Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide

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Hartsfield, Cynthia L., Jawed Alam, Julia L. Cook, and Augustine M. K. Choi. Regulation of heme oxygenase-1 gene expression in vascular smooth muscle cells by nitric oxide. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L980–L988, 1997.—Heme oxygenase (HO)-mediated heme degradation is the primary mechanism for production of cellular carbon monoxide (CO). Analogous to nitric oxide (NO), CO mediates physiological and cellular functions such as vasodilatation, stimulation of guanylate cyclase, and neuronal transmission. In view of accumulating data demonstrating a correlation between the activity of these two gaseous molecules and that the predominant source of CO is via HO catalysis, we hypothesized that NO regulates HO expression. We demonstrate that the NO donor spermine NONOate (SNN) increases steady-state levels of HO-1 mRNA in aortic vascular smooth muscle cells (aSMC) in both a time- and dose-dependent manner. The accumulation of HO-1 mRNA that correlated with increased HO-1 protein synthesis resulted from both an increased rate of gene transcription and a decreased rate of mRNA turnover. Inhibition of the NO-induced HO-1 mRNA expression by cycloheximide suggests that new protein synthesis is required for increased HO-1 gene expression. Induction of HO-1 expression by SNN occurs in a guanosine 3',5'-cyclic monophosphate (cGMP)-independent manner because exposure of cells to 8-bromoguanosine 3',5'-cyclic monophosphate, a cGMP analog, did not increase HO-1 mRNA levels, and pretreatment of cells with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, a selective guanylate cyclase inhibitor, did not prevent SNN-induced HO-1 mRNA accumulation. The antioxidant N-acetyl-L-cysteine markedly inhibited SNN-induced HO-1 mRNA expression, whereas peroxynitrite did not induce HO-1 expression in aSMCs. Interestingly, CO did not attenuate NO-induced HO-1 expression through an autocrine negative feedback mechanism as had been observed for hypoxia-induced HO-1 expression. These data provide evidence for an important regulatory network between NO and CO via HO-1.

Heme oxygenase (HO) catalyzes the rate-limiting step in the oxidative degradation of heme to biliverdin, releasing equimolar amounts of carbon monoxide (CO) and iron (6). Biliverdin is subsequently reduced to bilirubin by bilirubin reductase (6). Released iron from HO catalysis is sequestered into ferritin. Two isoforms of HO exist and are products of distinct genes (6). HO-2 is a constitutively expressed isofrom predominantly found in the central nervous system, whereas the HO-1 isofrom is inducible and ubiquitously distributed in mammalian tissues (6). In addition to heme, its major substrate, HO-1 synthesis is upregulated by a variety of nonheme inducers, including heavy metals, cyto-

kines, hormones, endotoxin, and heat shock (3, 5, 6). Recent studies also demonstrate that HO-1 is highly induced in response to various agents causing oxidative stress, including ultraviolet (UV) irradiation, sodium arsenite, hyperoxia, and glutathione depletors (1, 6, 14). Accumulating evidence suggests that HO-1 induction may mediate cellular protection against oxidant insults both in vitro (6, 11, 15, 22) and in vivo (23, 24).

In view of increasing evidence that HO-1 confers protection against oxidant-induced cellular and tissue injury, much attention has focused on the antioxidant properties of the catalytic by-products of HO catalysis, namely biliverdin and bilirubin. In addition, the concomitant increase in ferritin synthesis stimulated from iron released by heme degradation has also been targeted as a potential mediator of cytoprotection. CO, another catalytic product of HO activity, however, may also mediate important cellular functions. CO, a gaseous messenger similar to nitric oxide (NO), has been demonstrated to share many properties with NO, including activation of guanylate cyclase, signal transduction, and gene regulation (20, 25, 28, 34). Recent evidence (33) demonstrating similar localization of NO synthase (NOS) and HO-2 in blood vessels and autonomic nervous system strongly implies a possible coordinated physiological role for these two molecules. Indeed, CO from HO catalysis has been shown to modulate the NO-guanosine 3',5'-cyclic monophosphate (cGMP) signaling pathway in neuronal tissue (10) as well as hypoxia-induced HO-1 expression in cultured vascular cells (21). Conversely, recent studies have demonstrated that NO can modulate HO activity in brain and spleen tissue (30) and cultured vascular endothelial cells (22). Many biological processes mediated by NO are manifested through the activation or inhibition of metalloproteins such as guanylate cyclase (27). Although HO itself is not a heme-containing protein, it forms a transitory heme-protein complex when bound to its substrate (6) and could therefore be subjected to activation or inhibition by NO.

To improve our understanding in the coordinated regulation between NO- and HO-induced CO in vascular smooth muscle cells, we examined the mechanisms by which NO regulates HO-1 gene expression. This study demonstrates that NO donors are potent inducers of HO-1 mRNA and protein expression in aortic vascular smooth muscle cells (aSMC). Increased HO-1 gene expression by NO resulted from both an increased rate of HO-1 gene transcription and increased stability of HO-1 mRNA transcript. Whereas the induction of HO-1 expression by NO was indepen-
placental ribonuclease inhibitor, 0.5 mM each ATP, CTP, and GTP, and 150 μCi of [α-32P]UTP. The mixture was incubated at 30°C for 10 min, and the reaction was terminated by the addition of 600 μl of a 40 μg/ml deoxyribonuclease I solution. After 15 min at 30°C, SDS, EDTA, and proteinase K were added to achieve final concentrations of 1%, 2.5 mM, and 200 μg/ml, respectively. The reaction mixture was further incubated at 37°C for 30 min and then was extracted twice with a 1:1 mixture of phenol-chloroform in the presence of 100 μg of RNA. The labeled RNA was ethanol precipitated three times in the presence of unlabeled 0.1 mM UTP. The final RNA pellet was dissolved in 100 μl of diethyl pyrocarbonate-treated H2O.

Five micrograms of plasmid DNA in 200 μl of 0.2 N NaOH were heated at 95°C for 5 min, cooled on ice, and applied to Zeta-Probe membranes in a dot-blot apparatus to prepare filters for nuclear transcription assays. The DNA-bound filters were preincubated in hybridization solution [3× SSPE (1× SSPE = 0.15 M NaCl, 1 mM EDTA, and 10 mM sodium phosphate buffer, pH 7.4) containing 40% (vol/vol) deionized formamide, 7% (wt/vol) SDS, and 50 μg/ml Escherichia coli tRNA] for 1 h and then were incubated with labeled RNA (3 × 16000 counts/min (cpm)) in 1 ml of hybridization solution for 48 h at 45°C. After hybridization, filters were washed with solutions of 2× SSC (1× SSC = 0.15 NaCl and 15 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 30 min and 0.1× SSC and 0.1% SDS at 50°C for 60 min. Filters were exposed to X-ray film (Kodak X-Omat AR) for 6 h.

cDNA and oligonucleotide probes. A full-length rat HO-1 cDNA, generously provided by Dr. S. Shibahara of Tohoku University, Japan (26), was subcloned into a pBluescript vector, and Hind III/Eco RI digestion was performed to isolate a 0.9-kb HO-1 cDNA insert. A 24-base pair oligonucleotide (5’-ACGGTTACTGGATGCTTCCGACC-3’) complementary to 18S rRNA was synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA). HO-1 cDNA was labeled with [α-32P]ATP using a random primer kit (Boehringer Mannheim, Mannheim, Germany). The 18S rRNA oligonucleotide was labeled with [α-32P]ATP at the 3’-end with terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Gaithersburg, MD). The construction and isolation of two of the plasmid pA1 containing chicken β-actin used in nuclear run-off experiments has previously been reported (7).

Western blot analyses. For HO-1 immunoblots, cells were homogenized in lysis buffer [1% Triton X-40 (NP-40), 20 mM tris(hydroxymethyl)aminomethane (Tris; pH 8.0), 137.5 mM NaCl, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml aprotinin]. Protein concentrations of the lysates were determined by Coomassie blue dye-binding assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of 2× SDS-sample buffer [0.125 M Tris-HCl (pH 7.4), 4% SDS, and 20% glycerol] was added, and the samples were boiled for 5 min. Samples (100 μg) were subjected to electrophoresis in a 12% SDS-polyacrylamide gel (Novex, San Diego, CA) for 2 h at 20 mA. The proteins were then transferred electrophoretically (Bio-Rad Laboratories) onto a polyvinylidene fluoride membrane (Immobilon, Bedford, MA) and were incubated for 2 h in TTBS buffer (Tris-buffered saline and 1% polyoxyethylene sorbitan monolaurate) containing 5% nonfat powdered milk. The membranes were then incubated for 2 h with rabbit polyclonal antibody against rat HO-1 (1:1,000 dilution). Rat HO-1 antibody was purchased from Stress Gen (Vancouver, BC, Canada). After three washes in TTBS for 5 min each, the membranes were incubated with goat anti-rabbit immunoglobulin G antibody (Amersham, Arlington Heights, IL) for 2 h. The membranes were then washed three times in TTBS.
for 5 min each, followed by detection of signal by using an enhanced chemiluminescence detection kit (Amersham).

Cellular nuclear protein extraction. Cells were scraped in cold phosphate-buffered saline, centrifuged at 5,000 g at 4°C for 10 min. After the supernatant was discarded, the cell pellet was lysed in lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.9), 1 mM EDTA, 60 mM KCl, 1 mM DTT, 0.5% NP-40, and 1 mM PMSF. The lysate was chilled in ice for 5 min and then was centrifuged at 1,500 g to obtain nuclei. The nuclei were washed in lysis buffer without NP-40 and were centrifuged again at 1,500 g for 5 min. The supernatant was removed, and the pellet was resuspended in nuclear resuspension buffer containing 25 mM Tris (pH 7.8), 60 mM KCl, 1 mM DTT, and 1 mM PMSF. The nuclei were then frozen and thawed three times to obtain nuclear protein. The protein was kept in nuclear resuspension buffer and was stored at −80°C.

Electrophoretic mobility shift assay. Mobility shift assays were performed as previously described (14). DNA binding activity was determined after incubation of nuclear protein extract (3 µg) with 10 fmol (20,000–50,000 cpm) of a32P-labeled DNA probe (5′-CTAGTGATGAGTCAGCCGGATC-3′; Stratagene) in reaction buffer containing 10 mM HEPES (pH 7.9), 1 mM DTT, 1 mM EDTA, 80 mM potassium chloride, 1 mg polydeoxyinosine-deoxycytidylic acid, and 4% Ficoll. After a 20-min incubation, the reaction mixture was electrophoresed on a 6% polyacrylamide gel. The gel was transferred to DE81 ion-exchange chromatography paper (Whatman, Maidstone, UK) and was dried down before exposure to autoradiographic film.

Results

NO induces HO-1 mRNA levels in vitro. Northern blot analyses were performed to examine the steady-state levels of HO-1 mRNA in primary cultures of rat aSMC after exposure to the NO donor SNN. Total RNA was isolated at 1, 2, 4, 8, 24, and 36 h after 500 µM SNN treatment and was analyzed for HO-1 mRNA expression (Fig. 1A). HO-1 mRNA levels were induced in a time-dependent manner. A low basal level of HO-1 transcript was detected by densitometer to allow determination of degree of induction. Quantitation of relative HO-1 mRNA levels (Fig. 1B) showed an initial rise at 1 h (7-fold), a peak induction at 4 h (105-fold), and a return to baseline by 36 h. Induction of the HO-1 mRNA of aSMC is also dose dependent, as evidenced by linear increases in mRNA expression by doses of SNN ranging from 100 µM to 1 mM (Fig. 2A). Quantitation of relative HO-1 mRNA levels (Fig. 2B) showed an induction of HO-1 mRNA at 0.1 mM SNN (34-fold), at 0.5 mM SNN (82-fold), and at 1.0 mM SNN (123-fold). All subsequent experiments involving SNN were performed using a concentration of 500 µM. Cells did not exhibit cytotoxicity at this dose.

HO-1 protein is increased after exposure to NO. Western blot analyses were performed to determine if enhanced HO-1 gene expression after SNN correlated with increased HO-1 protein levels. An increase in HO-1 protein levels was evident at 4 h, with sustained levels at 8 and 24 h, before returning to baseline by 72 h after SNN treatment of aSMC (Fig. 3).

Induction of HO-1 mRNA expression by NO is dependent on both transcriptional and posttranscriptional mechanisms. To examine the mechanism(s) for increased expression of HO-1 in response to NO, we first examined whether HO-1 mRNA induction was dependent on gene transcription. Induction of HO-1 mRNA was completely abolished in the presence of 0.5 µg/ml
actinomycin D, a potent inhibitor of RNA transcription (Fig. 4). We further confirmed this observation by performing nuclear run-off experiments. As shown in Fig. 5A, aSMC treated with SNN exhibited a 31-fold increase in rate of gene transcription. The β-actin gene was used as a housekeeping gene, and the pBluescript vector served as control for vector control. In addition to increased HO-1 gene transcription (Fig. 5A), SNN could also increase HO-1 gene expression by enhancing the stability of mRNA transcripts. We tested this possibility by determining whether SNN affected the stability of HO-1 mRNA transcript. Cells were pre-treated with SNN for a 4-h incubation, washed, and subsequently exposed to actinomycin D either in the presence or absence of SNN for 1, 2, 4, and 8 h. The decay of HO-1 transcripts in actinomycin D-treated cells was delayed in the presence of SNN (Fig. 5B), as demonstrated by higher levels of HO-1 mRNA in cells exposed to actinomycin D in the presence of SNN (4- and 8-h time points) when compared with cells exposed to actinomycin D alone. These data suggest that SNN increased HO-1 gene expression by enhancing both gene transcription and mRNA stability. To further determine regulation of HO-1 expression by SNN, cells were pretreated with the protein synthesis inhibitor cycloheximide (5 µg/ml) before treatment with SNN and then were analyzed for HO-1 mRNA expression. Cycloheximide completely blocked the upregulation of HO-1 mRNA steady-state levels in response to SNN treatment, suggesting that new protein synthesis was required for SNN-induced HO-1 mRNA expression (Fig. 4).

NO induces HO-1 gene expression via a cGMP-independent pathway. The activation of guanylate cyclase and cGMP by NO mediates many of the biological functions of NO. To determine if HO induction by NO was mediated through the activation of guanylate cyclase or cGMP, we first exposed cells to 8-bromoguanosine 3′,5′-cyclic monophosphate, a cGMP analog (Fig. 6A). Surprisingly, no HO-1 induction was observed, even at concentrations of up to 100 µM. Additionally, treatment of cells with 10 µM ODQ, a selective inhibitor of guanylate cyclase, did not prevent the induction of HO-1 gene expression by SNN (Fig. 6B). Collectively, these data suggest that SNN induces HO-1 gene expression via a cGMP-independent pathway. SNN is a mem-
member of a relatively new family of NO donor drugs that have become available for the biological release of NO that yield several advantages over older compounds, including release of two NO molecules per reaction, increased half-life, and NO yield without redox activation. To eliminate the possibility that the HO-1 induction was occurring in response to a unique element of the SNN composition rather than the NO released, we stimulated aSMC with two additional widely used NO donors, SNAP and SNP. Both of these NO-generating compounds induced HO-1 gene expression similar to that observed with SNN (Fig. 7).

NO-induced HO-1 gene expression is modulated by the antioxidant NAC. In addition to modulating the enzymatic activity of various metalloproteins, NO as a free radical can react with other free radicals, altering the redox state of the cell. To determine if reactive O₂ species play a role in the SNN induction of HO-1 expression, cells were pretreated with the antioxidant NAC (20 mM) for 1 h before 500 µM SNN treatment. NAC markedly inhibited SNN-induced HO-1 gene expression, suggesting that reactive O₂ species play an important role in NO-mediated induction of HO-1 expression (Fig. 8A). NO is known to preferentially combine with superoxide anion to form the deleterious free radical ONOO⁻. To explore the possibility that HO-1 induction was modulated by the formation of ONOO⁻, we treated cells directly with 100 µM ONOO⁻. As shown in Fig. 8B, ONOO⁻ alone is insufficient to induce HO-1 mRNA in aSMC. Furthermore, cells treated with both ONOO⁻ and SNN exhibited similar levels of HO-1 mRNA expression compared with cells exposed to SNN alone.

SNN treatment increases AP-1 DNA binding activity in aSMC. We have previously demonstrated that oxidants such as hyperoxia and endotoxin (6) activate the transcription factor AP-1 and that AP-1 may mediate HO-1 gene transcription (5, 14). To examine whether NO can also activate AP-1, we performed electrophoretic mobility shift assays of nuclear cellular proteins from aSMC after SNN treatment. Increased AP-1 binding activity in aSMCs was evident after 1, 2, and 4 h of SNN exposure (Fig. 9).
HO-1 induction by hypoxia but not NO is downregulated in an autocrine fashion by the production of CO. Studies have demonstrated that CO is involved in the regulation of hypoxia-induced gene expression (13, 20). Specifically, Morita et al. (21) reported that CO inhibited induction of HO-1 gene transcription during hypoxia via a negative feedback mechanism. We were interested whether CO could function in a similar manner to downregulate SNN-induced HO-1 gene expression. Our experimental design included five groups: normoxia, hypoxia control, 10% CO, SNN, 10% CO, and SNN as detailed in METHODS. As shown in Fig. 10, the presence of CO suppressed hypoxia-induced HO-1 gene expression by ~54%; however, there was no effect on the induction of HO-1 steady-state mRNA levels in response to exogenous NO.

**DISCUSSION**

The role of HO in heme and heme-iron metabolism is well established. The chemical diversity of HO-1 inducers in conjunction with the properties of the catalytic by-products, however, argues for a broader physiological role for HO in cellular and systemic functions. Consistent with this hypothesis, HO-1 has been shown to provide cellular protection against oxidative damage (6, 15, 22) as well as to modulate inflammation (29). Our laboratory recently demonstrated that overexpression of HO-1 in human pulmonary epithelial cells resulted in cell growth arrest and increased resistance to hyperoxia (15). One or more of these effects may be mediated by CO, which, like NO, is an endogenous gas that can activate guanylate cyclase, regulate vascular...
that, at least with other gaseous molecules, such as O₂, not been extensively studied; however, we have shown various HO-1 inducers on HO-1 mRNA turnover has increase the stability of HO-1 transcripts. The effect of ing agents. In addition, we also observed that NO can transcription is the principal mechanism by which 13-acetate, hypoxia, and hyperoxia (1, 6, 16), giving evidence supporting a possible coordinated role between these two gaseous molecules was demonstrated by Zakhary et al. (33), who recently reported that HO and NOS were colocalized in endothelial cells as well as in the adventitial nerves of blood vessels.

We demonstrate here that NO was a potent inducer of HO-1 gene expression in vascular smooth muscle cells. Inhibition of NO-induced HO-1 mRNA expression by prior treatment of cells with the RNA synthesis inhibitor actinomycin D and the increased rate of HO-1 transcription by nuclear run-on assays confirm that HO-1 induction by NO is regulated at the level of gene transcription. This dependency on gene transcription for HO-1 expression has also been observed in cells treated with other inducers, including cadmium, UV irradiation, endotoxin, arsenite, 12-O-tetradecanoylphorbol-13-acetate, hypoxia, and hyperoxia (1, 6, 16), giving further credence to the fact that modulation of gene transcription is the principal mechanism by which HO-1 expression is regulated by most, if not all, inducing agents. In addition, we also observed that NO can increase the stability of HO-1 transcripts. The effect of various HO-1 inducers on HO-1 mRNA turnover has not been extensively studied; however, we have shown that, at least with other gaseous molecules, such as O₂ (hypoxic conditions), mRNA stability was not affected (14).

Many of the biological actions of NO are mediated through the activation of guanylate cyclase and cGMP production; however, we demonstrate that induction of HO-1 mRNA by NO is independent of the cGMP signaling pathway similar to observations by Kim et al. (12) in hepatocytes. We speculate that induction of HO-1 expression by NO may be a consequence of oxidative stress. Indeed, cells exposed to an NO donor in the presence of the antioxidant NAC demonstrated a significant inhibition of HO-1 induction during NO exposure, suggesting that reactive O₂ species may mediate NO-induced HO-1 gene expression. It is well established in the literature that NO as a free radical can participate in reactions with other free radicals. The most prominent reaction of this type is the formation of ONOO⁻ when NO combines with superoxide anion (4, 27). We considered the possibility that formation of ONOO⁻ was responsible for the modulation in NO-induced HO-1 gene expression; however, when vascular smooth muscle cells were treated directly with ONOO⁻, no induction of HO-1 expression was observed.

It should be noted that, independent of its reaction with free radicals, NO can exist in several redox-activated forms. The predominant redox form in human plasma is S-nitrosothiols, which are believed to create a bioactive pool to buffer the concentration of free NO. Interestingly, HO-1 has been shown to be induced by thiol reagents (1, 6), and it is possible that induction of HO-1 via NO may be regulated through the formation of nitrosothiols rather than oxidant species. Yee et al. (31) recently demonstrated that the induction of HO-1 expression by NO appears as a result of glutathione depletion. In light of this observation, it is possible that the inhibition of HO-1 expression by NAC may be due to increased glutathione levels and unrelated to scavenged oxidants. Another possible mechanism for HO-1 induction includes NO-induced heme loss that was demonstrated by Kim et al. (12) in cultured rat hepatocytes; however, this observation did not extend to sheep pulmonary artery endothelial cells, suggesting that this pathway might be cell specific (31).

Although NO is known to modulate the activity of several genes, such as HO-1, little is known about the transcription factors that mediate these responses. Our laboratory has previously demonstrated that HO-1 gene expression is primarily regulated through activation by various transcriptional activators for which the specificity is dependent on particular stimuli. For example, we have observed that the transcription factor hypoxia inducible factor-1 was critical for the induction of HO-1 mRNA during hypoxia (16), whereas the transactivator AP-1 mediated lipopolysaccharide-induced HO-1 gene expression (5). In addition, we have characterized the activation of AP-1 under conditions of oxidative stress in macrophages during hyperoxic exposure (14). It was recently reported that immortalized human bronchial epithelial cells transformed to constitutively express inducible NOS exhibited increased levels of c-fos, one of the subunits of AP-1 (8). Based on these observations, we speculate that the AP-1 transcriptional complex may be a strong candidate to...
mediate this upstream pathway because preliminary experiments in our laboratory suggest activation of AP-1 by NO in aSMC. We are currently attempting to delineate this pathway, and future experiments will be necessary to fully define the cis- and trans-acting elements mediating NO-induced HO-1 gene transcription.

Increasing evidence in the literature suggests a coordinated role between the activities of NO and CO. For example, it has been shown that NO may function as an inhibitory modulator for the NO–cGMP signaling system in the brain (10). Recently, Morita et al. (21) demonstrated that late-phase suppression of HO-1 mRNA during acute hypoxia was facilitated, in part, by a negative feedback mechanism mediated by the accumulation of CO. The upregulation of HO-1 mRNA by NO was transient, returning to baseline by 36 h, similar to the observed biphasic response of HO-1 gene expression during hypoxia. In an effort to determine if CO could also function as a reciprocal modulator to NO-induced HO-1 gene expression as had been observed for hypoxia-induced HO-1 gene expression (21), we exposed cells to 10% CO in the presence and absence of SNN. NO-induced HO-1 expression was not affected by CO in vascular smooth muscle cells in contrast to the observations reported by Morita et al. (20, 21) during hypoxia. These results support our data suggesting NO-induced HO-1 gene expression is mediated through a different signaling pathway from hypoxia. Furthermore, the complexity of regulation of HO-1 expression is highlighted by a recent study demonstrating that interleukin-1β-induced HO-1 expression is independent of NO (32).

It is interesting to note that NO is one of the most potent inducers of HO-1 known to date. We hypothesize that the release of CO may be contributory to the regulation of various cellular functions currently attributed solely to the action(s) of NO. It is prudent to remember that, despite the apparent similarities between these two messengers, there are significant differences that should not be ignored, such as 1) biological half-lives, 2) requirements for enzyme activation, and 3) reactivity with O2 and metals, suggesting each messenger maintains a functional autonomy (17, 19). Collectively, our data strongly implicate a coordinated interaction between NO and HO and its byproduct CO that extends to the level of gene expression. In view of the increasing use of NO in preclinical and clinical pathophysiological states and the increasing realization that both HO and CO possess important cellular function in maintaining both cellular and organ homeostasis, it would be of extreme importance to augment our understanding of the biochemical and molecular mechanism(s) regulating this coordinated interaction, a process still largely uncharted.

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