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Original Contribution

Nitric oxide blocks cellular heme insertion into a broad range of heme proteins

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ABSTRACT

Although the insertion of heme into proteins enables their function in bioenergetics, metabolism, and signaling, the mechanisms and regulation of this process are not fully understood. We developed a means to study cellular heme insertion into apo-protein targets over a 3-h period and then investigated how nitric oxide (NO) released from a chemical donor (NOC-18) might influence heme (protoporphyrin IX) insertion into seven targets that present a range of protein structures, heme ligation states, and functions (three NO synthases, two cytochrome P450's, catalase, and hemoglobin). NO blocked cellular heme insertion into all seven apo-protein targets. The inhibition occurred at relatively low (nM/min) fluxes of NO, was reversible, and did not involve changes in intracellular heme levels, activation of guanylate cyclase, or inhibition of mitochondrial ATP production. These aspects and the range of protein targets suggest that NO can act as a global inhibitor of heme insertion, possibly by inhibiting a common step in the process.

Heme proteins perform widespread and essential roles throughout biology, including electron transfer, redox transformations, small-molecule transport, metabolism, catabolism, and signal transduction [1–3]. To perform these roles, heme proteins have evolved into a number of structural classes. The most common group utilizes protoporphyrin IX bound within the protein. Members of this group include globins such as hemoglobin and myoglobin, heme–thiolate enzymes such as cytochrome P450's (CYP)[2] and NO synthases (NOS), peroxidases, and per-arnt-sim-type (PAS) signaling proteins [4–7].

The steps of heme biosynthesis and how heme functions in proteins are well studied [8–10]. However, with the exception of cytochrome *c* biogenesis [11] and some aspects of heme acquisition and catabolism [12,13], relatively little is known about how heme is transported within eukaryotic cells and becomes inserted into soluble protein targets or how these processes might be regulated. These steps are critical, given that free heme is bioactive and potentially cytotoxic [14] and is normally kept at low intracellular levels [15]. A number of cytosolic heme-binding proteins have been described and include liver fatty acid binding protein [16], glutathione *S*-transferase [17], heme-binding protein HBP23 (peroxiredoxin 1) [18], heme-binding protein HBP22 [19], and PAS-domain proteins [20,21]. However, their possible roles in heme transport or insertion reactions have not been tested. Heme insertion into catalase was shown to possibly occur within the peroxisome [22], and myeloperoxidase trafficking in cells was found to depend on its heme content [23]. Hsp90 is required for heme insertion into neuronal NOS [24], implying that there is an active cellular mechanism that involves multiple proteins. Regarding regulation, a general deficiency in iron or heme can down-regulate heme protein biosynthesis at the level of gene transcription or translation [25–27], which is upstream of the heme insertion process [28].

We previously reported that nitric oxide (NO) could block heme insertion into inducible NO synthase (iNOS) [29]. In that study, iNOS protein expression was induced in the RAW264.7 macrophage cell line by cytokine immune activation, and thus the effect involved NO that was generated by iNOS within the cells. After some of the early expressed iNOS protein had formed an active dimer, the NO it produced was able to progressively antagonize heme insertion into any subsequently generated iNOS protein, such that by 8 h post-induction, heme insertion into any new iNOS protein was essentially blocked and the iNOS accumulated as heme-free monomers (apo-iNOS) within the cells. A variety of experiments established that the NO did not affect heme availability within the cells but was rather acting to prevent heme insertion [29]. After this study, the possibility that NO has a broader role in controlling cellular heme insertion was not pursued and is still unclear.

To address this issue we sought to examine the effects of NO on heme insertion into several target proteins. We first developed a cell

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[2] *Abbreviations used:* NOS, nitric oxide synthase; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; SA, succinyl acetone; CYP, cytochrome; PIF, pyrimidine imidazole FITC; LPS, *Escherichia coli* bacterial lipopolysaccharide; IFN-γ, interferon-γ; L-NAME, N^{ω}-nitro-L-arginine methyl ester; HEL, human erythroid leukemia.

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culture method that can test the ability of NO (generated in a controlled manner by a chemical NO donor) to inhibit cellular heme insertion into any soluble protein target. We utilized our method to test whether NO inhibits heme insertion into iNOS and six other heme proteins that utilize protoporphyrin IX and present a diversity of protein structures and functions, namely, two constitutively expressed mammalian NOS enzymes (endothelial NOS, eNOS; neuronal NOS, nNOS), two cytochrome P450 enzymes (CYP 3A4 and 2D6), catalase, and hemoglobin. We found that NO blocked cellular heme insertion into all of these target proteins and therefore seems capable of global effects on cellular heme insertion. The possible mechanisms and biological impacts are discussed.

Experimental procedures

Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) or Fischer Scientific (Springfield, NJ, USA). Molecular mass markers were purchased from Bio-Rad (Hercules, CA, USA). Enzymes were purchased from New England Biolabs (Beverly, MA, USA). PIF was provided by Dr. John Parkinson of Berlex Biosciences. Hemin was purchased from Sigma. Stock solutions of hemin were freshly prepared as described elsewhere [30]. Mouse catalase cDNA [31] was a gift from Dr. Serpil Ezurum (Lerner Research Institute, Cleveland Clinic). cDNAs for soluble forms of cytochrome P450 2D6 and 3A4 [32,33] were a gift from Dr. F. Peter Guengerich (Vanderbilt University). Human embryonic kidney HEK293T cells that stably express either eNOS [34] or nNOS [35] were gifts from Drs. Bill Sessa (Yale University) and Solomon Snyder (Johns Hopkins University), respectively. The human erythroid leukemia (HEL) cell line K562 was purchased from ATCC (Manassas, VA, USA). Mouse IFN-γ was purchased from Genentech (South San Francisco, CA, USA).

Antibodies

Rabbit polyclonal antibody specific to hemoglobin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody specific to Flag was purchased from Sigma. A monoclonal antibody specific to catalase was obtained from Santa Cruz Biotechnology. Anti-eNOS, -nNOS, and -iNOS antibodies were obtained from BD Transduction Laboratories (San Jose, CA, USA). These antibodies were used in Western blots and in immunoprecipitation assays following the procedures outlined by the manufacturers.

Cell culture methods, heme depletion, and preparation of centrifuged cell lysates

All cell lines were grown in 100-mm tissue culture dishes. Mouse macrophage RAW264.7 cells and HEK293T cells were cultured in DMEM containing 10% FBS and 5000 units of penicillin–streptomycin. HEK293T cells that were stably transfected to express eNOS or nNOS were cultured in a 1:1 mixture of DMEM and HAM's F-12 containing l-glutamine and pyruvate, 4.5 g/L glucose, 5000 units/L of Pen–Strep, 10% FBS, and 250 μg/ml of Geneticin (G418). HEL K562 cells were grown in RPMI 1640 medium containing 10% FBS and 5000 units/L penicillin–streptomycin.

To inhibit heme biosynthesis and deplete stores of intracellular heme the cell lines were cultured with 250 μM succinyl acetone (SA) for 48 to 72 h before use [36]. Afterward, the RAW264.7 cells were given fresh medium + SA and induced to express iNOS with *Escherichia coli* LPS (50 μg/ml) and mouse IFN-γ (10 ng/ml) in the presence of 3 mM L-NAME (a NOS NO synthesis inhibitor) as described previously [29]. After 16 h of induction, the cells were given fresh medium that also contained the protein synthesis inhibitor cycloheximide (10 μg/ml), incubated for 30 min, and then further incubated with the indicated concentrations of hemin for variable times as described in the text. In some cases, the NO donor NOC-18 alone or with hemin was also added. In other cases, after iNOS induction, RAW264.7 cells were given antimycin A (10 μM) or 8-bromo-cGMP (1 mM) for 30 min before hemin addition. At the point of cell harvest, the monolayers were washed twice with 4 ml cold PBS containing 1 mg/ml glucose, and cells on each plate were collected by scraping in the presence of 500 μl of lysis buffer (40 mM EPPS buffer, pH 7.6, 10% glycerol, 3 mM dithiothreitol, 150 mM NaCl, and 1% NP-40). The collected cells were lysed by three cycles of freeze–thawing (in liquid nitrogen and at 37 °C, respectively). The lysates were centrifuged for 30 min at 4 °C and the supernatants were collected and stored at −70 °C. Total protein contents of the supernatants were determined using the Bio-Rad protein assay kit. Similar procedures of culture with SA, cycloheximide, hemin, and NOC-18 and cell supernatant production and storage were utilized for the K562 (HEL), HEK293T, and stably transfected HEK293T cells.

Transient transfection of cells

Cultures (50–60% confluent) of SA-treated RAW264.7 or HEK293T cells were transfected with the pTRE plasmid containing cDNA of CYP 2D6 and CYP 3A4 or the pS3 plasmid containing cDNA for human catalase, using Lipofectamine and following protocols as described by the manufacturer (Invitrogen, San Diego, CA, USA). The transfected cells were cultured for up to 42 h in the presence of SA to allow for expression of the apo-proteins. Afterward, the culture medium was replaced with fresh medium containing cycloheximide, NOC-18, L-NAME (when needed), and/or hemin, and the cells were further processed as required in each experiment. The transfection experiments were carried out in duplicate or triplicate plates and the experiments were repeated at least three times.

Confocal microscopy

SA-pretreated RAW264.7 cells were cultured on glass coverslips. Cells were induced with cytokines for 16 h in the continued presence of SA. PIF (1 μM) and, as indicated, hemin (5 μM) and NOC-18 (125 μM) were added to the induced cells for 3 h at 37 °C. Cells on coverslips were washed three times with PBS and distilled water and fixed with 4% formaldehyde. Coverslips were mounted on glass microscope slides with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) to visualize nuclei. Confocal images were taken under a 63× objective lens (zoom 3) of a Leica TCS-SP_AOSB laser confocal microscope and processed using Leica Confocal software version 2.5.

UV–visible spectroscopy

UV–visible wavelength scans were recorded at room temperature between 350 and 700 nm on a Cary or Shimadzu spectrophotometer. To measure heme incorporation into NOS or CYP proteins the supernatants were diluted one- to threefold in EPPS buffer (40 mM EPPS buffer, pH 7.6, 10% glycerol, and 150 mM NaCl) in a cuvette, bubbled with CO, and then reduced by adding a small amount of dithionite. Spectra of each sample were recorded before the CO bubbling and after dithionite addition to obtain a calculated difference spectrum. The concentrations of heme-containing NOS were determined from the Soret absorbance peak at 444 nm using the extinction coefficient 74 $mM^{-1}\,cm^{-1}$ [37]. The heme content for hemoglobin was determined from the Soret absorption peak at 414, using the extinction coefficient of 342.5 $mM^{-1}\,cm^{-1}$ for the tetramer. The heme content of CYP enzymes in cell supernatants was estimated from the absorbance difference at 450 nm of the