings, we demonstrated that increased vascular contractility by phenylephrine is totally abolished by up-regulation of the HO-1/CO/ cGMP pathway and restored by low concentrations of SnPPIX but not by the NOS inhibitor, L-NMMA.[6] Despite the higher affinity of NO for guanylate cyclase compared to CO, the experimental evidence supporting a direct role of endogenously produced CO in the regulation of cGMP could also be explained by the existence of a molecule which sensitizes guanylate cyclase to CO. Indeed, the benzylindazole derivative, YC-1, has been shown to possess such biochemical properties. Studies on purified guanylate cyclase demonstrated that YC-1 potentiates CO-mediated stimulation of cGMP production to about the same extent as its best known activator, NO.[78] In addition, increased cGMP levels by YC-1 in the presence of NO or CO led to complete inhibition of platelet aggregation at concentrations that were ineffective by themselves.[13] The mechanism underlying YC-1 action appears to be the stabilization of guanylate cyclase in its active conformation. The discovery of a naturally synthesized substance, which structurally and functionally resembles YC-1, would unequivocally establish the role of the heme oxygenase/CO system as stimulator of guanylate cyclase and modulator of biological activities. An endogenous YC-1-like molecule would also account for the discrepancy between the cGMP-increasing effects of CO *ex vivo* or *in vivo*[5,6] and the rather low stimulatory effects of CO on purified soluble guanylate cyclase *in vitro*[74,75]

A second, cGMP-independent, mode of action employed by NO to exert its function in transduction mechanisms relies on nitrosation of cysteine residues of target proteins.[79] S-nitrosation appears to serve as a functional switch in control of gene expression and regulation of protein activity. For instance, both exogenous

and endothelium-dependent NO can directly activate smooth muscle calcium-dependent potassium (K_{ca}) channels, thereby causing hyperpolarization and relaxation of vascular smooth muscle.[80] This effect is mediated by NO which chemically modifies sulfhydryl groups present on the channel. Similarly, reversible activation of the cardiac calcium release channel (ryanodine receptor) is achieved by poly-S-nitrosation of several free thiols contained in the four channel subunits.[81] S-nitrosation can also inhibit protein activity as in the case of caspases, a family of cysteine proteases that execute programmed cell death.[82,83] Accordingly, very recent published observations demonstrate that Fas-induced denitrosation of the catalytic-side cysteine is partly responsible for caspase stimulation.[84]

NO, possibly via S-nitrosation, redox events and/or other yet unidentified mechanisms,[79] could constitute an interactive biological signal modulating stress and adaptive responses in tissues. Interestingly, Nunoshiba *et al.* provided evidence that NO induces an oxidative stress response that defends *Escherichia coli* against activated macrophages.[85] In addition, it has been shown that the prokaryotic transcription factor OxyR not only responds to oxidative stress (hydrogen peroxide) but is also sensitive to activation by S-nitrosation (nitrosative stress); this activation results in the induction of antioxidant and anti-nitrosative genes acting as a highly specialized defense stratagem in bacteria.[86] We were interested to analyze whether NO is implicated in the induction of analogous responses in mammalian cells. Indeed, in a variety of cell types and tissues, we found that diverse NO releasing agents possess the ability to increase HO-1 (HSP32) protein expression and heme oxygenase activity in *in vitro*, *ex vivo* and *in vivo* models.[6,37,38,87–89] Other authors have published similar findings confirming that NO-mediated induction of HO-1 and HSP70 occurs in cultured hepatocytes[90] and rat organs *in vivo*.[91] The regulation of gene expression by NO may find an elegant application in *preconditioning*, a phenomenon wherein exposure of tissues to certain agents and conditions results in up-regulation of endogenous defensive proteins (HSPs) that confer resistance to subsequent insults. Consistent with this hypothesis, both NO donors and endogenously produced NO seem to trigger late preconditioning against myocardial stunning and infarction in conscious rabbits.[92–94]

In analogy with NO, CO may also act as an effector molecule in signaling processes by interacting with targets different from guanylate cyclase. Interestingly, Wang and co-workers have performed studies showing that K_{Ca} channels are susceptible to activation by CO. Both extracellularly and intracellularly applied CO increased the probability of single high conductance K_{Ca} channels opening in a concentration-dependent fashion[95] and this effect may rely specifically on histidine residues localized in the channel.[96] The same authors have also provided evidence that CO-induced vascular relaxation in rat tail artery tissue is mediated by both cGMP pathway and activation of K_{Ca} channels.[97] Additional studies are, however, necessary to address the role played by endogenously produced CO on K_{Ca} channels. It has also been proposed that CO-mediated relaxation in the lamb ductus arteriosus results from the inhibition of a contractile process by reducing the synthesis of the vasoconstrictor endothelin-1.[51]

Little is known about how CO is sensed biologically. However, the photosynthetic bacterium *Rhodospirillum rubrum* has the ability to respond to CO, and the CooA protein is required for this response.[98,99] CooA is a heme-containing protein which, upon exposure to CO, functions as a DNA-binding transcriptional activator of the gene encoding for CO dehydrogenase (CODH), the key enzymatic step in the oxidation of CO to CO_2.[100–102] Thus, CooA senses CO through its heme moiety and transduces the signal by controlling the DNA binding activity. Whether a similar transcriptional factor sensitive to CO exists in eukaryotic cells is currently unknown. Yet, it is intriguing that heart cytochrome *c* oxidase

possesses CO-oxygenase activity by catalyzing the oxidation of CO to CO_2[103,104] and that oxygen-dependent oxidation of small amounts of CO has been detected in heart and skeletal muscle.[105] Further investigation is required to discover selective targets of this heme oxygenase product in eukaryotes and unravel other biological activities of endogenously produced CO.

Sulfhydryl Groups: Important Modulators of NO Function and HO-1 Expression

NO interacts readily with thiol groups of proteins and glutathione producing S-nitrosoproteins and S-nitrosoglutathione, respectively.[25,106] As mentioned above, S-nitrosation of proteins mediates signaling functions similar to protein phosphorylation;[79] indeed, thiols seem to be involved in NO binding, stabilization and transport in biological systems.[107,108] Nitrosothiols possess endothelium-derived relaxing factor-like characteristics and, in virtue of their NO releasing capacities, may invoke many and possibly all NO actions.[109] In this respect, glutathione is likely to be a preferential target of NO as the intracellular concentrations of this non-protein antioxidant are in the range of 5–10 mM. A correlation between heme oxygenase and glutathione has been documented, where HO-1 levels are augmented in conditions of depleted endogenous glutathione.[34,36,110] The exact mechanism by which decreased glutathione affects heme oxygenase levels remains to be fully elucidated. However, we have shown that thiol groups are important modulators of NO-mediated HO-1 induction in vascular endothelial cells.[38] Exposure of cells to sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP) resulted in elevated HO-1 protein expression and heme oxygenase activity; this effect was abolished by increased intra- and extra-cellular thiols and partially involved both superoxide and peroxynitrite ($ONOO^-$) anions.[38] We proposed the existence of a dynamic equilibrium among free NO, superoxide and endogenous glutathione in their ability to regulate the expression of the stress protein HO-1. That is, under physiological conditions a balance exists between produced NO, its intracellular association with glutathione and its target proteins and enzymes. Stress situations, such as oxidative stress and endotoxic shock, may alter this balance leading to glutathione depletion, enhanced superoxide formation and up-regulation of iNOS. The overall result would be an increased amount of NO and NO derivatives, which may act as intracellular signals to mediate the expression of HO-1 (see Figure 1). NO could also regulate the enzymatic function of HO-2 and HO-3, δ-aminolevulvinate synthase (the rate limiting enzyme in heme biosynthesis) and biliverdin reductase (which oxidizes biliverdin to bilirubin). In fact, those enzymes contain cysteine residues that are potentially susceptible to oxidation and S-nitrosation by NO and its redox-activated forms. Indeed, preliminary data from our laboratory show that certain NO donors inhibit biliverdin reductase activity in a concentration-dependent manner *in vitro* (R. Foresti and R. Motterlini, unpublished observations).

Pharmacological Agents Used to Inhibit Heme Oxygenases, NO Synthases and Guanylate Cyclase Activities

One of the major criticisms encountered by scientists working in the field of heme oxygenase comes from the use of inhibitors of heme oxygenases activity (i.e. SnPPIX and ZnPPIX), which at high concentrations also show inhibitory action on guanylate cyclase and NO synthase. Furthermore, metalloporphyrins are very sensitive to light and, once photosensitized, their effects seem to be independent from heme oxygenase inhibition.[111] We found that SnPPIX also interacts directly with peroxynitrite,[39] which is not surprising, since a group of metalloporphyrins capable of catalyzing peroxynitrite decomposition has recently been identified.[112–114] We feel

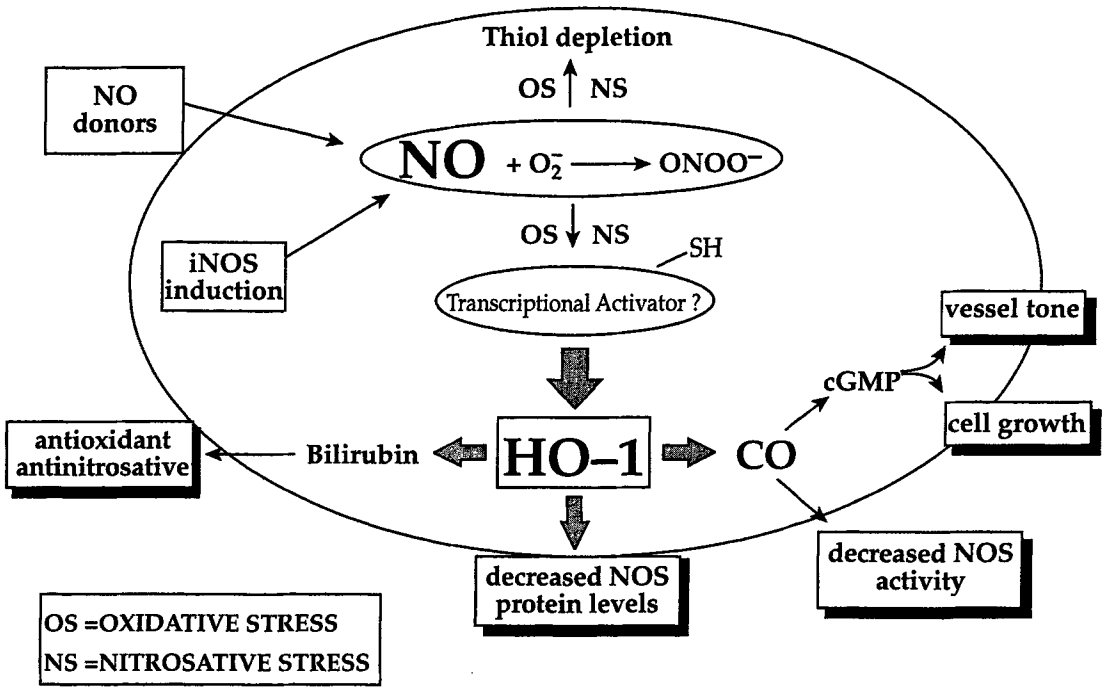

FIGURE 1 Possible mechanisms underlying heme oxygenase-1 (HO-1) induction by nitric oxide (NO) and NO derivatives in eukaryotic cells. Increased NO, either derived from NO donors or from iNOS, and peroxynitrite ($ONOO^-$) may cause an oxidative/nitrosative threat by interacting with specific intracellular targets. This effect results in depletion of thiols, the prime line of defense, and cells counteract this challenge by inducing highly specialized antioxidant/anti-nitrosative genes, including HO-1. A transcriptional factor(s) sensitive to activation by both nitrosative and oxidative stress is likely involved in this response. Increased HO-1 activity is translated into augmented production of carbon monoxide (CO) and bilirubin. These effector molecules may have important feedback mechanisms on NO synthase activity and NO-mediated cytotoxicity. In addition, HO-1 may directly decrease NO synthase levels by degrading the cofactor heme. O_2^-, superoxide anion; cGMP, guanosine 3′,5′-cyclic monophosphate; SH, sulfydryl group.

that this issue deserves careful consideration, in view of the fact that several studies implicate the involvement of heme oxygenase, CO and bilirubin in physiological functions by using these compounds exclusively; the following considerations are made on the assumption that metalloporphyrins are prepared and used in darkness. As already discussed by Maines,[19] the heme oxygenase pocket is not occupied by heme; therefore, minute concentrations of metalloporphyrins (2–10 μM) can inhibit heme oxygenase activity by readily gaining access to the pocket. On the other hand, guanylate cyclase and NO synthase are classified as heme-containing enzymes; that is, heme is an integral part of the protein. Thus, it is expected that high concentrations of SnPPIX and ZnPPIX will be needed to compete and displace the heme bound to the protein. Indeed, it has been shown that reconstitution of purified soluble guanylate cyclase with metalloporphyrins, such as ZnPPIX and MnPPIX, results in inhibition of enzymatic activity[77] *in vitro*. However, Zachary *et al.* reported that SnPPIX and ZnPPIX have no significant inhibition on NO synthase at the concentration of 50 μM but the same compounds elicited $< 50\%$ inhibition of guanylate cyclase activity at 100 μM in cultured endothelial cells.[4] Accordingly, we have observed that SnPPIX (10 μM) does not affect the basal production of cGMP in aortic isolated rings and vascular contraction to phenylephrine suggesting that,

at 10 μM, the metalloporphyrin does not inhibit the NOS pathway.[6] It should be pointed out that the study by Zachary *et al.* was performed in normal conditions, where heme oxygenase activity is mainly attributed to HO-2. Therefore, one wonders whether the same concentrations of metalloporphyrins can be applied to *ex vivo* and *in vivo* models and to conditions characterized by high levels of HO-1 in order to obtain a complete inhibition of heme oxygenase activity. For example, we have shown that 50 μmol/kg ZnPPIX and 40 μmol/kg SnPPIX injected intraperitoneally do not change control blood pressure and cGMP levels in rat aorta[5] and maintained coronary perfusion pressure unaltered in isolated perfused rat heart (J.E. Clark and R. Motterlini, unpublished observations). Maines also recommends to use 40 μmol/kg ZnPPIX *in vivo* to achieve selectivity for heme oxygenase activity inhibition.[115] Interestingly, bilirubin production measured in the medium 24 h after exposure of cultured endothelial cells to 50 μM heme (a treatment that results in strong HO-1 induction), is decreased by no more than 40–50% when using 50 μM SnPPIX (R. Foresti and R. Motterlini, unpublished observations); this indicates that considerably high concentrations of metalloporphyrins are required to block heme oxygenase activity once the HO-1 isoform is up-regulated.

Pharmacological agents used to inhibit guanylate cyclase also show limited specificity. Methylene blue, in addition to blocking guanylate cyclase activity, oxidizes thiols and transition metals[116] and the recently marketed 1 H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), has been reported to lack specificity[82] and to interfere also with NO synthase activity.[117] Concerning the various inhibitors of NO synthases activity, it is interesting to describe the mechanism of action of N^6-(1-iminoethyl)-L-lysine (NIL), a relatively selective inhibitor of iNOS. NIL inactivation of the iNOS protein primarily targets the heme residue at the active side, resulting in destruction and loss of heme.[118] Because the porphyrin ring is a substrate for heme oxygenase activity and the hemoprotein NOS could represent a source of heme for degradation by heme oxygenase (see below), it cannot be excluded *a priori* that NIL may also inhibit indirectly heme oxygenase activity. Furthermore, we have observed that another selective inhibitor of iNOS, S-(2-aminoethyl)isothiourea (≥ 40 μM), directly reduces heme oxygenase activity in an *in vitro* assay (R. Foresti and R. Motterlini, unpublished observations). We should also keep in mind that cyclooxygenase and cytochrome P450, two other enzymes which produce vasoactive molecules, are heme containing proteins.

Taken together, these findings indicate that caution must be taken when interpreting results based on the use of heme oxygenases, NOS and guanylate cyclase inhibitors. In addition, as investigators interested in the heme oxygenase pathway must be aware of the undesired effects of metalloporphyrins on guanylate cyclase and NOS, it would be sensible to apply the same awareness in respect to heme oxygenases when working with NOS and guanylate cyclase activity inhibitors, especially in view of the increasing experimental evidence supporting a mutual and close relationship of these three enzymatic systems in the control of similar physiological functions.

NO AS A MODULATOR OF CO PRODUCTION

Four years ago we found that incubation of aortic endothelial cells with various NO releasing agents resulted in a marked increase in heme oxygenase activity and that this effect was associated with reduced cell death mediated by hydrogen peroxide.[119] After the submission of our data for publication,[37] Kim and colleagues reported that NO donors were capable of inducing HO-1 gene expression and increasing heme oxygenase activity in isolated rat hepatocytes.[120] Since then,

a number of relevant publications have confirmed these findings in a variety of tissues and experimental models,[6,9,37,38,70,87–89,121,122] indicating that this may be a generalized cellular response resulting from exposure to these agents. That NO is the initial element in the cascade of events leading to HO-1 up-regulation was ascertained by the use of hydroxocobalamin, an NO scavenger that considerably decreased heme oxygenase activation by NO donors.[38,89] Most importantly, we demonstrated for the first time that NO-mediated enhancement of heme oxygenase activity results in augmented CO generation in aortic tissue.[6] This effect was accompanied by almost complete suppression of the vascular contractile response to phenylephrine and was reversed by 10 μM SnPPIX. We postulate that, in addition to regulating biological processes through its rapid pharmacological action, NO exerts delayed and long-lasting effects via induction of the HO-1/CO/bilirubin pathway. Although cGMP seems to directly affect HO-1 expression in cultured hepatocytes,[123] NO-mediated HO-1 induction appears to be a cGMP-independent mechanism[9,37,70] and the specific events underlying this response are still unknown. In our experiments we employed various NO releasing agents which are characterized by distinct key biochemical properties. SNP, SNAP, GSNO (S-nitrosoglutathione) and SIN-1 (3-morpholinosydnomine) caused increased heme oxygenase in cultured endothelial cells[38] and the actual absolute values of enzyme activity correlated with the stability of the different NO donors and their capability to generate NO; that is, heme oxygenase induction was dependent upon the rate of decomposition of the NO releasing agent and consequently on the amount of NO delivered into the medium. However, SNP is an iron nitrosyl with strong NO^+ character[124] and contains cyanide and iron (a heavy metal known to induce HO-1); indeed we have observed that the iron chelator desferrioxamine partially attenuated SNP-mediated increase in heme oxygenase activity whereas cyanide did not produce any evident effect.[37] SNAP and GSNO are S-nitrosothiols which, depending on the reaction conditions, can act as donors of NO, NO^- and NO^+; trans-nitrosation reactions (transfer of bound NO from one thiol group to another) also distinguish S-nitrosothiols from other NO donor classes. SIN-1 releases stoichiometric amounts of NO and superoxide anion, which react yielding peroxynitrite.[124] Accordingly, we reported that the presence of superoxide dismutase attenuated SIN-1-mediated endothelial HO-1 induction[38] and that peroxynitrite itself produces a concentration-dependent increase in HO-1 protein expression.[39] Considering that NO, NO^+ and NO^- show distinctive chemistries and reactivities,[125] we cannot exclude at present that multiple mechanisms of action are implicated in the up-regulation of HO-1 by NO donors.

Intracellular augmented levels of NO produced following stimulation of iNOS also seem to regulate HO-1 expression. We and others have reported that the activation of iNOS by bacterial lipopolysaccharide and cytokines resulted in increased heme oxygenase in endothelium, smooth muscle and, most recently, in Kupffer cells.[9,37,120,122] This effect was abolished by inhibitors of iNOS activity. It is interesting to note that both neuronal NOS (nNOS) and iNOS can generate superoxide; however, while nNOS produces superoxide when the heme center is not occupied by L-arginine and 100 μM L-arginine can totally block superoxide generation from nNOS, iNOS-mediated superoxide formation is essentially unaltered by 100 μM L-arginine and only partially blocked by high levels of the substrate (1 mM).[126] These data strongly suggest that superoxide and NO synthesis can occur simultaneously within iNOS. This could lead to the formation of peroxynitrite and it is postulated that this mechanism may enhance the killing activity of iNOS in host defense. In view of our results showing HO-1 upregulation by peroxynitrite,[39] it is tempting to speculate that iNOS-derived peroxynitrite may also play a role in the induction of the stress response.

HEME OXYGENASE–CO AS MODULATORS OF NO PRODUCTION

NOS is a cytochrome P-450 type hemoprotein and as such is susceptible to inhibition by CO.[127] In fact, exogenously applied CO (80% CO/20% O_2) has been reported to inhibit macrophage and rat cerebellar NOS activity[127] and therefore to decrease NO formation. Endogenous CO derived from HO-2, the isoform of heme oxygenase present in normal conditions in the vast majority of tissues, has also been suggested to control constitutive NOS activity. Under physiological conditions, ZnPPIX (1 μM) increases NO production in internal anal sphincter smooth muscle strips suggesting that, in the resting state, the heme oxygenase pathway exerts important counter-regulatory effects on NOS.[128] In addition, CO may interfere with NO binding to guanylate cyclase, since Ingi *et al.* demonstrated that endogenous CO modulates the NO-cGMP signaling system in cerebellar granule cell cultures.[11] Heme oxygenase may also regulate NO generation because of its function in the control of intracellular heme levels and, as part of the normal protein turnover, the heme-containing NOS likely constitutes a substrate for heme oxygenase activity. The heme oxygenase pathway may have even stronger relevance for the maintenance and/or restoration of cellular homeostasis under conditions characterized by high iNOS levels. Although NO generated by iNOS is responsible for the defense against invading microorganisms, it also has multiple tissue damaging effects and is involved in the pathogenesis of inflammation and graft rejection.[129] In virtue of being very sensitive to up-regulation by stress stimuli, including NO and peroxynitrite, HO-1 might represent the feedback mechanism for controlling iNOS activity and iNOS-derived NO (see Figure 1). This hypothesis is sustained by recent findings of Turcanu and colleagues showing that heme oxygenase inhibition (heme oxygenase inhibitors do not discriminate between HO-1 and HO-2) increases NO production in mouse macrophages exposed to endotoxin.[130] Most importantly, the authors reported that HO-1 induction 24 h prior to endotoxin treatment suppresses generation of NO. Similarly, we observed that NO production is greatly enhanced by SnPPIX (1–10 μM) in cultured smooth muscle cells stimulated with IL-1β, a cytokine which induces both iNOS and HO-1 protein expression (J. Dulak, R. Foresti and R. Motterlini, unpublished observations). Again, inhibition of iNOS would be attributed to both the action of CO in decreasing iNOS activity and to the degradation of the iNOS cofactor heme as a consequence of the high heme turnover expected when HO-1 is up-regulated (see Figure 1). However, further investigation is required to confirm this hypothesis.

HO-1 is well recognized as an important antioxidant inducible system. It is intriguing that recent reports describe the existence of metabolic pathways responsible for NO detoxification in *Escherichia coli*. Apparently, the bacterium possesses both constitutive and inducible anti-nitrosative systems; bacterial hemoglobin (flavohemoglobin) seems to be central to the inducible response and to the resistance to nitrosative stress.[131,132] These findings and the above considerations lead us to suggest that the heme oxygenase enzyme family, albeit through a different biochemical mechanism, may be regarded as a fundamental NO-detoxifying system under normal and pathophysiological conditions in eukaryotic cells, ultimately regulating the action of NO in signal transduction (see Figure 1).

HEME OXYGENASE-DERIVED BILIRUBIN: AN ENDOGENOUSLY PRODUCED ANTIOXIDANT AGAINST OXIDATIVE AND NITROSATIVE STRESS?

Bilirubin has always been considered a cytotoxic waste product, mainly because of its association with jaundice in neonates and because at high concentrations (> 300 μM) it causes severe brain

damage.[133] It is only in recent years that a physiological property of bilirubin as an efficient antioxidant has emerged. Stocker and colleagues showed that physiological levels of the bile pigment effectively protect against lipid peroxidation of cellular membranes, rivaling and probably surpassing vitamins E and C with its potent antioxidant properties.[134] Furthermore, serum antioxidant activities are selectively associated with bilirubin in neonatal Gunn rats, a particular rat strain that contains abnormally high levels of serum bilirubin.[135] We have also reported that exogenous bilirubin (5–15 μM) prevents cell death mediated by hydrogen peroxide in cultures of aortic endothelial cells.[37] Despite these promising features, little has been done to elucidate the inherent cytoprotective characteristics of bilirubin derived from heme oxygenases. The specific role of endogenously generated bilirubin as a potent antioxidant is, however, sustained by recent published observations in which direct measurements of bilirubin produced by heme oxygenases have been performed. We have reported that increased levels of HO-1-derived bilirubin in cultured endothelial cells is associated with marked reduction of apoptosis mediated by peroxynitrite, a NO derivative with strong oxidant and nitrosative characteristics.[39] Accumulation of bilirubin due to enhancement of HO-2 catalytic activity by phosphorylation is also protective against hydrogen peroxide-induced cytotoxicity in neuronal cultures.[33] Moreover, nanomolar concentrations of bilirubin were sufficient to provide a neuroprotective effect. We observed that enhanced cardiac bilirubin levels following stimulation of HO-1 ameliorates post-ischemic myocardial function and tissue viability upon reperfusion of isolated rat hearts (J.E. Clark *et al.*, manuscript submitted). Remarkably, this cardioprotective effect was simulated by exogenous bilirubin administered to heart tissue at low nanomolar concentrations. These findings together suggest that intracellular bilirubin levels can be locally and transiently augmented by rapid activation of HO-2 and induction of HO-1 proteins. This peculiar ability of heme oxygenases may represent a fine stratagem evolved by mammalian cells to counteract short and long-lasting oxidative challenges (see Figure 1). Whether the antioxidant bilirubin also possesses anti-nitrosative properties is very much an open question; nevertheless, this possibility could partially account for the effect of NO, NO donors and peroxynitrite in up-regulating HO-1 expression.[38,39] It is noteworthy that uric acid, another waste product which naturally occurs from purine catabolism, has been reported to be a strong antioxidant and peroxynitrite scavenger.[136,137] We see in bilirubin, as in uric acid, the expression of the principle that the end product of a degradative metabolic pathway may be selected in evolution to exert a beneficial action.

CONCLUDING REMARKS

Until recently, investigations in the field of CO–heme oxygenase and NO–NO synthase have evolved independently from each other, posing particular emphasis on the role of one pathway or the other in the control of important biological activities. The experimental evidence showing that NO stimulates HO-1 gene expression and that the heme oxygenase/CO pathway affects NO synthase activity points to the existence of a mutual relationship between the two systems. This interdependence becomes evident in the cardiovascular system where a prominent localization of constitutive NOS and HO-2 is observed and the inducible isoforms (HO-1, iNOS) can be greatly over-expressed following stressful stimuli. In this respect, it is not surprising that NO and CO modulate multiple vascular functions such as control of vessel tone, blood pressure, platelet aggregation and cell proliferation. Although further investigation is required to clarify the exact action of these two gaseous molecules, we believe that NO and CO function interdependently, each dynamically influencing

the other to regulate signal transduction mechanisms. Moreover, the specific induction of the HO-1 pathway in certain disease states may represent a fine expedient to protect against tissue injury and, above all, to restore cellular homeostasis.[138] Remarkably, the first known human case of HO-1 deficiency was recently described.[139] The patient suffered from severe growth retardation, persistent hemolytic anemia, low serum bilirubin levels and extensive endothelial damage. The study provides strong ground for an essential key role played by this enzyme *in vivo* and suggests that a clear understanding of the specific biochemical factors involved in the regulation of heme oxygenases activity would allow the development of targeted therapeutic strategies to prevent or treat vascular dysfunction.

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