Nitric oxide (NO) has been reported to inhibit protein synthesis in eukaryotic cells by increasing the phosphorylation of the α-subunit of eukaryotic initiation factor (eIF) 2. However, the mechanism through which this increase occurs has not been characterized. In this report, we examined the effect of the diffusible gases nitric oxide (NO) and carbon monoxide (CO) on the activation of the heme-regulated eIF2α kinase (HRI) in rabbit reticulocyte lysate. Spectral analysis indicated that both NO and CO bind to the N-terminal heme-binding domain of HRI. Although NO was a very potent activator of HRI, CO markedly suppressed NO-induced HRI activation. The NO-induced activation of HRI was transduced through the interaction of NO with the N-terminal heme-binding domain of HRI and not through S-nitrosylation of HRI. We postulate that the regulation of HRI activity by diffusible gases may be of wider physiological significance, as we further demonstrate that NO generators increase eIF2α phosphorylation levels in NT2 neuroepithelial and C2C12 myoblast cells and activate HRI immunoadsorbed from extracts of these non-erythroid cell lines.

Over 2 decades ago, the heme-regulated inhibitor (HRI) of protein chain initiation in rabbit reticulocyte lysate (RRL) was identified as a heme-regulated protein kinase that phosphorylated the α-subunit of eukaryotic initiation factor eIF2 (reviewed in Refs. 1–3). eIF2 delivers Met-tRNA i in a complex with GTP to 43 S ribosomal initiation complexes and is released as a complex with GDP at the completion of the initiation cycle. Recycling of eIF2-GDP and the formation of eIF2-GTP-Met-tRNA i complexes requires the action of the guanine nucleotide exchange factor, eIF2B. Under heme-deficient conditions, HRI is activated and phosphorylates eIF2. Phosphorylated eIF2 avidly binds eIF2B, sequestering eIF2B in a poorly dissociable complex, which subsequently leads to the inhibition of the initiation of translation, as eIF2-GDP complexes that are present in excess of eIF2B fail to recycle. Thus, HRI functions to coordinate globin synthesis with heme availability in RRL.

The amino acid sequence deduced from HRI cDNA indicates that it is composed of at least five distinct domains (4). The unique N-terminal domain of HRI contains ~165 amino acids. The second and fourth domains contain conserved sequence motifs I–V and motifs VI–XI, which comprise the N-terminal and C-terminal catalytic lobes of protein kinases, respectively. The third and fifth domains are also unique, consisting of ~140 amino acids that are inserted between the two conserved kinase lobes and ~50 amino acids at the C terminus, respectively.

HRI is a hemoprotein that contains two distinct of heme-binding sites (5–7). Heme binding to the first site is stable, and remains associated with purified HRI, whereas heme-binding to the second site is reversible and appears to be responsible for the rapid heme-induced down-regulation of HRI activity. We have recently demonstrated that the N-terminal domain of HRI contains the stable heme-binding site, whereas the unique third domain of HRI appears to contain the reversible heme-binding site (6, 7), thus raising the question as to the function of the N-terminal heme-binding domain (NT-HBD) in the regulation of HRI activation.

Nitric oxide (NO) is now recognized as a major signaling molecule (8–10). Like NO, carbon monoxide (CO) has also been identified as an endogenous second messenger in the peripheral and central nervous system, and has been demonstrated to play an important role in hemodynamic regulation (10, 11). Both NO and CO have high affinity for both protein-bound and free heme-iron (12–14). Recently, the cytostatic activity of NO was found to correlate with NO-induced increase in eIF2α phosphorylation and inhibition of protein synthesis in a number of cell lines (15). However, the mechanism of this NO-induced increase in eIF2α phosphorylation was not characterized. In this report, we examined the possible function of the NT-HBD in the regulation of HRI activation, and present evidence that HRI activity is regulated by the binding of the diffusible gases NO and CO to its NT-HBD.

Experimental Procedures

Protein Synthesis and eIF2α Phosphorylation in Reticulocyte Lysates—Protein synthesis was determined by measuring the incorporation of [35S]methionine into the acid-precipitable protein in 5-μl aliquots taken from standard RRL mixtures containing 20 μM hemin at 30 °C as described (16, 17). The phosphorylation of eIF2α in 2 μl of protein synthesis mix was analyzed as previously described by Western blotting of one-dimensional vertical isoelectric focusing (IEF) slab gels using 1:1000 dilution of anti-eIF2α monoclonal antibody (18–20).

Treatment of Samples with NO and CO—The NO generator N0C-9 (Calbiochem) was prepared as a 100 mM stock solution in 20 mM Tris-HCl (pH 8.0) and used immediately for experiments in RRL or in vitro. NOC-9 breaks down to form NO with a half-life of 3 min at pH 7.4. For CO treatments, samples (RRL or immunoresins) were gassed in a fume hood with CO (99.0-1% CO; Sigma) for 3 min on ice by directing a
stream of CO through a 21-gauge needle at the surface of the sample with sufficient velocity to cause continuous mixing of the microcentrifuge tube contents. The tubes were then sealed with parafilm prior to any further incubations.

De Novo Synthesis, Maturation, and NO or CO Treatment of HRI in Situ—Coupled transcription/translation reactions to pulse-label \(^{[35S]}\)HRI and His-\(^{[35S]}\)HRI in nuclease-treated RRL (TnT RRL, Promega) were carried out as described previously (21, 22). After radiolabeling, one volume of TnT protein synthesis mix containing either \(^{[35S]}\)HRI and His-\(^{[35S]}\)HRI was mixed with seven volumes of normal heme-deficient or heme-supplemented (10 \(\mu\)M hemin) protein synthesis mix containing non-nuclease-treated RRL and the protein synthesis initiation inhibitor auranofin (60 \(\mu\)M). The samples were then incubated for 50 min at 30 °C to yield “mature-competent” HRI (50 min in heme-supplemented RRL), “transformed” HRI (50 min in heme-deficient RRL), or “repressed” HRI (40 min in heme-deficient RRL), followed by a 10-min incubation in the presence of 10 \(\mu\)M hemin (21, 22). The in situ effects of NO, CO, N-ethylmaleimide (NEM), or dithiothreitol (DTT) on HRI were then assayed by treating samples with NOC-9, gassing RRL with CO, or treatment of samples with NEM or DTT followed by an additional period of incubation at 30 °C as specified in the figure legends. HRI was then affinity-purified and assayed for kinase activity as described below.

Assay of the Kinase Activity of Affinity-purified His-\(^{[35S]}\)HRI—Immunoadsorption of His-\(^{[35S]}\)HRI by anti-His antibodies (Qiagen) and non-immune control antibodies were done as described previously (22). Assays for the elF2α kinase activity of His-\(^{[35S]}\)HRI bound to immunoaffini-gels were performed for 4 min at 30 °C as described (21, 22). Samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene difluoride membrane and autoradiography as described previously (21, 22). Autophosphorylation of HRI was assayed by the incorporation of \(^{32P}\)Pi, into HRI during elF2α kinase assays incubated with [\(^{32P}\)Pi]ATP. \(^{32P}\)-Labeled HRI and elF2α were detected by quantitatively quenching \(3^\text{S}\) emissions with three intervening layers of previously developed x-ray film (21, 22).

To study the effects of NO or CO on HRI activation in vitro, HRI was synthesized de novo and matured for 60 min in heme-supplemented or heme-deficient RRL, or in heme-deficient RRL (50 min) followed by the addition of 10 \(\mu\)M hemin (for 10 min) to yield mature-competent, transformed HRI and repressed HRI, respectively. HRI was then affinity-purified, and autophosphorylation of HRI was assayed by the incorporation of \(^{32P}\)Pi, into HRI incubated with [\(^{32P}\)Pi]ATP and treated or untreated with NO or CO as described above. A non-His-tagged \(^{[35S]}\)HRI was similarly studied for nonspecific interactions to the resin.

Spectral Analysis of NT-HBD of HRI—Recombinant NT-HBD of HRI was purified as previously described (7). Spectral analysis was done using a Shimadzu UV-160 spectrophotometer scanning the NT-HBD dissolved in 20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl from 200 to 650 nm. The NT-HBD was then reduced by the addition of several grains of dithionite to the cuvette. Immediately after the spectrum of NO-bound and CO-bound NT-HBD was obtained, NOC-9 was added to a concentration of \(1 \times 10^{-5}\) M hemin-HCl. The samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis with antibody specific to phosphorylated elF2α (Ref. 26); provided by Dr. D. DeGracia, Wayne State University, Detroit, MI.

RESULTS

Effect of NO and CO on Protein Synthesis and elF2α Phosphorylation in RRL—To determine the effects of NO on protein synthesis varying concentrations of the NO generator NOC-9 was added to heme-supplemented RRL (Fig. 1a). Low concentrations of NOC-9 stimulated protein synthesis slightly, while high concentrations of NOC-9 caused a rapid and complete shut-off of protein synthesis. To examine the mechanism by which NO inhibits translation, the phosphorylation status of elF2α in NOC-9-treated RRL was analyzed (Fig. 1b). NO generation stimulated elF2α phosphorylation in a concentration-dependent manner with 1 mM NOC-9 treatment of heme-supplemented RRL causing a near quantitative phosphorylation of elF2α.

To determine the effect of CO on protein synthesis, RRL was gassed with 100% CO for 3 min on ice. CO stimulated the rate of protein synthesis in heme-supplemented RRL by \(40\%\) during the first 5 min of incubation, but shut-off of translation was not prevented by CO (data not shown). Similarly, pre-gassing of heme-supplemented RRL with CO stimulated translation by \(40\%\) during the first 5 min of incubation in NOC-9-treated RRL, but did not prevent translational shut-off (Fig. 2a). These observations suggested that NO and CO have opposing effects on protein synthesis in the RRL.

Effect of NO on Protein Synthesis and eIF2α Phosphorylation in RRL—To determine whether NO modulated the activation of HRI, His-\(^{[35S]}\)HRI was synthesized by coupled transcription/translation in nuclease-treated RRL and then matured in normal RRL in the presence or absence of heme to generate specific populations of HRI molecules (see Refs. 21 and 22 for detailed descriptions of the HRI populations). Samples were then treated in situ with in-
Regulation of HRI by NO and CO

Mechanism of Regulation HRI Activity by NO and CO—The physiological effects of NO are mediated through a number of mechanisms, including (i) S-nitrosylation of proteins (30), (ii) the generation of free radicals and oxidative stress (31), or (iii) the coordination of NO by protein-bound heme (12, 32). The only known biological reactivity of NO is as a ligand for protein-bound heme, and CO is neither sulfhydryl- nor redox-active. Thus, the observations that CO both blocks and reverses NO-induced activation of HRI strongly suggest that NO induces the activation of HRI through its coordination by heme. However, since sulfhydryl-reactive compounds and oxidative stress are well known to cause the activation of HRI in hemin-supplemented RRL (1–3, 33), we carried out experiments designed to test the first two possible mechanisms further.

To examine whether NO activates HRI by modifying sensitive sulfhydryls of HRI, the effect of NO on the kinase activity of repressed His7-[35S]HRI that was affinity-purified from NOC-9-treated RRL was assayed in vitro (Fig. 2a). Autokinase activity of all the three forms of HRI was through a direct interaction of NO with His7-[35S]HRI that had been affinity-purified from NOC-9-treated RRL. Furthermore, in vitro treatment with NO markedly enhanced the autokinase activity of repressed His7-[35S]HRI that had been affinity-purified from CO-gassed RRL. These results suggest that CO and NO compete for a common binding site in HRI and have opposing effects on HRI activation.

To further test the hypothesis that the activation of HRI by NO was not mediated through its effect on sensitive sulfhydryls of HRI or through the generation of a generalized oxidative...
stress, the effect of the reducing agent DTT on NO-induced activation of repressed HRI was examined (Fig. 4b). DTT reverses the effect of NO on proteins that are mediated through S-nitrosylation (36). In addition, DTT protects HRI from activation induced by sulfhydryl-reactive compounds and reverses the activation of HRI induced by generalized oxidative stress and sulfhydryl-reactive heavy metal ions (3, 33, 37). Addition of DTT prior to NOC-9 treatment had no effect on NO-induced inhibition of protein synthesis (data not shown) or activation of repressed His$_7$-[35S]HRI (Fig. 4b).

**NO Modulates the Activation of HRI by Binding to HRI's NT-HBD**—To determine whether NO and CO might modulate the activation of HRI through their coordination by heme, a spectral analysis of the recombinant NT-HBD of HRI was carried out. Reduction of HRI's NT-HBD with dithionite caused a shift in the absorption maximum of the Soret band from 414 nm to 428 nm, with the absorption maximum in the a/b region shifting from 534 nm to distinct a- and b-bands with absorption maximum at 560 and 530 nm, respectively (Fig. 5A). Addition of NO caused the Soret peak to shift immediately to 421 nm and decrease in intensity by ~50%, with the a- and b-bands shifting to 572 and 542 nm, respectively (Fig. 5B). After 15 min, the Soret band broadened to give an absorption maximum at 402 nm. In contrast, gassing the reduced NT-HBD with CO (Fig. 5C) caused the Soret band to shift from 428 to 422 nm and increase in intensity by ~30%, with the absorption maximum of the a- and b-bands shifting to 568 and 536 nm, respectively. These results indicate that NO and CO become coordinated to the heme moiety of the NT-HBD of HRI.

To address the question of whether the activation of HRI by NO is due to the NO binding to NT-HBD, we studied the effect of NO on the activity of HRI from which the NT-HBD has been deleted (HRI/Met-3; Ref. 6). His$_7$-[35S]HRI/Met-3 was synthesized de novo, transformed in heme-deficient RRL, and repressed by the addition of hemin. Compared with wild type His$_7$-[35S]HRI, the kinase activity of His$_7$-[35S]HRI/Met-3 was only marginally activated upon NOC-9 treatment (Fig. 6a). In contrast, treatment with NEM markedly increased the kinase activity of both His$_7$-[35S]HRI/Met-3 and wild type His$_7$-[35S]HRI (Fig. 6a). These results support the hypothesis that the NT-HBD domain mediates NO-induced activation of HRI. However, we cannot currently rule out the possibility that NO may have additional effects mediated through an interaction with heme bound to the second regulatory heme-binding site in HRI, because NOC-9 treatment reproducibly caused a slight stimulation in kinase activity of HRI/Met-3.

To further confirm the involvement of NT-HBD domain of HRI in NO activation, we studied whether [35S]HRI/Met-3 could form a complex with His$_7$-[35S]NT-HBD, and what effect NO had upon the kinase activity of [35S]HRI/Met-3 in such a complex. [35S]HRI/Met-3 and His$_7$-[35S]NT-HBD were co-expressed in TnT RRL, followed by transformation of the [35S]HRI/Met-3 in heme-deficient RRL and treatment (or not) with NOC-9. The ability of anti-His-tag antibodies to co-adsorb [35S]HRI/Met-3 with His$_7$-[35S]NT-HBD indicated that the NT-HBD of HRI interacted with HRI/Met-3 in trans, although the two proteins did not interact quantitatively (Fig. 6b). [35S]HRI/Met-3 and non-His-tagged [35S]NT-HBD were co-expressed and treated with 1 mM NOC-9, as a control for nonspecific binding. Although the amount of [35S]HRI/Met-3 protein that co-adsorbed as a complex with His$_7$-[35S]NT-HBD was much less compared with the amount of His$_7$-[35S]HRI/Met-3 that was directly adsorbed by the anti-His-tag antibodies, assays of eIF2α kinase activity indicated that the specific activity of the [35S]HRI/Met-3 (32P incorporated into substrate per amount of [35S]HRI/Met-3) that was complexed with the NT-HBD was significantly greater than the specific activity of His$_7$-[35S]HRI/Met-3 alone (Fig. 6b). Furthermore, NOC-9 markedly enhanced...
samples of RRL containing repressed His 7-[35S]HRI were treated with NEM and NOC-9 (lane 1). Dithionite was added to reduce the bound hemin and the sample was rescanned immediately (A, spectrum 1) and after 15 min (B, spectrum 2). After the spectral analysis of the dithionite-treated NT-HBD, NO was generated by the addition of 1 mM NOC-9, and the sample was rescanned immediately (B, spectrum 1) and after 15 min (B, spectrum 2), or the dithionite-treated NT-HBD was gassed with 100% CO for 3 min followed by spectral analysis (C). Arrows indicate the Soret band, the insets within the panels show an expansion of the region of the αβ absorption spectrum, and the asterisk (*) indicates an absorption peak due to the presence of dithionite.

Effect of NO on HRI Activity in Non-erythroid Cells—Possible regulatory roles for HRI in nonerythroid cells have yet to be established (40, 41). A search of the data base of DBEST (Table I) indicated that HRI is expressed in a wide variety of nonerythroid cells and tissues, as well as a variety of tumors. 1862-base pair cDNA encoding HRI has been reported to be expressed in several nonerythroid tissues (40, 41). A search of the data base of DBEST (Table I) indicated that HRI is expressed in a wide variety of nonerythroid cells and tissues, as well as a variety of tumors. A 1862-base pair cDNA encoding HRI has been reported to be expressed in several nonerythroid tissues (40, 41).

FIG. 4. The effect of NEM and dithiothreitol on NO-induced activation of HRI. Repressed His-[35S]HRI was synthesized de novo as described under “Experimental Procedures.” a, RRL was then treated with 1 mM NEM (lane 4), 1 mM NOC-9 (lane 6), or not treated (control, lane 3) for 10 min followed by immunoadsorption with anti-His-tagged antibodies. Samples were then treated with 1 mM NOC-9 (NEM + NOC-9, lane 6) or treated with buffer (control, NEM, NOC-9) during the assay for autokinase activity. Lanes 1 and 2, nonspecific binding of activity from RRL expressing non-His-tagged [35S]HRI that was treated with NEM and NOC-9 (lane 2) or not (control, lane 1). b, samples of RRL containing repressed His-[35S]HRI were treated with 1 mM NOC-9 (NOC-9, lane 3), 1 mM DTT (lane 4), 1 mM DTT plus 1 mM NOC-9 (DTT + NOC-9, lane 5) or not treated (control, lane 2) for 10 min. His-[35S]HRI was affinity-purified and assayed for kinase activity. Lane 1, nonspecific binding of activity from RRL expressing non-His-tagged [35S]HRI that was treated with NOC-9. His-[35S]HRI was detected by direct autoradiography (upper panel, A and B), while 32P-phosphorylated HRI (middle panel, A and B) and 32P-phosphorylated eIF2α (lower panel, B) were detected after quenching 35S emissions.

The eIF2α kinase activity of [35S]HRI/Met-3 that was expressed in and affinity-purified from TnT extracts was below the limit of detection by Western blotting, however, the amount of HRI immunoadsorbed from the C2C12 extracts was below the limit of detection by Western blotting, so we could not determine whether the protein was HRI by Western blotting. In addition, our anti-HRI/NT-HBD antibody failed to adsorb any eIF2α kinase or NO-activable autokinase activity from extracts prepared from the blood of HRI-knockout mice. Therefore, we could not determine whether the protein was HRI by Western blotting. In addition, our anti-HRI/NT-HBD antibody failed to adsorb any eIF2α kinase or NO-activable autokinase activity from extracts prepared from the blood of HRI-knockout mice.
out mice, indicating that our antibody was not cross-reacting with other eIF2α kinases, particularly PKR, which is known to be present in blood cells (42).

To determine whether NO stimulated eIF2α phosphorylation in NT-2 (Fig. 8A) and C2C12 (Fig. 8B) cells, cells were cultured in the presence or absence of the NO donor SNAP. Treatment of both NT-2 (Fig. 8A) and C2C12 (Fig. 8B) cells with SNAP caused an increase in eIF2α phosphorylation in both cell lines. These results suggest that NO may be an important physiological regulator of HRI activation in non-erythroid cells.

**FIG. 6.** The ability of NO to induced activation of HRI is modulated through the NT-HBD. a, His7-[35S]HRI (wt HRI) or His7-[35S]HRI/Met-3 was synthesized de novo, and then matured and transfected in heme-deficient RRL, and repressed by the addition of 10 μM hemin as described under “Experimental Procedures.” RRL was then treated with 1 mM NEM (NEM, lanes 2, 4, and 7), 1 mM NOC-9 (NOC-9; lanes 2, 5, and 8) or not treated (control, lane 3) for 10 min. His-tagged wtHRI and HRI/Met-3 were affinity-purified and assayed for kinase activity. His7-[35S]HRI was detected by direct autoradiography (upper panel), while aP-phosphorylated HRI (middle panel) and 32P-phosphorylated eIF2α (lower panel) were detected after quenching 35S emissions. HRI*, transformed HRI. NS, non-specific binding of activity from RRL expressing non-His-tagged [35S]HRI that was treated with NEM plus NOC-9 (lane 2) or not (control, lane 1). B, His7-[35S]HRI/Met-3 (lanes 2 and 4) and non-His-tagged [35S]HRI/Met-3 (lanes 1, 3, and 5) were synthesized for 20 min in TrT RRL, followed by the addition of auranitin carboxylic acid. Non-His-tagged [35S]HRI/Met-3 was then mixed with RRL containing previously synthesized His-tagged [35S]NT-HBD. His7-[35S]HRI/Met-3 (lanes 2 and 4) and non-His-tagged [35S]HRI/Met-3 plus His-tagged [35S]NT-HBD (lanes 3 and 5) were then matured in heme-deficient RRL for 50 min following a 10-min treatment with 1 mM NOC-9 (lanes 4 and 5). His-tagged proteins were then affinity-purified and assayed for kinase activity. Upper panel, autoradiogram of [35S]-labeled proteins. Lower panel, autoradiogram of 32P-phosphorylated eIF2α. NS, non-specific binding of activity from RRL expressing non-His-tagged [35S]HRI/Met-3 and NT-HBD that was treated with NOC-9.

**TABLE I**  

| Tissue libraries | Brain (fetal/infant/adult) | Breast/Mammary gland | Liver-Spleen (fetal) | Lung (fetal) | Retina | Spleen (fetal) | Thyroid | Uterus (pregnant) |
|-----------------|---------------------------|----------------------|---------------------|-------------|--------|--------------|--------|------------------|
| Tumor libraries | Adrenal | Anaplastic oligodendroglioma | Colon carcinoma (metastatic to liver) | endometrial | Normal cell libraries | Endothelial cell (umbilical cord) | T cell (activated) | 2-Cell stage (mouse embryo) |
| Cell lines | C2C12 (myotubes) | F9 embryonal carcinoma | HeLa | HSC172 (lung fibroblast) | Jurkat (T cells) | NT2 (neuroepithelial-uninduced and retinoic acid-treated) | T84 (colon carcinoma) | WEHI-3 (macrophage) |

**DISCUSSION**

Mechanisms that control initiation of protein synthesis play significant roles in regulating cell growth (reviewed in Ref. 43). eIF2α phosphorylation performs a critical role in control of cell proliferation (reviewed in Refs. 44 and 45), with increased eIF2α phosphorylation (20, 46, 47) and down-regulation of protein synthesis correlating with cell growth arrest and entrance into G0 (48). Cell growth arrest is required for the subsequent terminal differentiation of a number of cell types (reviewed in Ref. 44). Increased eIF2α phosphorylation also mediates different forms of stress-related apoptosis (44, 45, 49, 50). Furthermore, suppression of eIF2α phosphorylation (44) or expression of a nonphosphorylatable mutant of eIF2α causes malignant transformation of cells (51). These observations suggest that eIF2α kinases act as tumor suppressors in the regulation of cell growth (reviewed in Ref. 44). Specifically, these studies have focused on the double-stranded RNA-activated eIF2α kinase (PKR), as constitutive or inducible expression of this eIF2α kinase occurs in most cell types.

Our results suggest that NO may be a physiological regulator of HRI activation. NO was as potent of an activator of HRI as heme deficiency in RRL. Although the 0.25–0.5 mM concentration of NOC-9 required to inhibit protein synthesis in heme-supplemented RRL likely falls outside of the physiological range of concentrations to which NO accumulates in vivo, the concentration range is reasonable considering the NO-binding capacity of the estimated millimolar levels of hemoglobin present in RRL. However, under physiological conditions, much lower levels of NO might be capable of activating HRI in reticulocytes, as recent work indicates that the reaction of oxygenhemoglobin with NO functions to maintain NO bioactivity (52, 53). A more likely physiological target of NO is HRI present in red cell precursors early during hematopoiesis, when significant levels of hemoglobin have yet to accumulate. NO sup-

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2 B.-G. Yun, A. Han, J.-J. Chen, and R. L. Matts, unpublished observations.
presses hemoglobin synthesis in K562 cells (54, 55). Although it was speculated that the NO-induced suppression of hemoglobin synthesis was a result of NO-induced inhibition of heme bio-

synthesis (54), our current results suggest it could be the result of NO directly activating HRI present in K562 cells. In addition, activation of nitric-oxide synthase has been implicated in tamoxifen-induced apoptosis of K562 cells (56), and NO has been reported to (i) influence the growth and differentiation of normal bone marrow cells (57), (ii) induce apoptosis in bone marrow progenitor cells (58), and (iii) be a mediator of cytokine-induced hematopoietic suppression (59). Our results suggest that these effects of NO could, in part, be mediated via NO-induced activation of HRI, considering the well-established role that eIF2α phosphorylation plays in suppressing cell growth and inducing apoptotic cell death.

The consequences of NO synthesis on the function and fate of cells varies depending on the rate of NO synthesis, cell type, presence of free radicals, and the anti-oxidant status of the cell (reviewed in Refs. 60–62). NO modulates signaling molecules in apoptotic pathways, contributing to the physiological balance between pro-apoptotic and anti-apoptotic stimuli (63). NO at high levels generally induces cell death, whereas low doses of NO rescues many cells from apoptotic death. NO is cytotoxic to many cells types (Ref. 64, and references therein), and the cytostatic activity of NO has been found to correlate with NO-induced increase in eIF2α phosphorylation in a number of cell lines (15).

Opposing effects of NO on protein synthesis and HRI autokinase activity were also observed in RRL. In situ, low levels of NO were observed to stimulate translation and suppress, to a degree, the autophosphorylation of HRI. These observations may reflect the ability of NO to displace CO from hemoglobin (see below), or a requirement for both HBDs to be liganded to NO within the HRI dimer for the NO-induced allosteric activation of HRI to occur.

A search of dbEST indicates that mRNA encoding HRI is present in many non-erythroid cell types. Our results indicate that the HRI mRNA present in NT-2 and C2C12 cells is translated into protein, albeit at levels much lower that that present in erythroid cells. Thus, NO-induced activation of HRI may contribute to NO-induced inhibition of protein synthesis in non-erythroid cells that express HRI. NO-induced activation of HRI, like the effects of PKR activation (44, 45), may also play a role in mediating NO-induced apoptosis in these cells. Furthermore, NO may be a principle activator of HRI in non-erythroid cells, as these cells are not likely to experience large fluctuations in their heme content.

Our data also suggest that CO is an important physiological regulator of HRI activation. CO is the product of the first step in heme catabolism catalyzed by heme oxygenase (HO), and has been proposed to be a physiological regulator akin to NO (10, 11, 65). HO-1 expression is activated in virtually all cell types, not only by the presence of free heme, but also in response to inflammatory cytokines, hypoxia, and many forms of oxidative stress (65, 66). Induction of HO-1 plays an important role in protecting cells from the adverse effects of oxidative stress (11, 36, 65–68). Furthermore, low concentrations of CO prevent tumor necrosis factor-α-induced apoptosis in L929 fibroblasts (69), suggesting that the anti-apoptotic effect of HO-1 expression may be mediated in part via CO. Here we demonstrate that CO suppresses the activity of HRI and stimulates protein synthesis in RRL, and has the capacity to reverse NO-induced activation of HRI. These results suggest that HRI may be regulated through the competition between NO and CO for a common binding site in the NT-HBD of HRI.

The spectral analyses of the binding of NO and CO indicate the potential mechanism through which NO and CO have opposing effects on HRI activation. Whereas CO binds and forms a 6-coordinate heme complex, the spectral changes induced upon the binding of NO to the NT-HBD are similar to
HO-1 expression appears to play a central role in an important regulatory network between NO and CO (11). The ability of CO to antagonize NO-induced activation of HRI leads us to propose a novel feedback mechanism through which protein synthesis may be regulated by these diffusible gases. Increases in cellular NO concentrations cause the release of protein-bound heme (71–73), and the induction of HO-1 (11). The HO-1-catalyzed breakdown of heme would not only be critical in protecting cells from oxidative damage that would result from the presence of elevated concentrations of free heme, but would also produce elevated concentrations of CO, which could act as a feedback inhibitor of HRI activation induced by NO. The overall effect of these diffusible gases on HRI activity and protein synthesis would ultimately be determined by the relative concentrations to which they accumulate within a cell. High levels of NO might stimulate pro-apoptotic pathways to a degree that could not be reversed by a subsequent elevation in CO. Indeed, NO readily nitrosylates intracellular free heme and prevents its degradation by HO (74). In contrast, low levels of NO might lead to the generation of sustained, elevated levels of CO, which might then promote cell growth. Furthermore, we speculate that the ability of elevated CO levels to inhibit the activity of HRI and suppress eIF2α phosphorylation may be a critical event in protecting cells from NO-induced apoptotic cell death.

In summary, we acknowledge that the question of whether HRI is present in cells from tissues of nonerythroid origin is contentious, as it difficult to assure that the source of the HRI protein synthesis may be regulated by these diffusible gases. Increases in cellular NO concentrations cause the release of protein-bound heme (71–73), and the induction of HO-1 (11). The HO-1-catalyzed breakdown of heme would not only be critical in protecting cells from oxidative damage that would result from the presence of elevated concentrations of free heme, but would also produce elevated concentrations of CO, which could act as a feedback inhibitor of HRI activation induced by NO. The overall effect of these diffusible gases on HRI activity and protein synthesis would ultimately be determined by the relative concentrations to which they accumulate within a cell. High levels of NO might stimulate pro-apoptotic pathways to a degree that could not be reversed by a subsequent elevation in CO. Indeed, NO readily nitrosylates intracellular free heme and prevents its degradation by HO (74). In contrast, low levels of NO might lead to the generation of sustained, elevated levels of CO, which might then promote cell growth. Furthermore, we speculate that the ability of elevated CO levels to inhibit the activity of HRI and suppress eIF2α phosphorylation may be a critical event in protecting cells from NO-induced apoptotic cell death.

In summary, we acknowledge that the question of whether HRI is present in cells from tissues of nonerythroid origin is contentious, as it difficult to assure that the source of the HRI mRNA or protein is not from occluded blood. Furthermore, the expression of HRI in cultured nonerythroid cells could be a result of the aberrant phenotype of immortalized cells. In addition, our results indicate that HRI expression in NT2 and C2C12 is an order of magnitude or more lower than its level of expression in reticulocytes. However, with the acknowledgment of these caveats, the results presented here suggest that the following hypotheses are worthy of further investigation: (i) that activation of HRI may contribute to pathophysiologies that result from chronic exposure of normal cells to elevated NO levels and (ii) that HRI may be a potential target for design of HRI plasmid constructs used in these studies.
Regulation of HRI by NO and CO

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