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Forum Review

Molecular Mechanisms Involved in Enhancing HO-1 Expression: De-Repression by Heme and Activation by Nrf2, The "One-Two" Punch

KLAOKWAN SRISOOK,* CHAEKYUN KIM, and YOUNG-NAM CHA

ABSTRACT

Heme oxygenase (HO)-1 is a stress response protein, which confers cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis. Molecular mechanisms involved in the inducible transcription of *ho-1* occurring in response to numerous and diverse stressful conditions have remained elusive. Since the discovery of E1 and E2, the two upstream enhancers regulating induction of *ho-1* transcription in 1989, there have been many studies dealing with molecular mechanisms involved in enhancing HO-1 expression. In this commentary, recent advances in our understanding of the mechanisms involved in the induction of HO-1 expression in mammalian cells are summarized with some supportive results reported by others. Currently available data indicate that activation of *ho-1* transcription involves both the heme (native substrate)-dependent selective alleviation of repressor and the oxidative stress-dependent activation of transcriptional activator. The stress-released free-heme (HO-1 substrate) from hemoproteins involved in causing oxidative stress itself appears to act as a molecular switch controlling the repressor–activator antagonism on the enhancer sequences of *ho-1*. Thus, induction of HO-1 appears to operate in a manner like a simple feedback loop. *Antioxid. Redox Signal.* 7, 1674–1687.

HEME AND HEME OXYGENASE (HO)

Heme or iron protoporphyrin IX (12) is a lipophilic and hydrophobic molecule and, as the prosthetic group in hemoglobin, plays a vital role in transporting and delivering oxygen to tissues in a most elegant and highly coordinated manner. The specific functional activity of other heme-containing enzymes essential for the life of aerobic cells, such as NADPH-oxidase, mitochondrial respiratory cytochromes, guanylate cyclase, and nitric oxide (NO) synthase (NOS), is also strictly dependent on the heme (112). These enzymes utilize heme as the catalytic center to achieve complex redox reactions producing superoxide anion (O_2^-), adenosine triphosphate, cyclic guanosine monophosphate, and NO, respectively. Because of its lipophilic nature, heme exists only in association with proteins both within the cell bound to intracellular heme enzymes and outside the cell bound to plasma albumin, lipoproteins, and hemopexin (63, 74). This association with protein allows the heme to exist in a relatively stable complex and limits the heme from getting involved in producing highly reactive hydroxyl radical ($HO^{\cdot}$) via Fenton chemistry. At the same time, this stable association with protein enables the heme–protein complex to be transported within various intracellular and extracellular compartments without causing oxidative damage to cellular constituents in many tissues (33).

Although protein-bound heme is integral to aerobic life, protein-unbound free heme is toxic because it can cause oxidative damage to cellular macromolecules. Indeed, an increased level of either intracellular or extracellular free heme can cause oxidative stress and injury by various mechanisms, most notably by lipid peroxidation (10, 33, 52). In higher eu-

Department of Pharmacology and Toxicology, College of Medicine, Inha University, Incheon, South Korea.
*Present address: Department of Biochemistry, Faculty of Science, Burapha University, Chonburi, Thailand, 20131.

karyotes, expression of HO-1 increases promptly in response to oxidative stress, and HO-1 degrades (eliminates) the free heme while producing antioxidants. Thus, aerobic cells can protect themselves from the toxic effects of free heme by enhancing the expression of HO-1 (63). HOs are the rate-limiting enzymes catalyzing oxidative degradation of heme. There are at least three isoforms of HO—the oxidative stress or heme-inducible HO-1 and the constitutively expressed non-inducible HO-2 and HO-3—all of which are present in most mammals (64, 65, 96, 98). They catalyze the oxidation of heme (iron-tetrapyrrole complex) to carbon monoxide (CO) and biliverdin and release ferrous (Fe^{2+}) iron (Fig. 1); in mammalian cells, biliverdin is subsequently reduced to bilirubin in the cytosol by biliverdin reductase, a rapidly NADPH consuming enzyme (63).

Heme-containing proteins release heme upon their structural alteration, destabilization, fragmentation, and proteolysis, and reactive oxygen species (ROS) promote all of these heme-releasing processes (27). As the released free heme is potentially toxic, it needs to be removed by degradation. Nearly 35 years ago, Tenhunen *et al*. (109) reported that heme can serve not only as the native substrate of HO activity but also as the specific stimulant increasing HO-1 expression. Thus, the free heme released from heme enzymes by the ROS-producing heme enzymes can be eliminated ironically by the enhanced HO activity occurring promptly in response to oxidative stress. As the result, free heme can be removed rapidly at the price of releasing bilirubin, CO, and iron. Ferritin synthesis also increases promptly in response to the free iron released from heme by HO activity, and ferritin chelates or stores the free iron to prevent additional production of $HO^{\cdot}$ catalyzed by the Fenton reaction (9, 15, 52). Thus, as shown in the classic view of metabolic control, expression of HO-1 is increased by its native substrate heme (109). In addition to the free heme, various other pro-inflammatory non-heme stimulants such as bacterial lipopolysaccharides, cytokines, heavy metals, physical stress, heat shock, and other oxidants can increase HO-1 expression. All these HO-1 inducers commonly cause oxidative stress, oxidation of intracellular reduced glutathione (GSH), and oxidative cell damage (17, 56, 80, 97, 100, 110, 118).

HO-1 is an oxidative stress-inducible defense enzyme that converts the toxic free heme into cytoprotective antioxidants (85). Products generated from HO activity—namely, the bile pigments (biliverdin, bilirubin) and CO—have been shown to have antioxidant and anti-inflammatory activities, respectively (21, 83, 92, 104). Thus, enhancing the HO-1 expression and HO activity will then increase (a) the elimination rate for the toxic free heme, (b) the production rate for the bile pigments (antioxidants) scavenging ROS and reactive nitrogen species (RNS), and (c) the production rate for the CO inhibiting further generation of ROS and RNS, all leading to protect

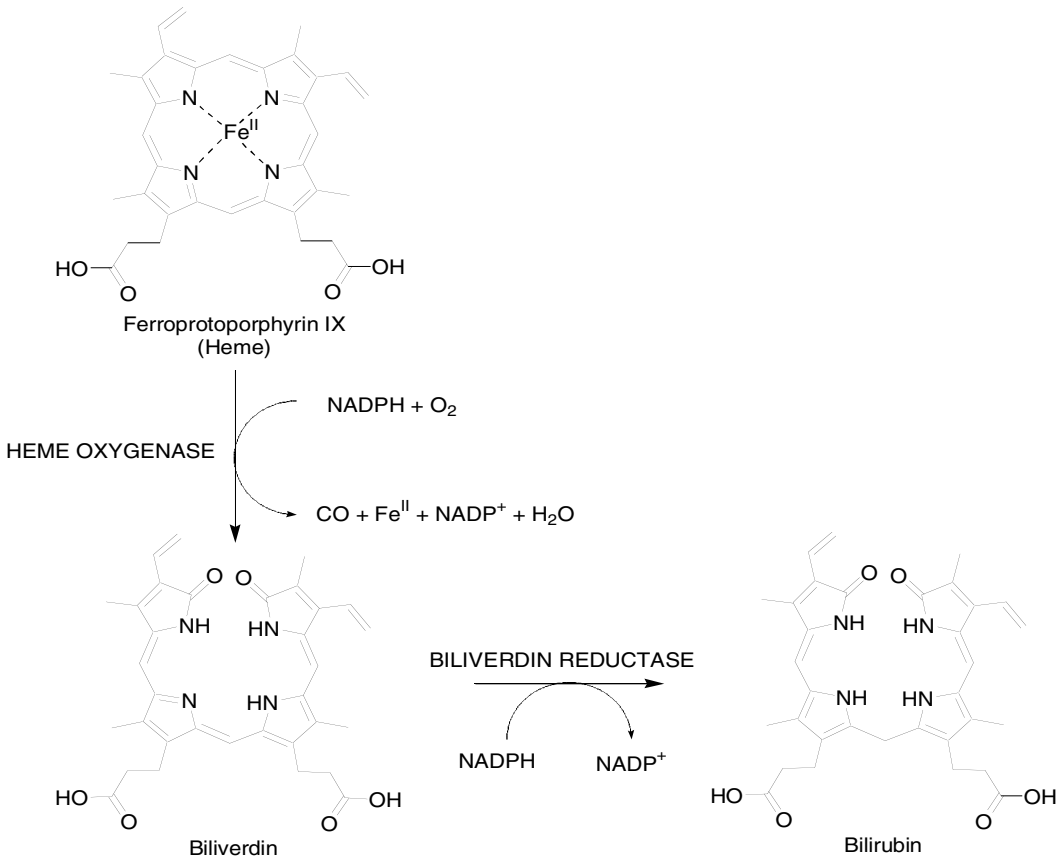

FIG. 1. Heme catabolism pathway. HO oxidizes heme to biliverdin, which then becomes reduced to bilirubin by biliverdin reductase.

the cells from ROS- and RNS-derived injuries (22, 26, 55, 62, 92, 101, 108). In support of this, overexpression of HO-1 has been associated with increased resistance to oxidative tissue injury in some clinical conditions (35, 75, 81, 99, 113, 119, 120). This led to a gene therapy approach employing the transfection of the *ho-1* gene (82).

INDUCTION OF HO-1 EXPRESSION BY OXIDATIVE AND NITROSATIVE STRESSES

ROS such as hydrogen peroxide (H_2O_2), O_2^-, and $HO^{\cdot}$ are generated even during normal course of cellular metabolism catalyzed by several heme-containing enzymes (*i.e.,* NADPH-oxidase, mitochondrial respiratory cytochromes, P-450). Production of ROS can increase markedly by exposure to noxious stimuli such as physical stress, heavy metals, ultraviolet (UV) irradiation, and bacterial endotoxins, the inducers of HO-1 expression. These ROS, although produced by the heme-containing enzymes, cause alteration of protein structures, destruction of enzyme functions, enhance proteolytic degradation of ROS-producing heme proteins themselves, and, finally, can promote the release of heme from heme-containing enzymes, as mentioned earlier (27). In response, cells detoxify the ROS by utilizing direct ROS scavengers, either obtained from exogenous sources (*e.g.,* vitamins and plant-derived phenolic antioxidants) or produced from endogenous biomolecules (*e.g.,* proteins, amino acids, GSH, and NO). Cells also induce antioxidant enzymes and use these enzymes to inactivate the ROS enzymatically (*e.g.,* superoxide dismutase, catalase, and glutathione peroxidase) in their attempt to survive from injuries that can be caused by excessive production of ROS.

As for the induction of these antioxidant enzymes occurring in response to excessive production of ROS, most notably, oxidation and depletion of intracellular GSH play the key role. In their effort to maintain redox homeostasis from lowered intracellular thiol/disulfide ratio, cells respond by enhancing the expression of a select set of early response genes that encode the cytoprotective antioxidant enzymes (90, 93). These cytoprotective enzymes are involved in enhancing the production of direct antioxidants, like GSH and NO, needed for rapid detoxification and scavenging of ROS, respectively. Among several cytoprotective enzymes induced by ROS, inducible NOS (iNOS) and HO-1 are most prominent. Thus, the ROS produced initially by variety of stimuli lead to enhancement of the synthesis of iNOS, and HO-1, the generators of NO and CO, respectively, the gaseous molecules needed for rapid scavenging of O_2^- and for suppressing additional production of O_2^-, respectively.

The NO radical is a gaseous signaling molecule involved in a wide variety of biological processes (23, 32, 46, 115). With a physiological level of stimulation, constitutively expressed endothelial NOS and neuronal NOS are activated immediately and produce small amounts of NO intermittently upon entry of Ca^{2+} (7). This NO can scavenge the small amount of O_2^- produced under normal physiological stimulation. However, when stimulation becomes excessive, a large amount of O_2^- is produced (*i.e.,* NADPH-oxidase-catalyzed oxidative burst), and this causes oxidative stress, oxidizing or depleting cellular GSH. In response, several redox-sensitive transcription factors like the nuclear factor-κB involved in enhancing iNOS expression are activated (31). Increased expression of iNOS causes excessive production of NO, perhaps to scavenge the large amount of O_2^- being generated by unphysiological and toxic stimuli. However, such NO-derived scavenging of excess O_2^- occurring rapidly at the rate of 6.7×10^9 *M*/s (43) will generate abundant peroxynitrite ($ONOO^-$), a highly reactive and strong GSH-oxidizing agent (14, 40, 116). This $ONOO^-$ will, in turn, cause rapid oxidation of GSH, lowering the thiol/disulfide ratio and depleting the GSH level (34, 87, 117). Alternatively, NO can also bind directly to heme-iron and -SH residues contained in a variety of heme and non-heme proteins with high affinity, causing nitrosative injury to cells (11, 25, 72, 102, 105). Thus, these RNS including NO and $ONOO^-$ can cause structural alteration of heme enzymes, destabilizing, inactivating, and enhancing their proteolytic degradation, as with ROS (27). This would cause the heme to be released from heme-containing enzymes like the NO-producing iNOS itself. Such RNS-released free heme may serve to enhance the expression of HO-1 and undergo accelerated degradation by enhanced HO activity, producing bilirubin and CO. In this connection, several recent studies reported that endogenously produced NO enhances HO-1 expression along with oxidation and depletion of GSH (16, 73, 100). GSH depletion alone without NO production was, however, able to induce HO-1 expression, and when this GSH depletion was combined with exposure to NO, either produced endogenously or delivered exogenously, synergistic potentiation on HO-1 expression was observed (8, 90, 100). This indicated that GSH depletion resulting from NO-derived nitrosative stress mediates the NO-derived induction of HO-1 expression.

Effective stimulation of HO-1 expression in many cell types can be achieved not only by exposure to compounds that release NO, but also by agents that liberate nitrosonium cation (NO^+; GSNO) or nitroxyl anion (NO^-; Angeli's salt [AS]), the one-electron oxidation and reduction products, respectively, of NO (13, 29, 47, 73, 76, 77, 101). Furthermore, when the cells were exposed to hemin (oxidized heme) together with these compounds releasing either NO or its redox metabolites, there was a synergistic potentiation on HO-1 expression, over and above the added sum of that induced either by NO and its redox metabolites alone or by hemin alone. In this context, a product of direct interaction between heme and NO or its redox activated forms (*i.e.,* NO^-, NO^+)—heme-nitrosyl complex—has been proposed as the molecular switch causing synergistic potentiation of HO-1 expression (77). Such superinduction of HO-1 will lead to faster elimination of toxic heme with greater production of antioxidants like bilirubin and CO. Thus, heme is serving not only as the required substrate of HO activity to produce these antioxidants but also as the synergistic potentiator of HO-1 expression, which has been stimulated by NO initially (77, 100). Such a magnified induction of HO-1 expression caused by the heme released from NO-producing heme proteins like iNOS itself would then increase the intrinsic antioxidant defensive capacity while eliminating the toxic free heme released from heme proteins. This renders the oxidatively stressed cells more tolerant to nitrosative stress as well.

Central to this response causing enhanced expression of HO-1 is the activation of redox-responsive transcriptional factors like heat shock factors (88) and members of activator protein-1 (AP-1) (16, 80, 120) and nuclear transcription factor erythroid 2p45-related factor 2 (Nrf2) (6, 77) families. Activation of these redox-sensitive transcription factors is promoted by both oxidative and nitrosative stresses, which oxidize or deplete intracellular GSH. Once activated, these transcription factors are transported into nucleus and bind to DNA sequences in heme-responsive elements (HREs), stress-responsive elements (StREs), antioxidant-responsive elements (AREs), and xenobiotic-responsive elements (XREs) localized in the promotor region of the genes coding for various stress-responsive antioxidant enzymes (16, 20, 49, 57, 69, 80, 88, 93, 114). Thus, stress-derived activation of these redox-sensitive transcription factors, their transport into nucleus, and their binding to these enhancer elements are involved in stimulating the transcription of early response genes that code for various stress-responsive antioxidant enzymes like iNOS and HO-1, the producers of NO and CO, respectively, in aerobic cells. Cellular events leading to such sequential induction of iNOS and HO-1 are depicted in Fig. 2.

INDUCTION OF HO-1 EXPRESSION BY ACTIVATION OF NRF2

Molecular mechanisms involved in the increased expression of HO-1 occurring in response to numerous toxins and diverse stressful conditions have remained elusive. However, one important clue came recently from detailed analysis of transcriptional regulatory mechanisms controlling the expression of HO-1 in mouse and human cells. Transcriptional activation of *ho-1* gene expression is regulated principally by two upstream enhancer sequences, E1 and E2 (1–3, 6, 17). Both E1 and E2 enhancer sequences contain multiple StREs (48), and they were all found have the core DNA sequences identical to that of Maf recognition element (MARE) (53). Additional studies have implicated that the redox-sensitive transcription factor Nrf2, which can combine with one or another of small Maf nuclear proteins (*i.e.,* MafK, MafF, and MafG), is also involved in the induction of HO-1. As heterodimers, the Nrf2/Mafs are involved in activating HO-1 expression through binding to these StREs and MAREs contained in E1 and E2 sequences (4–6, 54). Such Nrf2-Maf heterodimers bind to upstream *cis*-elements of many stress-inducible genes that have the core sequences known as MAREs (59, 68), AREs (50, 111), and StREs (60) (Fig. 3). Nrf2 binding to these enhancer elements up-regulated many of the genes that are activated by various oxidants, electrophiles, or xenobiotics. These include the genes that encode, in general, the Phase II detoxification enzymes such as NAD(P)H:quinone oxidoreductase, γ-glutamylcysteine synthase, glutathione *S*-transferase, HO-1, and thioredoxin (6, 36, 49, 50, 57), among others. Individually and collectively, these select set of Nrf2-regulated gene products function to disrupt the ROS-generating one-electron redox cycling reactions, maintain cellular levels of reducing equivalents, detoxify xenobiotics, counteract the effects of ROS, and reduce the oxidized proteins, respectively. Given these activities of Nrf2 target gene products, Nrf2 appears to be one of the key physiological regulators of cellular adaptive response to stresses caused by oxidants and xenobiotics. Consistent with this idea, Nrf2-deficient mice were found to be more prone to butylated hydroxytoluene-mediated pulmonary dysfunction (18), were more susceptible to acetaminophen-derived hepatotoxicity (19, 28), and exhibited a significantly higher burden of benzo[*a*]pyrene-induced gastric neoplasia with reduced chemoprotective efficacy (89). Moreover, macrophages derived from Nrf2-deficient mice had reduced resistance to toxic electrophilic compounds (49).

Although much is known about Nrf2 target genes, mechanisms by which xenobiotics and oxidants regulate Nrf2 activity are not well characterized and are under active investigation.

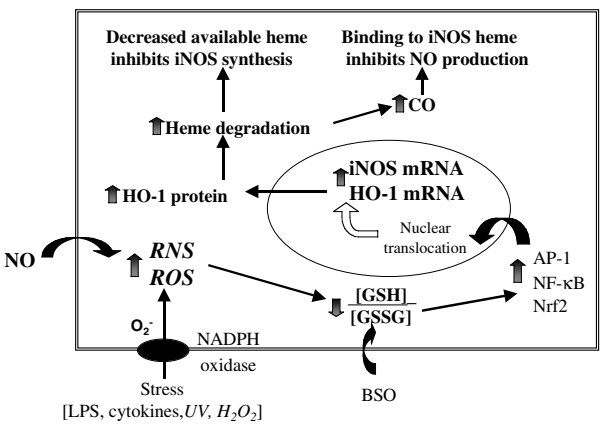

FIG. 2. Schematic diagram showing the pathway leading to inductions of HO-1 and iNOS initiated by external stress stimuli and the role of HO-1 induction in inhibiting iNOS synthesis and NO production (63). BSO, buthionine sulfoximine; GSSG, oxidized glutathione; LPS, lipopolysaccharide.

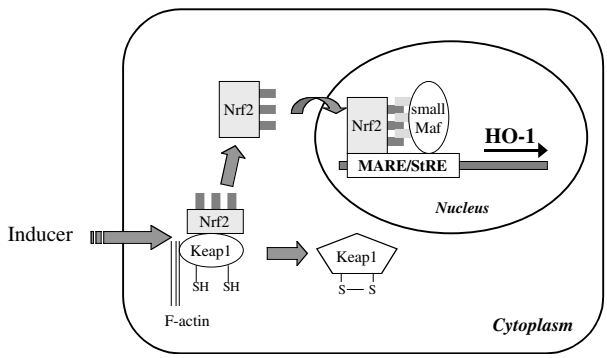

FIG. 3. Proposed mechanism for induction of HO-1 expression via activation of Nrf2 and its translocation into nucleus to bind MARE/StRE in association with small Maf nuclear protein. Nrf2 exists in an inactive state, normally sequestered by Keap1 in cytoplasm. Upon exposure to various oxidants, Nrf2 is liberated from Keap1, translocates into the nucleus, and forms a heterodimer with small Maf nuclear protein, and the heterodimer binds to MARE/StRE localized in the promoter region of *ho-1* and other target genes to activate their transcription.

Accumulated data suggest that Nrf2 activity is regulated, at least in part, at the level of its subcellular compartmentalization (6, 42, 58, 95, 124). According to this model, Nrf2 exists in cytoplasm in an inactive state bound to cytoskeleton-associated protein Kelch-like ECH-associated protein 1 (Keap1) under normal conditions (51, 58, 67). Upon stimulation by agents causing oxidative stress, -SH residues contained in Keap1 are oxidized and the Keap1-dependent cytoplasmic Nrf2 retention mechanism is inactivated. Consequently, Nrf2 is released, activated, and transported into the nucleus. In the nucleus, Nrf2 binds to several response elements like MAREs, AREs, XREs, and StREs in association with small-Maf nuclear proteins to activate the transcription of various Nrf2 target genes mentioned above (Fig. 3). Furthermore, some xenobiotics have been demonstrated to increase the Nrf2 level also by stimulating transcription of the *Nrf2* gene itself. In this connection, Kwak *et al.* (60) recently described a positive feedback mechanism in which the Nrf2 autoregulates its own expression in response to ^{3}H-1,2-dithiol-3-thione *via* an ARE-like sequence present in the promoter region of the *Nrf2* gene.

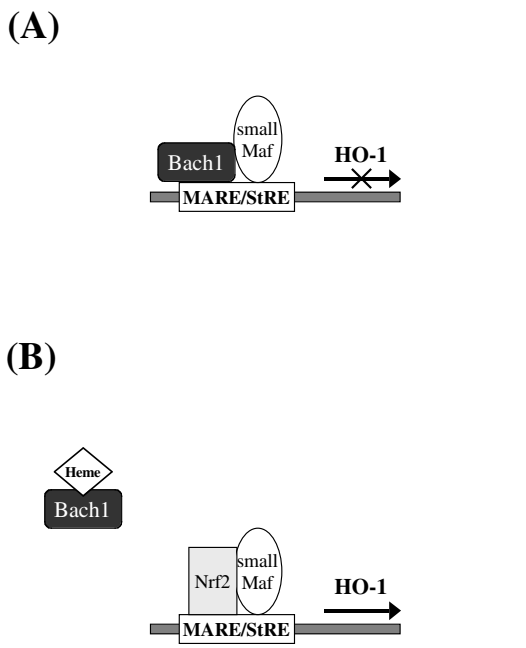

FIG. 4. Schematic model showing de-repression of Bach1 by heme (106). (**A**) Under the normal unstimulated condition, the heterodimer of Bach1 and small Maf is bound to MARE/StRE, and transcription of *ho-1* is inhibited or repressed. (**B**) Under stress conditions producing ROS and RNS, heme is released from hemoproteins. The released free heme binds to Bach1 via its heme regulatory motif and displaces Bach1 from MARE/ StRE, de-repressing *ho-1* transcription. Once MARE/StRE is relieved from Bach1-derived repression, Nrf2 replaces Bach1, forming the Nrf2–Maf heterodimer and binding to MARE/ StRE as a unit to stimulate the transcription of *ho-1*.

INDUCTION OF HO-1 EXPRESSION BY HEME-DERIVED DE-REPRESSION OF BACH1

As mentioned above, heme is an essential molecule in aerobic cells, and heme plays a central role in electron transfer reactions involving diatomic gases like molecular oxygen (O_2), NO, and CO and catalyzes their redox reactions. In addition, heme can participate in the regulation of gene transcription by acting as a ligand for several transcription factors in prokaryotes (70, 86) and yeasts (30, 41, 122, 123). Heme is proposed to perform similar regulatory roles in higher eukaryotes as well (94). Consistent with this hypothesis, it was found recently that heme binds to a mammalian transcriptional repressor, Bach1 (84), and interferes with its binding to DNA (79). Thus, heme can regulate the DNA binding activity of Bach1 negatively and can remove Bach1-derived repression, perhaps allowing the Nrf2-dependent transcription of the *ho-1* gene to be activated (106). Repressors can restrict the expression of activator-dependent target genes in many cell types, and several transcriptional activators can induce gene expression only in the absence of repressor function (38). An emerging concept suggests that transcriptional activators stimulate the expression of *ho-1* only upon heme (substrate)-specific de-repression of Bach1-derived repressor function (106).

Cysteine-proline dipeptide sequences contained in Bach1, a conserved heme regulatory motif (HRM) (61, 66, 79, 91), mediate the Bach1–heme interaction. Binding of heme to the HRM in Bach1 causes structural modification and makes the Bach1 unable to bind to MARE sequences. In the absence of free heme, however, Bach1 forms heterodimers with small Maf nuclear proteins (as does Nrf2), and together, the Bach1-Maf heterodimer binds to MAREs and represses the transcription of *ho-1* in a MARE-dependent manner (45, 84, 107) (Fig. 4). The above model suggests that the *ho-1* locus is situated in a chromatin environment that is permitted to undergo transcriptional activation only upon removal of Bach1-derived repression (de-repression), and this alleviation of repression is caused by heme-specific inhibition of Bach1 binding to the MARE sequence. In the presence of heme or in the absence of Bach1-derived repression, the MARE sequence can now bind various other Maf-dependent heterodimeric basic leucine zipper factors, including the Nrf2 and AP-1 families involved in activating the transcription of *ho-1*. However, random binding of these transcriptional activators to the MARE sequences contained in the E1 and E2 enhancers of the *ho-1* gene, which have been relieved from Bach1-derived repression, can cause an uncontrolled excessive HO-1 expression. This raises a paradox as to how cells can reduce and regulate the uncontrolled excessive transcriptional activation of the *ho-1* gene that can occur in the absence of metabolic or environmental stimulation. The presence of heme-removable repressors like Bach1, preventing nonspecific activation of *ho-1* transcription, can handle this problem. The repressor function of Bach1, which contains HRM, is regulated specifically by the availability of free heme, the native physiological substrate of HO-1 that is released by oxidative and nitrosative stresses. This may explain the reason for the lack of *ho-1* gene expression observed under normal physiological condition. Under normal condi-

tions, protein-unbound free heme is not available, and Bach1 binds the MAREs to repress the transcription of *ho-1*. Only when free heme becomes available by ROS- or RNS-derived liberation from heme enzymes, can it displace Bach1 from MAREs. This allows the binding of transcriptional activators (*e.g.,* Nrf2, AP-1) to MAREs and the activation of *ho-1* gene transcription (Fig. 4).

To investigate the physiological role of Bach1 in suppressing the uncontrolled transcriptional activation of the *ho-1* gene *in vivo*, Sun *et al.* (106) employed mice with a disrupted *Bach1* gene. Bach1 knock-out (*Bach1* −/−) mice were fertile and appeared grossly normal in size and morphology, and this indicated that development and reproduction of mice were not affected by deletion of the *Bach1* gene. These *Bach1* −/− mice, however, served as an ideal genetic tool to test the possible involvement of Bach1 in regulating *ho-1* gene expression. The levels of HO-1 expressed in various organs obtained from *Bach1*-deleted mice were much higher than that expressed in the same organs of wild-type normal mice. One exception was spleen, an organ normally involved in the degradation of hemoglobin delivered from senescent red blood cells. With such an abundant supply of heme released from hemoglobin, the spleen can express a high level of HO-1 even under normal physiological condition in wild-type mice. These results suggested that, when abundant heme is supplied by hemolysis, as is the case with spleen, high-level HO-1 expression can occur because of the effect of removal of Bach1-derived repression on *ho-1* gene transcription. HO-1 mRNA was expressed at much higher levels in thymus, heart, lung, and liver in the *Bach1* −/− mice than in the same organs obtained from wild-type or heterozygous *Bach1* mutant mice. These data suggested further that lack of HO-1 expression seen in various tissues of wild-type mice under normal physiological condition might have been due to the Bach1-dependent repression, preventing several transcriptional activators of *ho-1* expression from functioning.

BIFUNCTIONAL ROLE OF MARE IN HARBORING MAF-DEPENDENT REPRESSION AND ACTIVATION OF *HO-1* GENE TRANSCRIPTION

As with Bach1, Nrf2 also binds to the MAREs present in E1 and E2 enhancers of the *ho-1* gene, but unlike Bach1, Nrf2 modulates *ho-1* gene transcription in a positive manner. In an effort to examine the reciprocal regulatory relationship between Nrf2 and Bach1 on HO-1 expression, Sun *et al.* (106) employed co-transfection assays. Nrf2 activated the expression of *ho-1* reporter (luciferase) only in the absence of Bach1. When co-expressed, however, Bach1 antagonized the Nrf2-dependent activation of *ho-1* reporter activity and repressed the luciferase activity dominantly. By increasing the heme level, however, the dominant repressor activity of Bach1 was lost, shifting the balance of this dominant repression toward Nrf2-dependent activation of *ho-1* gene transcription. These data indicated that Bach1 alone antagonizes the activator activity of Nrf2 and that Bach1 repressed the Nrf2-dependent transcription of the *ho-1* gene in concert with pre-existing small Maf nuclear proteins. This suggested further that, in the absence of Bach1-derived repressor activity or in the presence of heme, some other redox-active basic leucine zipper transcription factors like AP-1 and activating transcription factor can also become active constitutively. This possibility needs to be tested in the future.

Results obtained from studies dealing with overexpression of Bach1, Nrf2, and Maf suggested that MARE regulates the expression of the *ho-1* gene both in positive and in negative ways. In other words, small Maf nuclear proteins can regulate the expression of the *ho-1* gene depending on the balance between small Mafs and their heterodimer partners binding to MAREs, either the transcriptional activator Nrf2 or the transcriptional repressor Bach1 (44, 71). To assess the involvement of small Maf proteins in *repressing ho-1* gene expression when it forms a heterodimer with Bach1, binding of small Maf proteins to MAREs present in the E2 enhancer was determined in cells obtained from either wild-type or *Bach1* −/− mice and also in the presence or absence of antibodies raised against small Maf. Binding of small Maf proteins to the E2 enhancer was clearly evident in thymocytes obtained from both wild-type and *Bach1* −/− mice. Alternatively, to assess the involvement of small Maf proteins in *activating ho-1* expression when it forms a heterodimer with Nrf2, binding of Maf proteins to MAREs present in the E2 enhancer was also determined in cells obtained from both wild-type and *Nrf2* −/− mice, again in the presence and absence of anti-Maf antibodies. Binding of small Maf proteins to the E2 enhancer was again evident in thymocytes obtained from both wild-type and *Nrf2* −/− mice. These results are consistent with a model in which the small Maf nuclear proteins can participate in *ho-1* gene transcription both in repression and in activation. The partner molecule for repression of *ho-1* gene expression is Bach1, whereas that for activation of *ho-1* gene expression is Nrf2 (or other basic leucine zipper transcriptional activators like AP-1 or activating transcription factor).

INHIBITING PROTEOLYTIC DEGRADTION OF ACTIVATED NRF2 LEADS TO ENHANCED HO-1 EXPRESSION

As mentioned earlier, Nrf2 appears to be one of the key physiological regulators of cellular adaptive response to oxidants, electrophiles, and xenobiotics. In the normal unstressed cell, Nrf2 exists in cytoplasm bound to the cytoskeleton-associated protein Keap1 in an inactive state protected from the proteolytic degradation catalyzed by the ubiquitin/ proteasome pathway (24, 58, 67). Once released, the activated Nrf2 undergoes rapid proteolytic degradation by the ubiquitin/proteasome pathway in the cytoplasm before entering the nucleus. Incidentally, the same oxidative and nitrosative stresses that are involved in releasing and activating Nrf2 can also enhance the proteolytic activity of the ubiquitin/proteasome pathway. Thus, only the Nrf2 surviving from this enhanced proteolytic degradation system can go into the nucleus by yet uncharacterized mechanisms. Once in the nucleus, Nrf2 can activate the transcription of *ho-1* and several other antioxi-

dant enzyme genes, which have the StRE and MARE sequences in their enhancer region. This suggested that protecting the activated Nrf2 from the ubiquitin/proteasome pathway is also of importance in enhancing *ho-1* gene expression.

Previously, it was demonstrated that cadmium-dependent *ho-1* gene activation required activation of Nrf2 in several cell types, including Hepa cells (5, 37, 103). Treatment of Hepa cells with cadmium chloride ($CdCl_2$) increased the steady-state level of activated Nrf2 in a time-dependent manner to a level that was greater than 20-fold above the basal values. Within 30 min after the $CdCl_2$ treatment, well before any observable increase in HO-1 mRNA (typically observed between 1 and 2 h after exposure to $CdCl_2$) and HO-1 protein (2–3 h), the Nrf2 released from Keap1 began to accumulate in cytoplasm. In contrast to the increased level of Nrf2 protein, cadmium exposure did not significantly alter the steady-state level of Nrf2 mRNA in Hepa cells. As expected, concomitant with the accumulation of released Nrf2, expression of HO-1 mRNA increased dramatically in a time-dependent manner in the cadmium-treated cells. In this regard, we observed that $CdCl_2$ increased the expression of HO-1 and protected C-6 neuroblastoma cells from injury caused by subsequent cadmium-driven oxidative stress (101). Taken together, these results suggested that cadmium enhances HO-1 expression by increasing the Nrf2 level, primarily by a post-transcriptional mechanism.

A recent study by Stewart *et al.* (103) showed that $CdCl_2$ increased the cytoplasmic level of Nrf2 in a time-dependent manner. Cadmium may increase the level of Nrf2 either by enhancing the rate of Nrf2 synthesis or by inhibiting the rate of Nrf2 degradation. Exposure to $CdCl_2$ did not increase the rate of [^{35}S]methionine-labeled Nrf2 accumulation, and this indicated that cadmium did not affect the rate of Nrf2 synthesis. This suggested that cytoplasmic accumulation of Nrf2 in $CdCl_2$-treated cells might be caused by stabilization or inhibition of Nrf2 from proteolytic degradation. To examine the role of cadmium in protecting Nrf2 protein from degradation, thus leading to its accumulation, Stewart *et al.* (103) monitored the decay rate of Nrf2 in Hepa cells after inhibition of protein synthesis with cycloheximide. After addition of cycloheximide, Nrf2 in the $CdCl_2$-untreated cells decayed rapidly, disappearing after 40 min, and the calculated half-life of Nrf2 was about 13 min. In the $CdCl_2$-treated cells, however, the elevated Nrf2 was more stable and had an estimated half-life of nearly 100 min. These results indicated that cadmium causes Nrf2 accumulation primarily by attenuating the rate of its proteolytic degradation (103).

Proteolytic activity of 26S proteasome, a highly conserved and multi-protein proteolytic system, which incidentally is increased markedly by oxidative stress, degrades many labile cytoplasmic proteins, including signal-activated transcription factors like Nrf2, to preserve homeostasis (39, 78). Upon treatment of Hepa cells with MG-132 or lactacystin, selective inhibitors of proteolytic activity of 26S proteasome, the steady-state level of Nrf2 was increased significantly, and in accordance, expression of HO-1 was enhanced markedly (78, 103). These proteasome inhibitors did not affect the steady-state level of Nrf2 mRNA, however. These results indicated that cadmium could enhance the expression of HO-1 by inhibiting proteasomal degradation of the released Nrf2 protein, but not by transcriptional activation of Nrf2 mRNA. Stewart *et al.* (103) demonstrated further that proteasome inhibitors and cadmium increase the stability of Nrf2 in cells by employing pulse-chase experiments. Under normal physiological condition, the [^{35}S]methionine-labeled Nrf2 undergoes rapid degradation and disappears by 30 min after termination of labeling. In the presence of the proteasome inhibitor MG-132 or $CdCl_2$, however, labeled Nrf2 remained at a constant level. Other non-proteasomal protease inhibitors like phenylmethylsulfonyl fluoride (inhibitor of serine proteases), PD-150606 (inhibitor of calpain), leupeptin (inhibitor of serine/cysteine proteases), and chloroquine (inhibitor of lysosomal proteases) did not protect the labeled Nrf2 from degradation. Taken together, these results indicated that 26S proteasome degrades Nrf2 and prevents prolonged activation of HO-1 expression.

Most proteins, but not all, selectively degraded by 26S proteasome are marked by covalent ligation of molecules of ubiquitin, a highly conserved 8-kDa polypeptide, to the ϵ-amino group of their lysine residues. GSH-oxidizing redox-stress signals provided by either ROS or RNS can promote this ubiquitination process. The phosphorylation-dependent ubiquitin-conjugating system usually generates proteasome-degradable proteins containing varying lengths of polyubiquitin chains (39). Thus, elevation of levels of ubiquitinylated Nrf2 following inhibition of the 26S proteasome proteolytic activity would then suggest that the ubiquitin–proteasome system is involved in the degradation of this transcription factor. In the $CdCl_2$-treated cells accumulating Nrf2, a ladder of mono- and polyubiquitinylated Nrf2 was detected readily (103), and this result indicated that the ubiquitin–proteasome pathway targets Nrf2 for a rapid degradation. The result suggested further that cadmium inhibited the proteolytic activity of proteasomes and delayed the rate of Nrf2 degradation and thus activated the *ho-1* gene transcription.

Cycloheximide, an inhibitor of protein synthesis, abrogated Nrf2 accumulation in cytoplasm and decreased the amount of Nrf2 binding to DNA in the nucleus. However, it did not alter the level of MafG, the dimerization partner of Nrf2 for binding to MARE sequences in the nucleus. Furthermore, although cycloheximide did not alter the steady-state level of Nrf2 mRNA, it abolished Nrf2 protein, suggesting that it allowed the degradation of existing Nrf2 by 26S proteasome-derived proteolytic attack. This cycloheximide-dependent abrogation of Nrf2 accumulation in cytoplasm and decreased Nrf2 binding to MARE in the nucleus correlated well with attenuation of HO-1 mRNA and protein accumulation (103). In contrast, results obtained with proteasome inhibitors indicated that they suppress the degradation of Nrf2 and thus enhance the expression of HO-1 mRNA and protein. With MG-132, the most potent proteasome inhibitor, highest accumulation of Nrf2 and HO-1 mRNA (17-fold) was observed. This proteasome inhibitor, however, did not enhance cadmium-dependent accumulation of HO-1 mRNA further. This suggested that MG-132 activates *ho-1* gene transcription by a similar mechanism as with cadmium. The observation that MG-132, like cadmium, promoted formation of the Nrf2/StRE complex and stimulated the expression of *ho-1* reporter gene and luciferase reporter activity by 13-fold pro-

vided further support for this hypothesis. Taken together, the above results suggested that cadmium or proteasome inhibitors can cause accumulation of activated Nrf2 in cytoplasm, allowing more of the transcriptional activator to be transported into the nucleus without degradation and permitting a greater amount of functional transcription factor to bind to its target enhancer sequences in E1 and E2. Thus, inhibition of proteasome activity can lead to highly efficient transcription of their target genes like *ho-1*.

In a separate study using renal epithelial cells conducted by Alam *et al.* (6), exogenously added heme was found to stabilize Nrf2 from degradation and stimulate *ho-1* gene transcription. Heme interfered with Nrf2 degradation by an unknown mechanism and allowed markedly higher accumulation of Nrf2 in the nucleus, permitting enhanced Nrf2 heterodimerization with MafG. High levels of the Nrf2-MafG heterodimer accumulated under such a condition then displaced some of the repressor proteins bound to the StRE and promoted a high rate of *ho-1* gene transcription. Thus, together with the direct heme-derived removal of Bach1 repressor from *ho-1* enhancers (106, 107), the indirect heme-promoted accumulation of the Nrf2-MafG heterodimer activator unit displacing the Bach1-Maf heterodimer repressor unit from *ho-1* enhancers was suggested to be involved in the transcriptional activation of *ho-1* gene expression (6). As the heme-mediated activation of *ho-1* gene expression is a ubiquitous phenomenon observed in several aerobic cell types studied, this heme-mediated derepression and activation of *ho-1* enhancers appear to be general mechanisms operating in many other cell types.

SYNERGISTIC INDUCTION OF HO-1 EXPRESSION BY COMBINED EXPOSURE TO HEMIN AND NO DONORS

In a recent study reported by Naughton *et al.* (77), the effects of exposing cardiac cells to hemin (oxidized heme), AS (a donor of NO^-), or a combination of both agents on the expression of HO-1 protein and HO activity were determined. Predictably, following treatment of cardiomyocytes with increasing concentrations of exogenous heme (hemin), the substrate and inducer of HO-1, expression of HO-1 increased in a dose-dependent manner. Alternatively, exposure to AS also increased the expression of HO-1, confirming earlier data on the high inducibility of *ho-1* gene transcription by exposure to NO^- (34). Of major interest was, however, the finding that simultaneous addition of AS and hemin potentiated HO-1 expression markedly in a synergistic fashion. However, HO-1 expression did not increase in the hemin-treated cardiac cells exposed to a conditioned medium obtained from preincubation of AS for 1 h at 37°C. Because AS undergoes rapid decomposition at 37°C and the conditioned medium may no longer contain NO, it was understandable that the conditioned medium did not enhance HO-1 expression in hemin-treated cells. Interestingly, however, when the hemin was present with AS in culture medium during this 1-h preincubation period prior to their addition to cells, this conditioned medium was able to enhance HO-1 expression markedly up to the level obtained when applied simultaneously with both agents. These data suggested that a stable nitrosyl–heme complex is formed during preincubation and the complex may dissociate after entering the cell. The dissociated heme could then act as a separate inducer of HO-1 by reacting with Bach1 and removing the Bach1-derived repression on the transcription of the *ho-1* gene. The dissociated NO^- may act as an oxidant stress leading to activation of Nrf2, thus causing synergistic induction of HO-1 expression in a manner like a "one-two" punch: one removing the repression, and the other activating the transcription of *ho-1*. In support of this hypothesis, analysis of HO-1 mRNA expression by reverse transcription-polymerase chain reaction revealed that cells exposed to AS in the presence of low micromolar concentrations of hemin were able to intensify the HO-1 induction markedly (77).

Naughton *et al.* (77) also found that the AS-mediated increase of HO-1 accumulation was associated with an activation of the redox-sensitive transcription factor Nrf2. Nrf2 was highly present in the nuclear fraction between 3 and 6 h after exposure of cardiac cells to AS, but this effect was totally abolished by prior exposure of cells to *N*-acetylcysteine, the membrane-diffusible form of cysteine. In the presence of *N*-acetylcysteine and with the ensuing elevation of intracellular GSH level, the cysteine residues in Keap1 may remain intact. This intact Keap1 may retain the Nrf2 in its inactive state, not allowing its release and translocation into the nucleus and thus not allowing the activation of *ho-1* gene transcription. Interestingly, cells treated with hemin alone did not result in any detectable increase of Nrf2 in the nuclear fraction, understandably because the added hemin is involved only in eliminating the repression caused by Bach1, but not activating or translocating Nrf2 into the nucleus. The nuclear presence of Nrf2 was markedly intensified only when the cells were exposed to both NO and hemin together. Furthermore, this intensified presence of Nrf2 in the nucleus correlated with synergistic amplification of HO-1 expression. These data suggested further that amplification of HO-1 expression achieved by NO-derived activation of Nrf2 might be intensified synergistically only upon heme-derived elimination of the repression caused by Bach1.

The hemin-derived amplification of AS-inducible HO-1 also occurred when AS was replaced by an NO-releasing agent, 2-(*N,N*-dethylamino)-diazenolate-2-oxide diethylammonium salt (DEA/NO) or (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate (spermine NONOate). In analogy with the data obtained with AS, both DEA/NO and spermine NONOate promoted a marked increase in HO-1 expression when applied directly to cells exposed to hemin (Fig. 5). Also, as with AS, both DEA/NO and spermine NONOate were ineffective when they were allowed to decompose spontaneously in the culture medium for 1 h at 37°C prior to addition to cells. However, the conditioned medium obtained after the 1-h preincubation of these NO-releasing agents together with hemin led to a dramatic increase in HO-1 expression, to the level significantly higher than the added sum of HO-1 expression obtained either with hemin alone or with NO donor alone. Thus, not only NO^- (AS) but also NO (DEA/NO, spermine NONOate) has the ability to interact directly with heme to generate heme–nitrosyl complex, causing a synergistic up-regulation of HO-1 expression in cardiomyocytes and macrophages.

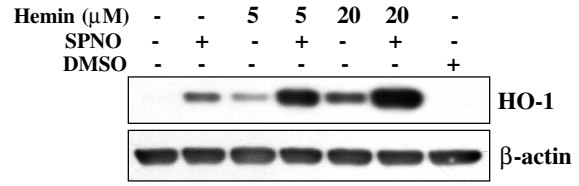

FIG. 5. Superinduction of HO-1 expression in RAW 264.7 macrophages by combination of heme and NO. Cells were treated with 20 μ*M* spermine NONOate (SPNO) in the presence or absence of 5 or 20 μ*M* hemin for 12 h. Hemin was dissolved in dimethyl sulfoxide (DMSO). The cellular content of HO-1 protein was determined and compared with the content of β-actin (used as an internal standard) by employing western blotting analysis. The blot shows a representative result obtained from three separate experiments.

CONCLUSIONS

The ability of heme to serve not only as a substrate of HO activity but also as a molecular switch in amplifying the *ho-1* gene expression initiated by NO in a synergistic manner may play a significant role in counteracting the injury that can be caused by the NO-derived nitrosative stress (29, 73, 76, 101). In support of this, results obtained in this and other laboratories (77) revealed that synergistic amplification of HO-1 protein level and HO activity observed in cells exposed to both hemin and NO is accompanied by marked increase in bilirubin production and survival. Products of heme degradation resulting from markedly enhanced HO-1 activity—namely, bilirubin (antioxidant) and CO (inhibitor of NADPH-oxidase and iNOS activity)—would then provide additional protection against $ONOO^-$. Increased chelation of $ONOO^-$ (by bile pigments) and inhibition of its generation (by CO) may provide additional cytoprotection against this highly reactive toxic product arising from concomitant oxidative (ROS) and nitrosative (RNS) stresses seen in many clinically important situations.

Synergistic amplification of HO activity by combination of heme and NO may have significant implications in those pathological states such as sickle cell anemia, inflammation, hemorrhagic shock, and tissue injuries caused by vascular ischemia and reperfusion. Local hypoxia, increased hemolysis, and augmented availability of free heme may occur under these pathological states. Liberated free heme, which is highly lipophilic, would activate the HO-1 pathway by the heme-dependent de-repression of Bach1 and also by serving as substrate for the elevated HO-1 activity, ultimately providing the cytoprotective antioxidants like bile pigments and CO (21, 83, 92, 104). Interestingly, these and other stressful conditions promoting the release and increasing the availability of heme may also be accompanied by induction of iNOS expression leading to overproduction of NO (47, 73, 106). The heme–NO interaction or generation of heme–nitrosyl complex arising under such a condition could then function as a potent signal to maximize the HO-1 induction providing maximal cytoprotection in those pathological states mentioned above. Thus, oxidatively stressed cells may enhance iNOS-derived NO production not only to scavenge O_2^- and activate Nrf2, but also to enhance the ROS- and RNS-derived liberation of heme from hemoproteins as well. Together, they promote synergistic amplification of HO-1 expression—first, by the heme-derived alleviation of repression caused by Bach1, and, second, by the NO-derived activation of Nrf2. It appears that oxidatively stressed cells are utilizing the heme—NO interaction, in a manner like a "one-two" punch to amplify the HO-1 expression maximally. Aerobic cells appeared to have adopted such an efficient regulatory mechanism to enhance HO activity and neutralize the potentially dangerous effects of concomitant oxidative and nitrosative stress reactions occurring in cells stressed by a variety of external stimuli.

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ABBREVIATIONS

AP-1, activator protein-1; ARE, antioxidant-responsive element; AS, Angeli's salt; CO, carbon monoxide; DEA/NO, 2-(*N,N*-dethylamino)-diazenolate-2-oxide diethylammonium salt; GSH, reduced glutathione; HO, heme oxygenase; $HO^{\cdot}$, hydroxyl radical; HRE, heme-responsive element; HRM, heme regulatory motif; iNOS, inducible nitric oxide synthase; Keap1, Kelch-like ECH-associated protein 1; MARE, Maf recognition element; NO, nitric oxide; NO^+, nitrosonium cation; NO^-, nitroxylanion; NOS, nitric oxide synthase; Nrf2, nuclear transcription factor erythroid 2p45-related factor 2; O_2^-, superoxide anion; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SPNO, spermine NONOate, (*Z*)-1-[*N*-(3-ammoniopropyl)-*N*-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate; StRE, stress-responsive element; UV, ultraviolet; XRE, xenobiotic-responsive element.

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Address reprint requests to:
Dr. Young-Nam Cha
Department of Pharmacology and Toxicology
College of Medicine
Inha University
Incheon, South Korea

E-mail: Youngnam@inha.ac.kr

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