Inhibition of Hemoglobin Expression by Heterologous Production of Nitric Oxide Synthase in the K562 Erythroleukemic Cell Line

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Recent studies have indicated that nitric oxide may affect iron metabolism through disruption of the iron-sulfur complex of iron regulatory protein-1, a translational regulator. In the present study, we report that heterologous expression of murine macrophage nitric oxide synthase (NOS-2) in the human erythroleukemic K562 cell line results in constitutive production of nitric oxide associated with inhibition of hemoglobin expression. K562 cells were transfected with an episomally-maintained, hygromycin-selectable expression vector bearing the coding region of NOS-2. Constitutive NOS expression was detected by Western blotting of cell lysates and by the accumulation of nitrite in the culture media. Although NOS-transfected cells grew more slowly than control cells, they were able to maintain constitutive expression of NOS and production of nitric oxide for more than 1 month following transfection. The hemoglobin content of NOS-transfected K562 cells was less than one-fifth that of control cells, but increased markedly if NOS inhibitor was included in the culture media. The nitric oxide-mediated inhibition of hemoglobin expression was reversed by supplementing the culture media with 20 μmol/L hemin or 0.5 mmol/L 5-aminolevulinate, indicating that nitric oxide did not directly inhibit hemoglobin synthesis, but likely acted on a step in heme synthesis. mRNA levels for globin and erythroid aminolevulinic acid synthase (eALAS) were the same in both NOS-transfected and control cells. Our observations indicate that hemoglobin expression is inhibited by nitric oxide in NOS-transfected K562 cells by posttranscriptional repression of eALAS, the first enzyme of the heme biosynthetic pathway. The most likely mechanism is a nitric oxide-mediated translational repression of eALAS, as was recently demonstrated for ferritin synthesis. These observations further illustrate the potential for endogenously produced nitric oxide to regulate cellular posttranscriptional events. In particular, our observations may be relevant to the role of nitric oxide in anemia and lowered blood hemoglobin concentrations that are associated with chronic infections, such as tuberculosis or parasitic disease.

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THE VARIETY OF cellular processes that involve nitric oxide is remarkable. Within the past 10 years, critical roles for nitric oxide in signal transduction, neurotransmission, and host defense have been revealed, whereas recent work suggests a role for nitric oxide in iron metabolism. Expression of several proteins of iron metabolism is controlled at the translational level by iron responsive elements (IREs) in the untranslated regions of the mRNAs of these genes. IREs are conserved sequences of approximately 30 nucleotides that form metastable stem-loop structures, which are stabilized by binding of a cytosolic iron regulatory protein (IRP-1). Formation of this protein-RNA complex in the 5' untranslated region hinders translation initiation; this is sufficient to provide iron-sensitive regulation of the iron storage protein ferritin and erythroid 5-aminolevulinate synthase (eALAS), the first enzyme of the heme biosynthetic pathway. Conversely, binding of IRP-1 to multiple IREs in the 3' untranslated region of transferrin receptor mRNA contributes to its stability and increases both the level of mRNA and the level of receptor. IRP-1 is an iron sensor by its ability to bind iron reversibly. Iron-loaded IRP-1 has cytosolic aconitase activity, and the protein shares 30% sequence identity with mammalian mitochondrial aconitase. The structure of mitochondrial aconitase is known, and it binds iron as an Fe₄S₄ cluster. Identity between IRP-1 and mitochondrial aconitase is highest in those residues that constitute the enzymatic active site of the mitochondrial protein and includes the three cysteine residues that the protein contributes to the iron-sulfur cluster. Moreover, the iron-sulfur cluster of mitochondrial aconitase, unlike other iron sulfur proteins, is relatively labile and exists in both active (Fe₃S₄) and inactive (Fe₄S₄) states. Based on this structural and functional similarity to mitochondrial aconitase, it was proposed that IRP-1 binds iron to form an Fe₃S₄ cluster with aconitase activity, but with low affinity for RNA; iron depletion leads to loss of iron and aconitase activity from the IRP-1 with a concomitant increase in its affinity for IREs. The characterization of recombinant IRP-1 and a constitutive mRNA-binding mutant in which serine replaces one of the conserved cysteine residues provided support for this mechanism.

Iron-sulfur clusters can be disrupted by conditions other than iron depletion. Nitric oxide, either directly or in combination with other oxidants, is capable of binding to iron-sulfur clusters and causing the release of iron. Murine B6 fibroblasts transfected with a nitric oxide synthase gene have repressed ferritin expression and increased transferrin receptor expression. A recent report demonstrated that treatment of the human erythroleukemic cell line K562 with exogenous nitric oxide results in an increase of transferrin receptor expression, while leaving ferritin levels unchanged. The K562 cell line is a useful model for the study of effects of nitric oxide expression on iron homeostasis, as these cells produce readily detectable quantities of hemoglobin that can be increased by the presence of agents such as sodium butyrate, heme, and the heme precursor 5-aminolevulinic. Globin expression is translationally controlled by...
the two PCR fragments, NOS-A and NOSC. As outlined in Materials and Methods, these two PCR fragments were cloned and then sequentially ligated together with the BamHI/EcoRV fragment internal to the coding region to achieve the final product, pTNG0. The pTNG0 was the source of NOS-2 coding sequence for ligation into the mammalian episomal expression vector (not shown) used in this study (see text). (I) Untranslated regions; (II) PCR-generated sequences.

MATERIALS AND METHODS

Plasmid constructions. Vector pCLBS-macNOS, a gift of Dr Charles Lowenstein (Johns Hopkins University, Baltimore, MD) contains the coding sequence of murine macrophage nitric oxide synthase (NOS-2) expression vector resulting in establishment of constitutive long-term production of nitric oxide by the genetically modified cells. In the present study, we report our observations on the ability of nitric oxide producing K562 cells to express hemoglobin. Our results demonstrate that nitric oxide production in these cells diminishes their ability to produce hemoglobin and are consistent with post-transcriptional repression of eALAS synthesis by endogenously produced nitric oxide.

NOS INHIBITION OF HEME AND GLOBIN SYNTHESIS

Fig 1. Construction of plasmid vector pTNG0 encoding only the coding region of murine iNOS (NOS2) flanked by several unique restriction sites; BglII and NotI at the 5’ end, and BclI, BglII, and SmaI at the 3’ end. Additionally, an Ncol site has been introduced at the start codon. All these restriction sites were introduced by generating the two PCR fragments, NOSA and NOSC. As outlined in Materials and Methods, these two PCR fragments were cloned and then sequentially ligated together with the BamHI/EcoRV fragment internal to the coding region to achieve the final product, pTNG0. The pTNG0 was the source of NOS-2 coding sequence for ligation into the mammalian episomal expression vector (not shown) used in this study (see text). (I) Untranslated regions; (II) PCR-generated sequences.

ATCCTCGGTTGCAGTTTTTCC and CCGGGAGATCTAAAGTGATCGTGCAGCGTCCGTCGGCTTGGGC were generated from a pCLBS-macNOS template. NOS-A and NOS-C were 630 and 102 bp in length, respectively. Each fragment was ligated into the EcoRV site of Bluescript-SK+ to generate pBS-NOS-A and pBS-NOS-C. Clones of pBS-NOS-C were screened for the insert in the desired orientation (retention of the EcoRV site) by restriction digestion with EcoRV/HindIII. pCLBS-macNOS was digested with EcoRV/BamHI and a 2.8-kb fragment corresponding to the central portion of the NOS gene, NOS-B, was ligated to EcoRV/BamHI digested pBS-NOS-C to generate pBS-NOS-BC. pBS-NOS-A was digested with BamHI/NotI and the 630-bp fragment corresponding to the 5’ end of the NOS gene was ligated to BamHI/NotI digested pBS-NOS-BC. The product, denoted pTNG0 (The NOS Gene), contains the entire NOS coding region devoid of native untranslated sequence, but flanked by the restriction sites introduced at either end as indicated in Fig 1. All PCR amplified fragments and cloning junctions used in the construction of the plasmid were confirmed by DNA sequencing.

The construct pTNG1 consists of the NOS gene from pTNG0 inserted into a mammalian expression vector, pCEP4 (Invitrogen, San Diego, CA), which can be maintained episomally in transfected cell lines by hygromycin selection. To make this construct, a 3.4-kb fragment containing the NOS coding region was isolated from pTNG0 after BglIII digestion and inserted into the BamHI site of pCEP4. The control plasmid pCEP4CAT, which contains the chloramphenicol acetyl transferase gene cloned into pCEP4, was purchased from Invitrogen. Plasmid DNA was prepared using a Qiagen Maxiprep kit.

Transfection and culture of K562 cells. The K562 cell line (American Type Culture Collection, Rockville, MD) was maintained in RPMI 1640 media (Biofluids) supplemented with 10% fetal calf serum (HyClone, Logan, UT), streptomycin, penicillin, and L-glutamine. Cells were transfected with pTNG1 by electroporation.22 Electroporated cells were added to 25 cm2 disposable culture flasks containing 10 mL of culture media and were incubated at 37°C, 5% CO2. After 2 days, the cells were pelleted and were resuspended to 104 cells/mL in selective media containing 275 μg/mL of hygromycin (Calbiochem, San Diego, CA) to kill cells not containing the episomal plasmid vector and to maintain the episomal element. Following this, further studies were performed in which hygromycin selected cells were cultured in the presence of the NOS inhibitor N’-methyl-L-arginine (L-NMA, 0 to 2 μmol/L) or inducers of hemoglobin expression: sodium butyrate (1 mmol/L), hemin (20 μmol/L), and 5-aminolevulinate (ALA, 0.5 mmol/L). The four additives were purchased from Sigma Chemical Co (St Louis, MO). Transfected cells were seeded at 2 × 105 cells/mL in the presence of these additives and were grown for 4 to 5 days before being harvested. Parallel transfections with the vector pCEP4CAT, which expresses chloramphenicol acetyl transferase but otherwise shares all of the other properties of pTNG1, were included in all experiments as controls.

Nitrite assay. The accumulation of nitrite, a breakdown product of nitric oxide, in the culture medium of the cells was measured colorimetrically by mixing 100 μL each of culture media and Griess’s reagent (0.5% naphthylethylenediamine in water: 1% sulfanilamide in 5% phosphoric acid). Absorbances at 550 nm were recorded on a microplate reader against standard solutions of sodium nitrite (Baker Chemical, Phillipsburg, NJ) prepared in media.

Preparation of cell lysates. Transfected K562 cells were centrifuged at low speed, washed twice in phosphate buffered saline, and resuspended to 2.5 × 105 cells/mL in lysis buffer (5% glycerol, 50 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 0.1 mg/mL aminothio-benzensulfonyl fluoride). The cells were subjected to three cycles of freeze-thaw (ethanol-dry ice/37°C water bath) with
a 5-minute incubation at each temperature. The lysed cells were microfuged for 10 minutes at 4°C and the supernatants were decanted into fresh cryovials. Lysates for recording of ultraviolet (UV) visible spectra were further clarified by centrifugation on a Beckman Airfuge for 30 minutes at 4°C. Protein concentrations were determined by the Bradford microassay against bovine serum albumin standards (Sigma Chemical). Lysates not used immediately were flash frozen in an ethanol-dry ice bath and stored at -80°C.

**Immunoblotting of cell lysates.** A total of 100 μg of protein from cell lysates were solubilized in 2X sodium dodecyl sulfate (SDS) buffer (final concentrations 450 mmol/L Tris-HCl, pH 8.45, 12% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.0025% Coomassie Blue G, 0.0025% Phenol Red), loaded onto tricine-buffered gels (Novex, San Diego, CA) and subjected to electrophoretic separation under denaturing conditions (100 mmol/L Tris base, 100 mmol/L Tricine, 0.1% SDS, pH 8.3) at 125 V. The gel-separated proteins were transferred onto nitrocellulose membranes (transfer buffer: 48 mmol/L Tris base, 39 mmol/L glycine, 1.3 mmol/L SDS, 20% methanol) using a KEM-EN-TEC semidry blotter at 0.8 mA per cm² of gel surface; the membranes were stained for protein (0.1% Fast Green, 10% methanol, 5% acetic acid) to verify transfer. The membranes were rinsed with water to remove the dye and were blocked for 1 hour with 5% powdered skim milk in Tris-buffered saline (TBS), pH 7.5. The membranes were incubated overnight in a solution of the primary antibody, diluted in 4% powdered skim milk/TBS. Antimurine macrophage iNOS (Transduction Laboratories, Lexington, KY) and anti-γ-hemoglobin (Accurate Laboratories, Westbury, NY) were diluted 1,000-fold and 500-fold, respectively. The membranes were washed with 0.05% Tween 20/TBS and were incubated for 1 minute in the presence of chemiluminescence reagents (Amerham, Arlington Heights, IL). The membranes were washed twice at 68°C before exposure to film. Hemoglobin contents relative to unstimulated, control transfected cells were estimated by densitometry of the bands (NIH Image software).

**UV-visible spectroscopy.** Cell lysates were placed in a 100-μL quartz cuvette, and their UV-visible spectra (700 to 350 nm) were acquired on a Beckman DU640 recording spectrophotometer. The difference spectrum was obtained using lysate solutions of equivalent cell lysates; the membranes were stained for protein (0.1% Fast Green, 10% methanol, 5% acetic acid) to verify transfer.

**Estimation of hemoglobin content.** A colorimetric assay for the latent peroxidase activity of hemoglobin in the presence of hydrogen peroxide and 2,7-diaminofluorene (DAF) substrate was used to measure hemoglobin levels in the cell lysates. A total of 8 μL of dye solution (5 mg/mL DAF in 90% acetic acid) were added to 100 μg of cytosolic protein in 200 μL of lysis buffer/6 mmol/L urea. Reactions were initiated with 10 μL of 1.5% hydrogen peroxide and after 20 minutes, absorbances at 600 nm were measured on a microplate reader. Human methemoglobin (Sigma) was used to prepare standard solutions.

**Northern blotting.** Total RNA was obtained from cells using RNAzol (TEL-Text Inc). RNA samples (10 μg) were loaded onto formaldehyde-agarose gels and electrophoresed. The gel-separated RNA was blotted onto a nylon membrane and cross-linked to the membrane with a Stratalinker 1800 (Stratagene, La Jolla, CA). After prehybridization at 37°C the membrane was hybridized at 37°C with a [32P]-end labeled probe specific to either γ-globin mRNA, (AGC-TCTGACATCATGGCCAGGAGCTCAGTTGTACTCGAGGAGAACGGCACTGGCC) or eALAS mRNA (ATCAAGTGTGTTGACCTCCCAAGAGATCGTCCTGCCTCGCTCGGGACCCGGA). After an 18-hour wash, the membranes were washed twice (5X SSPE/0.1% SDS) for 30 minutes at 37°C, and exposed to X-ray film (Eastman-Kodak, Rochester, NY). Blots were stripped and reprobed with an antisense 48-mer β-actin probe. The membranes were washed twice at 68°C before exposure to film.

**RESULTS**

**Growth characteristics and NOS expression.** K562 cells were transfected with either the NOS episomal vector, pTNG1 (NOS-transfected), or the CAT control episomal vector, pCEP4CAT (control-transfected), by electroporation as described in Materials and Methods. After 2 days, hygromycin was added to the culture to maintain the episomal plasmid and to eliminate cells not containing the episomal plasmid carrying the resistance element. After 5 days of selection and for as long as the transfected cells were maintained in culture, murine macrophage NOS-2 could be detected by Western immunoblotting of cell lysates prepared from NOS-transfected K562 cells, but not the control-transfected cells. Conversely, chloramphenicol acetyl transferase could be detected in the control-transfected cells, but not the NOS-transfected cells.

When cells surviving the initial 5 days of selection were seeded at 200,000 viable cells per milliliter in fresh selective medium and cultured for an additional 5 days, NOS-transfected cells did not proliferate and the culture medium contained 28 ± 6 μmol/L nitrite (average of three experiments). In contrast, the control-transfected cells increased fivefold in number, and the culture medium contained only background levels of nitrite, 0.2 ± 0.2 μmol/L.

However, if the control-transfected cells were maintained in culture, proliferation of cells resumed by about day 14 and continued thereafter. As shown in Fig 2, the growth
doubling time of NOS-transfected cells was about 72 hours in contrast to a doubling time of about 30 hours for the control transfected K562 cells. The growth rate of the NOS-transfected K562 cells doubled when 1 mmol/L of the NOS inhibitor L-NMA was included in the culture medium, but this arginine analog had no effect on the growth of the control-transfected K562 cells (not shown).

Surprisingly, the resumption of growth of NOS-transfected K562 cells was not associated with a loss in their ability to express NOS as measured by Western blot, and nitric oxide production continued as inferred by the accumulation of nitrite in the culture media. Furthermore, accumulation of nitrite in the medium was significantly lowered by the presence of 1 mmol/L L-NMA, and nitrite accumulation did not occur in the medium of control-transfected cells. NOS-transfected cells maintained their ability to produce nitrite for more than 1 month, which was the maximum length of time for which the cells were monitored following transfection. These results suggest that the resumption of proliferation by NOS-transfected cells resulted from some adaptation to continuous exposure to nitric oxide rather than to suppression of nitric oxide synthesis.

We noticed that the maximum concentration of nitrite in the culture media did not exceed 25 to 30 μmol/L. However, if the cells were pelleted and resuspended in fresh media, nitrite accumulation resumed, suggesting the depletion of a component(s) of the medium critical to NOS activity. We investigated in more detail the time course of nitrite accumulation following seeding of cells into fresh medium. These studies showed three phases of nitrite accumulation: an initial lag period after seeding the new culture of approximately 2 hours, a 10- to 15-hour period in which nitrite accumulation was linear, and a plateau at which no further significant accumulation of nitrite occurred despite continued proliferation of the cells (Fig 3). In the middle period, nitrite accumulated at a rate of 0.8 to 1.1 mmol/hour/×10⁶ cells, corresponding to a 1 μmol/L/hour change in nitrite accumulation in cultures seeded at a cell density of ×10⁶ cells/mL. In fresh medium, the nitrite accumulation rate was proportional to the initial density of NOS-transfected K562 cells in the culture.

Hemoglobin expression. We noticed a marked difference between NOS-transfected and control-transfected cells in the color of the cell pellets. This is observed both early after transfection and in NOS-transfected cells that have resumed proliferation. Untransfected and control-transfected K562 cells give cell pellets that are pale orange-pink, while cell pellets of NOS-transfected cells are white. As K562 cells express low levels of fetal and embryonic hemoglobin,24 this difference suggested an effect of NOS activity on hemoglobin expression. The UV-visible spectra and the resulting difference spectrum of cytosols from control and NOS-transfected cells are presented in the upper and lower panels of Fig 4, respectively. The difference spectrum (lower panel of Fig 4) closely matches the spectrum of oxyhemoglobin with absorbance maxima at 414 nm (Soret), 542 nm (α-band), and 577 nm (β-band).25

The hemoglobin content of control cells was estimated by peroxidase assay to be fivefold to 14-fold higher than in NOS-transfected cells (Table 1). Immunoblotting of the cell lysates with an antibody to human fetal γ-hemoglobin chain confirmed that the hemoglobin content of NOS-transfected cells was diminished (compare the two left lanes of Fig 5, top panel). To confirm that this effect was due to NOS activity, the NOS-transfected cells were cultured in the presence of 0.5 to 2 mmol/L of the NOS inhibitor L-NMA, and the lysates were immunoblotted with anti–γ-hemoglobin (see the four right lanes of Fig 5, top panel). Even at the lowest concentration of inhibitor, hemoglobin content was increased to a level approaching that of control cells.

To determine if NOS expression was affecting globin expression at the level of transcription, total RNA from NOS-transfected and control-transfected cells were isolated and probed with a labeled synthetic oligonucleotide specific for the 3’ end of human γ-globin mRNA (Fig 6, top panel). In both cases the level of expression of the message is similar. Thus, NOS activity is not inhibiting transcription of fetal globin mRNA.

Effect of butyrate induction or heme synthesis intermediates on hemoglobin expression. Sodium butyrate produces a variety of morphological and biochemical changes in cell cultures, including erythroid differentiation in erythroleukemic cell lines.26,27 With control-transfected K562 cells addition of sodium butyrate to the growth medium results in a 1.8-fold increase in hemoglobin content. However, this general induction of enhanced hemoglobin production was not sufficient to reverse the profound inhibition of hemoglobin synthesis in the NOS-transfected cells (Fig 5, bottom panel; compare lanes from cultures with no additives, "None," to that with butyrate, "NaOBu").
Hemoglobin expression in erythrocyte precursors is coordinated to the availability of heme and intermediates of the heme biosynthetic pathway, such as 5-aminolevulinate. Hemin and 5-aminolevulinate stimulate a 1.8-fold and 2.4-fold increase in hemoglobin levels, respectively, in control-transfected K562 cells. We next examined the effects of hemin and 5-aminolevulinate on hemoglobin levels in NOS-transfected K562 cells. In contrast to butyrate, which did not restore hemoglobin levels, both hemin and 5-aminolevulinate reversed the profound inhibition of hemoglobin levels in NOS-transfected K562 cells (Fig 5, lower panel; compare lanes from cultures with no additives, “None”, to that with 5-aminolevulinate, “ALA”, or with Hemin). NOS-transfected cells stimulated with hemin had a hemoglobin level identical to unstimulated, control transfected cells, while the hemoglobin level of ALA-stimulated, NOS-transfected cells was 1.5-fold greater than in unstimulated, control transfected cells.

The ability of 5-aminolevulinate to overcome inhibition of globin expression suggests that NOS activity is affecting erythrocyte 5-aminolevulinate synthase (eALAS), the first enzyme of the heme biosynthetic pathway. We measured the level of eALAS mRNA present in both NOS-transfected and control cells by Northern blotting and found that the level of this mRNA was similar in both cases (Fig 6, middle panel).

**DISCUSSION**

Continuous endogenous expression of nitric oxide synthase in K562 cells diminishes both their hemoglobin expression and growth rate. Both observations may share the same chemical basis in the interaction of nitric oxide with iron sulfur proteins.

The slower growth of NOS-transfected K562 cells relative to control cells may be attributed to inhibition of respiratory proteins that contain iron-sulfur clusters, particularly complexes I (NADH: ubiquinone oxidoreductase) and II (succinate: ubiquinone oxidoreductase) of the respiratory chain. Additionally, nitric oxide may also inhibit complex IV (cytochrome c oxidase), possibly through competition with molecular oxygen. Despite these possible effects of constitutive nitric oxide production on the respiratory chain, NOS-transfected K562 cells are able to adapt to the expression of active NOS and survive and proliferate in long-term culture. How NOS-transfected K562 cells manage to do this is unknown. It is unlikely that the reaction of nitric oxide with oxyhemoglobin to produce nitrate and methemoglobin is a factor in the survival of NOS-transfected K562 cells, as hemoglobin production in these cells is greatly suppressed. Other protective mechanisms may be at work; constitutive expression of macrophage NOS in stably transfected B6 fibroblasts has been reported, although details of their growth characteristics were not given. We have also generated fibroblast and macrophage cell lines that constitutively produce large amounts of nitrite with similar growth patterns (Domachowske et al, in preparation).

When NOS-transfected K562 cells are transferred to fresh culture media, there is a brief lag period in which no nitrite accumulates, followed by a period in which nitrite accumulation is linear. However, nitrite eventually ceases to accumulate, and we did not observe nitrite concentrations greater than 30 μmol/L in the culture media. This suggests depletion of a component required for enzyme activity. It is unlikely that arginine is a limiting factor, as it is present at an initial

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**Table 1. Peroxidase Colorimetric Determination of Hemoglobin Content in NOS-Transfected and Control-Transfected K562 Cells**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hemoglobin Content, ng/100 μg Protein</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-transfected</td>
<td>400 ± 10</td>
<td>5.7 ± 4</td>
</tr>
<tr>
<td>NOS-transfected</td>
<td>70 ± 3</td>
<td></td>
</tr>
<tr>
<td>Control-transfected</td>
<td>250 ± 12</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>NOS-transfected</td>
<td>17 ± 2</td>
<td></td>
</tr>
<tr>
<td>Control-transfected</td>
<td>110 ± 5</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>NOS-transfected</td>
<td>10 ± 3</td>
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Shown are the results with cells selected and grown following each of three separate transfections. The first column of values is the hemoglobin content in the cytoplasam, while the second column shows the fold decrease in hemoglobin expression by NOS-transfected K562 cells relative to the control-transfected K562 cells.
Both panels show Western immunoblots detecting γ-hemoglobin with specific antibody following SDS-PAGE separation of cytosols from either control-transfected K562 cells or NOS-transfected K562 cells. In the top panel starting from the left are cytosols from control-transfected K562 cells and NOS-transfected K562 cells, and from NOS-transfected K562 cells grown in the presence of increasing concentrations of N-methyl-L-arginine (L-NMA). In the bottom panel are the same two types of transfected K562 cells grown in the presence of (starting from the left) no additives (None), 0.5 mmol/L 5-aminolevulinate (ALA), 1 mmol/L sodium butyrate (NaOBu), or 20 μmol/L hemin. On the vertical axis is indicated the M, in thousands. Cells were grown in the presence of these additives for 4 days before the preparation of cytosols. Above each lane is the level of hemoglobin, relative to untreated, control-transfected K562 cells, as estimated by densitometry. The nitrite concentrations in the culture media at the time of harvesting are also shown along the top of each immunoblot. In each case the error in nitrite measurements was ±0.5 μmol/L.

Concentration of 1.15 mmol/L in the culture medium. Nor is it likely that we are measuring a steady-state concentration of nitrite in which its production is balanced by its conversion to nitrate, as we have found that nitrite concentrations in stored media samples do not change significantly over a period of 1 week. Ironically, heme itself may be a limiting factor in the ability of the cells to accumulate nitrite, as nitric oxide synthases require heme. Serum contains small quantities of hemoglobin, which could serve as a source of heme; the concentration of serum-derived hemoglobin in the culture medium is about 10 μg/L. Although we have not measured this, the plateau in nitrite accumulation may be correlated to depletion of heme from the media.

The iron sensitive regulatory element IRP-1 is a potential target for nitric oxide-derived oxidants. However, there is controversy whether nitric oxide reacts directly with iron-sulfur clusters, or whether the reactive species is peroxynitrite that is produced by the prior reaction of nitric oxide with superoxide. Formation of peroxynitrite from nitric oxide and superoxide in solution is rapid (6.7 × 10³ M⁻¹ s⁻¹)⁹ and thus may be the agent responsible for removing iron from IRP-1. Experiments performed in vitro to address this question give contradictory results and provide support for either peroxynitrite¹⁰ or nitric oxide¹¹ as the reactive agent. Regardless of the nature of the active species, nitric oxide that has been generated endogenously¹² or by exogenous donors¹³ alters the expression of genes that are translationally controlled by IRE-IRP interactions in a manner that is consistent with the disruption of the iron-sulfur cluster of IRP-1. However, our results do not exclude the possibility that endogenously produced nitric oxide acts upstream of IRP-1 by lowering the availability of iron from the iron regulatory pool.
The effect of nitric oxide on expression of transferrin receptor and ferritin in K562 cells has been examined recently, with 10 μmol/L S-nitroso-N-acetyl penicillamine (SNAP) as the source of nitric oxide. The presence of SNAP increased transferrin receptor expression, yet did not decrease ferritin expression, as would be expected by models of nitric oxide's interaction with IRP-1. This failure to affect ferritin expression may be a consequence of the transient nitric oxide release from the rapid decomposition of SNAP. In contrast, we noted decreased ferritin expression in a K562 cell line that constitutively expresses macrophage NOS from a plasmid integrated into the genomic DNA (Domachowske et al, in preparation). Oria et al did not examine eALAS expression, but suggested that this was also a potential target for suppression by nitric oxide.

eALAS is the first enzyme of the biosynthetic pathway to protoporphyrin IX and subsequently to heme in erythroid cells (see outline of pathway in Fig 7). The mRNA for eALAS is known to contain an iron-responsive element in its 5' untranslated region and translation is known to be regulated in an iron-dependent manner by reversible binding of IRP-1. Globin expression, in turn, is tightly regulated by the availability of heme, which binds to and inactivates a translational inhibitor called hemin controlled repressor, or HCR.

Fig 7. Outline of heme and hemoglobin biosynthesis. Heme synthesis is translationally regulated by reversible, iron-sensitive binding of IRP-1 to an iron-responsive element in the 5' untranslated region of mRNA of eALAS. The present study suggests that this first step (indicated at the upper left of the scheme) is the likely site of action of nitric oxide (indicated by "NO"). The nitric oxide is postulated to convert IRP-1 to its iron-depleted mRNA-binding conformation resulting in repression of eALAS translation. Globin biosynthesis is translationally regulated by the availability of heme, which binds to and inactivates a heme-controlled repressor (HCR). This explains the ability of hemin to bypass the nitric oxide-mediated block in γ-hemoglobin synthesis in the present study.
eALAS mRNA levels are similar in both NOS-transfected and control cells. Nitric oxide could repress hemoglobin biosynthesis at the level of eALAS by several possible mechanisms: depriving the enzyme of substrate; by direct inhibition of enzymatic activity; or by translational repression as described in the introduction. With respect to substrate depletion it is conceivable that nitric oxide inhibition of mitochondrial aconitase, a member of the Kreb’s cycle, could deprive eALAS of succinyl-CoA. However, succinyl-CoA can also be produced by the degradation of the amino acids glutamine, glutamic acid, histidine, arginine, and proline via α-ketoglutarate, as well as from methionine, valine, and isoleucine via propionyl-CoA. While there have been no reports on any effects of nitric oxide on eALAS activity, direct inhibition of enzyme activity cannot be excluded. It is most likely that nitric oxide decreases the translation of eALAS message in the same manner as it affects ferritin expression, through the known iron regulatory sequence in its mRNA. Thus, the observation of diminished hemoglobin expression in NOS-transfected K562 cells is the most visible example of the effect of nitric oxide on translationally regulated iron homeostasis.

It was recently demonstrated that mammalian ferrochelatase, which catalyzes the last step of heme biosynthesis (the insertion of iron into porphyrin), possesses a noncatalytic Fe₆S₄ cluster that may regulate enzyme activity through reversible binding of iron. Our observations suggest that the cluster is either resistant to disruption by nitric oxide, or that it does not serve as a regulator of activity, as 5-aminolevulinate is sufficient to restore hemoglobin expression in NOS-transfected cells.

There is evidence that nitric oxide plays a role in the control of proliferation of the myelomonocytic cell compartment in the bone marrow, with the granulocytes and/or macrophages as the source of nitric oxide. It has also been demonstrated that nitric oxide has an antiproliferative effect, manifested as anemia, on erythroid cells. This study found a direct association between anemia and nitric oxide production in Trypanosoma-infected mice. Blood hemoglobin concentrations were lower in infected mice, but could be restored by treatment with the nitric oxide synthase inhibitor LNAME. An antiproliferative effect may partially explain the results of this study, the reduction of hemoglobin synthesis may be occurring by repression of eALAS, as suggested by our studies. In this way, nitric oxide produced in the bone marrow by macrophages or chondrocytes may suppress the production of hemoglobin in the developing erythrocytes of infected animals. Additionally, it has been suggested that interferon-γ and tumor necrosis factor-α act as indirect inhibitors of hematopoiesis by stimulating NOS activity in highly purified CD34+ cells from bone marrow. Further work will be needed to determine if bone marrow-derived nitric oxide has a specific role in anemia, particularly in the anemia of infection and chronic disease.

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