Cytokines induce the mouse macrophage cell line RAW 264.7 to express cytokine-inducible nitric oxide synthase (iNOS), which is active only in dimeric form. Because dimerization of purified iNOS subunits requires tetrahydrobiopterin, heme, and L-arginine, we investigated if availability of these factors also influences intracellular assembly of dimeric iNOS. Following exposure to cytokines, iNOS protein was found to accumulate in a near linear manner over 16 h of further culture. In contrast, dimeric iNOS accumulated at a slower rate that continuously decreased during culture, resulting in only 25% of the accumulated iNOS protein being in dimeric form by 16 h. Further experiments argued against dimer instability or L-arginine and tetrahydrobiopterin availability as factors limiting iNOS dimer accumulation. Blocking cellular NO synthesis with N\(^{\text{N}}\)-nitro-L-arginine methyl ester (L-NAME) greatly increased iNOS dimer assembly, indicating NO synthesis limited iNOS dimerization. NO synthesis was found to prevent an increase in soluble heme level that was associated with iNOS induction in N\(^{\text{N}}\)-nitro-L-arginine methyl ester-treated cells and also diminished heme insertion into iNOS. These NO-related defects were not reversed by adding heme precursors or hemin to the activated cell cultures. Measurement of iron release from activated cells demonstrated that endogenous NO synthase substantially increased the release of \(^{54}\)Fe to the medium. These observations suggest that iNOS dimerization is limited to a large extent by iNOS NO synthesis. NO appears to limit intracellular assembly of dimeric iNOS by preventing heme insertion and decreasing heme availability.

Nitric oxide (NO)\(^1\) is an important bioregulatory molecule involved in signal transduction, vascular resistance, platelet aggregation, and destruction of microbes and tumor cells (1, 2). NO is generated by three distinct NO synthases (NOSs) whose genes are located on different chromosomes and code for proteins that display — 65% sequence homology to one another (3, 4). The NOSs isolated from rat neurons (nNOS) and from cytokine-stimulated mouse macrophages (iNOS) have been the most intensively studied and are known to share some fundamental catalytic and structural properties. For example, both isoforms are homodimeric and catalyze a stepwise NADPH-and O\(_2\)-dependent oxidation of L-arginine to generate NO and citrulline (for reviews, see Refs. 5 and 6). Both NOSs have a bidomain structure in which contains a reductase domain that contains binding sites for NADPH, FMN, FAD, and calmodulin is linked to an oxygenase domain that contains binding sites for iron protoporphyrin IX (heme), tetrahydrobiopterin (H\(_4\)biopterin), and substrate (L-arginine) (7, 8). During NO synthesis, the flavins accept electrons from NADPH and transfer them across domains to the heme iron, which then binds and activates O\(_2\), to catalyze the stepwise oxidation of L-arginine (9–11). The heme iron in both NOSs is coordinated to the protein through a cysteinethiolate and has an overall ligand environment similar to the heme iron in the cytochrome P-450s (12–15). Thus, the NOSs are considered to be the only soluble cytochrome P-450-like enzymes found in eukaryotes.

Cellular expression of the NOS isoforms is subject to varied and multi-level control. For example, nNOS is constitutively expressed in an inactive form and requires Ca\(^{2+}\)-promoted calmodulin binding to activate its NO synthesis (16). Calmodulin binding activates NOSs by triggering electron transfer to the heme iron (10). In contrast, iNOS is not normally present in cells but is synthesized in response to immunomacromolecules and bacterial products such as interferon-\(\gamma\), interleukins 1 and 2, tumor necrosis factor, and bacterial lipopolysaccharide (LPS) (1). Once formed, iNOS can generate NO independent of the intracellular Ca\(^{2+}\) level due to its containing tightly bound calmodulin (17).

Although cellular expression of the iNOS protein is primarily controlled at the transcriptional level (18, 19), studies by Baek et al. (20) suggest that post-translational modifications are also needed to generate the active enzyme. Specifically, iNOS is synthesized as a monomer in cytokine-activated macrophages and must dimerize to generate NO. Purified iNOS monomers require the coincidental presence of H\(_4\)biopterin, L-arginine, and heme to associate into active dimers, with heme and H\(_4\)biopterin becoming bound to the protein in the process (Scheme 1). Curiously, cytokine-activated macrophages contain about a 50–50 mixture of iNOS monomers and dimers after 8 h of induction (20), suggesting that dimer assembly may somehow be limited in activated macrophages.

To address this possibility, we have studied iNOS dimer assembly over time in RAW 264.7 macrophage cells activated to express iNOS with interferon-\(\gamma\) and LPS, paying particular attention to whether a deficiency in any of the three factors known to promote subunit dimerization in vitro (L-arginine,
H₂biotin, and heme) was limiting subunit assembly in cells. Our results show that iNOS dimerization is indeed limited in these cells due to a deficiency in heme insertion and availability brought on by NO synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials—** Monoclonal antibody raised against macrophage iNOS was obtained from Transduction Laboratories (Lexington, KY). Interferon-γ was a gift from Genentech (South San Francisco, CA). CO gas was obtained from Gas Technics (Cleveland, OH). Fe₅⁹ was obtained from Amersham Corp. All other reagents and materials were obtained from Sigma or from sources previously reported (20).

Cell Culture and Preparation of Cell Lysates—The mouse macrophage cell line RAW 264.7 was grown to a density of 1–2 × 10⁶ cells/ml in 0.5–3-liter spinner bottles containing RPMI 1640 with 8% calf serum and induced to express iNOS with interferon-γ and LPS as described previously (20). In some cases, additives such as L-NAME, hemin, δ-aminolevulinic acid (ALA), or dioxoheptanoic acid were also added to the cultures at the point of cytokine addition. Cells were harvested at different times after induction, washed, and lysed by 3 cycles of freezing and thawing in a lysing buffer containing 40 mM Bis-Tris, pH 7.4, 3 mM dithiothreitol, 4 μM H₂biotin, 10% glycerol, and 100 mM NaCl. In some cases, H₂biotin was omitted. The cell lysates were subjected to ultracentrifugation, and the supernatants were collected and stored at −70 °C.

Measurement of NO Synthesis Activity—NO synthesis by cell cultures was estimated by measuring nitrite accumulated in the culture medium over time. Nitrite is a stable oxidation product of NO, and its measurement serves as a convenient assay for NO production. Aliquots (3–0.1 ml) of cell culture fluid were removed at various times, centrifuged, and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, 2.5% H₂O₂) at room temperature for 5–10 min. Nitrite was estimated using a microplate reader, and the concentration was calculated using standard curve of a range of 10 μM–1 mM sodium nitrite. Standards.

Quantitation of iNOS Dimer/ Monomer Ratios—We first developed a method to measure the relative amounts of iNOS dimers and monomers present in macrophage cell supernatants. Size exclusion chromatography was carried out at 4 °C using a Pharmacia Superdex 200 gel filtration column. The column was equilibrated at 0.5 ml/min with 40 mM Bis-Tris buffer, pH 7.7, containing 3 mM dithiothreitol, 1 mM L-arginine, 1 mM NADPH, and 4 μM each of FAD, FMN, and H₂biotin to give a final volume of 0.1 ml. Reactions were incubated for 90 min at 37 °C and terminated by enzymatic depletion of remaining NADPH (20).

Gel Filtration Chromatography—To determine the relative amounts of iNOS dimers and monomers present in macrophage cell supernatants, size exclusion chromatography was carried out at 4 °C using a Pharmacia Superdex 200 gel filtration column. The column was equilibrated at 0.5 ml/min with 40 mM Bis-Tris buffer, pH 7.7, containing 3 mM dithiothreitol, 1 mM L-arginine, 4 μM H₂biotin, 4 μM FAD, 10% glycerol, and 200 mM NaCl. Equal amounts of protein (1–3 mg) from cell supernatants were diluted to 200 μl prior to injection, and the protein in the column effluent was collected at 280 nm using a flow-through detector. Fractions were assayed for iNOS activity as above. The molecular weights of the protein fractions were estimated relative to gel filtration molecular weight standards.

SDS-Gel and Western Blot—Aliquots from the gel filtration fractions that fell within a molecular mass range of 600 to 20 kDa (around 16–1 ml fractions) were electrophoresed on 8.5% SDS-polyacrylamide gel, transferred to a nytran membrane, blocked with 5% non-fat dry milk, and probed with an iNOS monoclonal antibody raised against a 21-kDa protein fragment corresponding to amino acids 961–1144 of mouse iNOS. Anti-mouse horseradish peroxidase-conjugated antibody was used as a secondary antibody, and the films were developed using an ECL system (Amersham International, United Kingdom). The intensity of the individual iNOS bands was quantified by densitometry, and the ratio between monomers and dimers was calculated from these values.

Biopterin Measurements—The concentration of total biopterins in the macrophage cell supernatants was measured by high pressure liquid chromatography analysis using a Rainin C18 reverse phase high performance liquid chromatography column as described previously (20). The column was equilibrated with 5% methanol flowing at 0.7 ml/min. Aliquots (0.5–0.1 ml) from cell supernatants that were prepared with H₂biotin-free lysing buffer were used for each analysis. Samples were oxidized and analyzed by a published fluorometric method (21) and quantitated using curves generated with authentic biopterin and H₂biotin.

**Total Soluble Porphyrin, Soluble Heme, and Soluble Porphyrin Measurements—** Total soluble heme in the cell supernatants was measured through the formation of a pyridine heme chromogen (12). Briefly, 150 μl of cell supernatant was mixed with 240 μl of heme chromogen reaction (40:60 pyridine/H₂O, 200 mM NaOH) and heme iron was reduced by adding a few grains of sodium dithionite. Heme chromogen formation was monitored at 556 nm, and its concentration was calculated from an extinction coefficient of 34.6 mM⁻¹ cm⁻¹. To measure total soluble porphyrin (soluble heme plus soluble porphyrin) in the cell supernatants, a fluorometric method was used (22, 23). Briefly, in an Eppendorf tube, 980 μl of 2 M oxalic acid was mixed with 20 μl of cell supernatant and boiled for 1 h. The tubes were allowed to cool to room temperature, after which porphyrin was measured by its fluorescence emission at 662 nm (excitation 400 nm) relative to standard curves generated with freshly prepared authentic heme solutions that had been subjected to oxalic acid treatment. P-450 heme concentrations were measured by mixing 150 μl of cell supernatants with 150 μl of 40 mM Tris buffer, pH 7.4, in a 500-μl cuvette. Heme iron was reduced by adding a few grains of sodium dithionite to the cuvette, and a baseline spectra was recorded between 400 and 500 nm. CO was bubbled into the cuvette, and the sample was rescanned. The P-450 heme concentration was calculated from the difference spectrum using an estimated extinction coefficient of 74,000 M⁻¹ cm⁻¹ per heme as determined for authentic dimeric iNOS (12).

**Fe Release—** RAW 264.7 cells were added to 6-well culture plates to give confluent monolayers. FeCl₃, was added to the medium at the medium at a concentration of 2 μl/ml, which contained 9 μl of freshly prepared authentic heme solutions that had been subjected to 1% solution of 4 M oxalic acid from added calf serum. At the end of the labeling time, cell monolayers were washed five times with RPMI medium and activated with medium containing LPS and interferon-γ in the presence and absence of 3 μg/ml l-NAME. Spontaneous release of Fe was determined from identical cultures of unactivated cells. Aliquots of culture medium were taken every 4 h for measurement of total release. Total Fe release was determined by measuring aliquots from labeled cells that had been lysed with 0.2% SDS. The percentage of Fe-specific release was determined by the following equation:

\[
\text{% specific release} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100
\]

Cell viability during the iron release experiment was monitored by trypan blue exclusion. Activated cells examined at 7 and 16 h remained 97% viable.

Protein concentration was measured colorimetrically using the Bio-Rad assay kit and bovine serum albumin as a standard.

**RESULTS**

Quantitation of iNOS Dimer/ Monomer Ratios—We first developed a method to measure the relative amounts of iNOS monomers and dimers present in macrophage supernatants that did not rely on purifying iNOS. Our method involves separating iNOS dimers and monomers in macrophage supernatants by gel filtration chromatography followed by SDS-polyacrylamide gel electrophoresis of the column fractions, Western transfer, and visualization of iNOS protein using a commercially available monoclonal antibody. Fig. 1, panel A depicts the iNOS immunoreactive bands of estimated molecular mass 130 kDa that represent iNOS dimers and monomers.
present in a supernatant prepared from macrophages that had been activated to express iNOS for 8 h prior to cell lysis. Panel B quantitates band intensity as determined by densitometry, which indicates that iNOS dimers and monomers were present in an approximate 40:60 ratio in the 8-h supernatant, consistent with earlier findings (20). A replica experiment in which purified dimeric iNOS was substituted for cell supernatant and then processed through the chromatography, Western transfer, and quantitation steps showed that the dimer does not dissociate into monomers under the conditions of analysis (data not shown). Also shown in panel B are the NO synthesis activities for each gel filtration fraction, which confirm that only dimeric iNOS is capable of NO synthesis (20).

Dimer Formation in Activated Macrophages Over Time—We next examined how dimer formation in activated macrophages progressed over time in a culture of interferon-γ and LPS-activated RAW 264.7 cells. Dimer/monomer ratios were calculated as noted above on aliquots of cells removed from the culture every 2 h after activation, and the values were used in conjunction with the measured specific activities of each supernatant to determine the amounts of iNOS dimer and total iNOS present at each time point. Fig. 2 shows that the amount of total iNOS increased in an approximately linear manner over the entire 16-h culture period, such that at 16 h iNOS represented 1.6% of the total soluble protein (assuming 7.7 nmol of protein per mg for purified iNOS). In contrast, the rate of iNOS dimer accumulation was much slower and generally decreased over the culture period such that only a small amount of dimer accumulated after 10 h in the experiment shown. Together, this suggests that the activated cells gradually lose either their ability to dimerize iNOS subunits or their ability to stabilize the iNOS dimer once it has formed.

iNOS Dimer Stability—To investigate whether instability of the iNOS dimer prevented its accumulation, cells were induced to express iNOS for 8 h and then were either lysed or had cycloheximide (10 μg/ml) added and were cultured an additional 8 h prior to lysis. We did not find any significant differences in dimer/monomer ratio or specific activity between the two groups (data not shown), indicating that the iNOS dimer once formed is stable for an additional 8 h of culture. This suggests that the poor rate of dimer accumulation observed in Fig. 2 is probably due to a deficiency in dimer assembly rather than dimer instability.

Influence of L-Arginine and H4Biopterin—Because L-arginine...
and H₄biopterin are required for iNOS subunits to dimerize in vitro (20), we examined whether availability of these molecules became limiting during the 16-h cell culture period. The initial L-arginine concentration in our culture medium was 0.4 mM and could conceivably be depleted by arginase, which is also induced in RAW 264.7 cells by LPS (24). However, as panel A of Fig. 3 shows, nitrite continued to accumulate over the entire 4–16-h culture period. Because nitrite is a stable product derived from NO, its continuous accumulation in the culture indicates that L-arginine was present at all times to support NO synthesis by iNOS. We also tested if adding excess L-arginine to the cell culture would increase dimer formation. Addition of L-arginine to give 5 mM both at the point of cell activation and again after 4 h failed to increase the dimer/monomer ratio or specific activity observed at the 8-h time point (n = 3; data not shown), indicating that L-arginine availability was probably not limiting dimer formation in the activated cells.

To investigate whether H₄biopterin availability was limiting intracellular iNOS dimerization, we measured intracellular levels of total biopterins during the 16-h culture. Panel B of Fig. 3 shows that the concentration of total biopterins increased substantially over the resting intracellular value, reaching 45 pmol/mg by 16 h in the experiment shown. In replicate experiments, maximum H₄biopterin levels in cells generating NO ranged from 45 to 90 pmol/mg (n = 3). These biopterin concentrations achieved during activation are somewhat lower than a previous study with RAW 264.7 macrophage cells, which reported that intracellular biopterin levels increased from 40 to 140 pmol/mg following exposure to interferon-γ and LPS (25). Inhibiting cellular NO synthesis with L-NAME did not greatly alter the kinetics or magnitude of increase in intracellular biopterins in our system, given that maximum biopterin concentrations reached in two experiments were 70 and 110 pmol/mg. Notably, supplementing an NO-producing cell culture with 50 µM H₄biopterin during activation increased its intracellular biopterin content at 10 h relative to a non-supplemented activated culture (135 versus 95 pmol/mg of biopterins at 10 h, respectively) but did not lead to an increase in dimeric iNOS content as determined by comparing the specific activities of the two lysates. Thus, we conclude that H₄biopterin availability is unlikely to limit intracellular iNOS dimerization in our system.

Effect of NO Synthesis—Given the above results, we were left to consider heme as a possible limiting factor. However, because induction of NO synthesis is known to alter iron and heme metabolism in a variety of cells (26, 27), we first examined how blocking NO synthesis in activated RAW 264.7 cells would affect accumulation of total iNOS protein and iNOS dimer. Fig. 4 shows the results of a typical experiment in which NO synthesis over the entire 16-h culture period was inhibited approximately 80% by inclusion of 3 mM L-NAME at the point of activation (time 0). A comparison of Figs. 4 and 2 reveals that blocking NO synthesis markedly enhanced iNOS dimer assembly but also reduced by half the total amount of iNOS protein formed compared to activated cultures not receiving NAME. Identical results were obtained when a different inhibitor of NO synthesis (N-aminoguanidine) substituted for L-NAME (data not shown). Together, the data suggest that iNOS dimer assembly is being limited by cell-generated NO.

Heme Distribution in the Soluble Cell Fraction—We next examined if NO-mediated inhibition of iNOS dimer assembly might be due to effects on heme accumulation and distribution in the soluble cell fraction. We therefore measured the levels of total soluble porphyrin (soluble heme + porphyrin), soluble heme, and soluble P-450 heme in supernatants prepared from cells induced to express iNOS in the absence or presence of L-NAME.

As shown in the upper panel of Fig. 5, the levels of total soluble porphyrin and soluble heme remained relatively constant during a 16-h induction period in cells whose NO synthesis was not blocked. About two-thirds of the total soluble porphyrin was in the form of heme at each time point. Soluble P-450 heme, which represents dimeric iNOS (12, 20), increased over time to reach 13 pmol/mg in this experiment, 4 which is

---

4 In activated RAW 264.7 cells, H₄biopterin represents over 80% of the total intracellular biopterins (25).

---
about 50% of the soluble heme.

In the presence of the NO synthesis inhibitor L-NAME (Fig. 5, lower panel), the levels of total soluble porphyrin and heme increased 4-fold during the 16-h induction period in this experiment and 1.5-4-fold in general. Also, the proportion of total soluble porphyrin that was heme increased throughout the culture period and approached 100% by 14 h. Third, the percentage of heme that was P-450 increased over time such that it represented 90% of the total soluble heme by 10 h. Thus, we conclude that NO synthesis affects heme accumulation and utilization in activated RAW 264.7 cells.

59Fe Release from Activated Macrophages—Because NO synthesis is known to promote iron loss from cells (26), we wished to determine if iron loss occurred in our cultures and might coincide with the defect in iNOS dimerization seen above. We measured iron release over time from cultures of 59Fe-preloaded activated cells in the presence and absence of L-NAME. Fig. 6 shows that the rate of 59Fe loss from cells generating NO was 3 times greater than that of identical cultures receiving L-NAME over the first 12 h and more than 10 times greater between 12 and 16 h. The levels of 59Fe release from activated cells in the presence of L-NAME were very similar to levels released from non-activated cells over the 16-h period (15-20% release). Thus, NO-mediated iron loss occurred and was associated with the inhibition of iNOS dimer formation in our system.

Effect of Adding a Porphyrin Precursor—Addition of ALA to bacterial cultures expressing neuronal iNOS has been shown to increase heme biosynthesis and insertion into the enzyme (8). However, adding ALA to control, activated, or activated plus L-NAME cell cultures did not increase the intracellular levels of soluble heme or P-450 heme by 8 h (Fig. 7), although it did increase intracellular porphyrin levels 3-4-fold over non-ALA-treated cultures in each case. Added ALA also did not increase iNOS specific activity or the dimer/monomer ratio in the activated cells (data not shown). These results indicate that increased porphyrin levels do not lead to increases in soluble heme or dimeric iNOS assembly in our system.

Effect of Adding Hemin—We next assessed if hemin addition to the cultures at the point of activation would increase intracellular heme levels and thereby increase dimerization of iNOS. As shown in Fig. 8, groups 2 and 3, supplementing an activated cell culture for 10 h with hemin did not increase the amount of dimeric iNOS formed relative to an unsupplemented activated culture, as determined by measuring P-450 heme (Fig. 8) or specific activities (not shown), although adding hemin doubled the soluble heme level. A control experiment in which activated cells were exposed to hemin for only 1 min prior to washing and lysis did not exhibit an increased level of soluble heme (data not shown), indicating that uptake of hemin into the cells was time-dependent. This data suggested that NO may prevent insertion of added heme into iNOS.

To further examine this possibility, we tested if added hemin would increase the heme content of iNOS expressed in cells
whose NO synthesis was blocked by L-NAME. Because iNOS in L-NAME-treated cells is normally 80–95% dimeric, we artificially decreased intracellular heme levels by adding the heme biosynthesis inhibitor dioxoheptanoic acid (42) to the L-NAME-treated cells at the point of activation. A comparison of groups 4 and 5 in Fig. 8 shows that dioxoheptanoic acid treatment significantly lowered soluble heme levels in activated cells treated with L-NAME and also decreased their dimeric iNOS content. Addition of hemin under these circumstances led to increases in both intracellular heme content and dimeric iNOS (Fig. 8, group 6). As estimated by Western analysis, the concentration of total iNOS in groups two and three were equivalent to one another, as was the concentration of total iNOS in samples 4–6. The values shown are the mean ± standard deviation of two or three determinations, and the experiment shown is representative of two.

**FIG. 8.** Effect of added hemin on soluble heme levels and distribution. Levels of total soluble heme and P-450 heme were determined in supernatants of control cells (group 1), activated cells cultured in the absence (group 2) or presence (group 3) of 10 μM hemin, or activated cells cultured in the presence of 3 mM L-NAME (group 4). L-NAME plus the heme biosynthesis inhibitor dioxoheptanoic acid at 200 μM (group 5), or L-NAME plus dioxoheptanoic acid plus hemin (group 6). As estimated by Western analysis, the concentration of total iNOS in groups two and three were equivalent to one another, as was the concentration of total iNOS in samples 4–6. The values shown are the mean ± standard deviation of two or three determinations, and the experiment shown is representative of two.

**DISCUSSION**

Macrophage iNOS is a hemeprotein that is catalytically active only in its dimeric form (20). We have studied iNOS dimerization in an activated macrophage cell line to understand what might control or limit this process. We found that activated macrophages become increasingly unable to assemble iNOS into dimers, such that in most cases limited iNOS dimerization takes place in cells 10 h post-induction. This defect arises despite a continuous synthesis of iNOS subunits by the cells and results in a majority of iNOS (50–80%) accumulating in the cells as inactive monomers by 16 h.

Because our previous work showed that L-arginine, H₂bipterin, and heme promote assembly of dimeric iNOS (20), we wished to determine whether availability of these factors might limit dimer assembly in activated macrophages. The evidence suggests that L-arginine availability did not limit iNOS dimerization in our system. Nitrite accumulated at a constant rate over the entire experimental period, and addition of excess L-arginine to the cells did not increase iNOS specific activity. In contrast, although intracellular concentrations of bipterins increased upon cell activation, there was some indication that the amount of iNOS monomers generated may in
some cases meet or exceed the amount of intracellular H$_4$biopterin available, especially at later time points (compare Figs. 2 and 3B). This was not the case for activated l-NAME-treated cells because they displayed a similar increase in intracellular H$_4$biopterin level while consistently producing approximately half the amount of total iNOS. In any case, adding H$_4$biopterin to activated cells that were producing NO did not increase their iNOS dimer formation, although it did significantly elevate their intracellular H$_4$biopterin level. This suggests that factors other than H$_4$biopterin availability are limiting in this system. Indeed, iNOS dimerization appeared to be inhibited by cellular NO synthesis. Blocking NO synthesis with l-NAME enabled macrophages to maintain a constant level of dimer assembly such that between 60 and 90% of the iNOS was present as a dimer at all times during the 16-h incubation.

The l-NAME experiments also identified three cellular processes associated with heme biosynthesis and utilization that appear to be inhibited by NO. Specifically, in activated cells whose NO synthesis was blocked by l-NAME, the total soluble porphyrin and soluble heme levels increased 1.5-4-fold over the course of the induction period, iron insertion into soluble porphyrin became nearly complete, and most of the cell’s soluble heme became incorporated into dimeric iNOS. These processes were either markedly reduced or absent in induced cells that were allowed to generate NO.

Because sufficient heme must be made available for iNOS to assemble into dimers (20), it is conceivable that an NO-mediated reduction in either heme accumulation or insertion into iNOS could be responsible for the poor iNOS dimerization that we observe in activated RAW 264.7 cells generating NO. Regarding heme accumulation, our data indicate that the amount of total iNOS generated during a 16-h induction period approaches or exceeds the amount of soluble heme present in activated macrophages that are generating NO. There are several mechanisms by which NO synthesis may prevent accumulation of porphyrin and heme in activated macrophages. In rat hepatocytes and RAW 264.7 cells, inducing NO synthesis inhibits the enzymes δ-aminolevulinic acid synthase and ferrochelatase (28, 38), which are responsible for porphyrin synthesis and iron insertion to form heme. NO synthesis also elevates the activity of the heme-degrading enzyme heme oxygenase by causing heme liberation from proteins (28). NO may inhibit the heme biosynthetic enzymes directly (i.e. ferrochelatase has an NO-sensitive FeS cluster (28, 38) or indirectly through causing increased release of intracellular iron (29). Indeed, NO-mediated iron loss is known to activate the cytoplasmic iron regulatory protein, which in turn down-regulates the expression of 5-aminolevulinic acid synthase (30).

Although NO synthesis did lead to reduced heme availability in our system, this effect alone probably does not limit dimer assembly for two reasons. First, soluble heme levels remained in excess of P-450 levels in NO-generating cells. Second, increased heme insertion into iNOS was still not observed when the intracellular heme level of activated cells was increased by adding hemin. Together, this suggests that NO reduces dimer assembly primarily by inhibiting heme incorporation into the iNOS protein. Our finding that added hemin did not increase iNOS heme content in NO-generating cells is in apparent contrast with that of Mayer and co-workers (31), who showed that added hemin greatly increased the heme content of neuronal NOS being expressed in an insect cell system, which is known to become heme-limiting (31). However, neuronal NOS was being expressed in its calmodulin-free form, which is inactive regarding NO synthesis. Indeed, our work shows that adding hemin to l-NAME-treated macrophages whose heme biosynthesis had been pharmacologically inhibited increased iNOS heme content 2–3-fold. This suggests that added hemin can be inserted into iNOS in activated macrophages but only in the absence of NO synthesis.

How NO might prevent heme insertion into iNOS is unknown and will require further study. However, it is clear from our results that NO does not cause heme loss from dimeric iNOS itself. This conclusion can be inferred from the finding that the iNOS dimer once formed is stable for an additional 8 h in cells actively generating NO. NO was recently shown to bind reversibly to the heme iron of iNOS and neuronal NOS without affecting their subsequent catalytic activity (32, 33). A different finding was observed in hepatocytes, where NO synthesis down-regulates the activities of many P-450 hemeproteins (34, 35), apparently by causing destabilization, loss, and degradation of their heme groups (28). Different cytochrome P-450 enzymes show different sensitivities to the inhibitory effect of NO (35, 39–41). Thus, heme loss due to endogenous NO depends mainly on the susceptibility of the particular hemeprotein involved.

In light of these studies, we propose a working model to explain why dimeric iNOS assembly is limited in activated macrophages. Initial formation of some dimeric iNOS leads to NO synthesis. NO prevents a buildup of soluble heme levels either through transcriptional mechanisms related to increased iron release or by direct reaction with heme biosynthetic enzymes themselves. Reduced heme availability, coupled with an NO-mediated inhibition of heme insertion into iNOS, results in limited iNOS dimer assembly and a buildup of iNOS monomers. This condition is manifested as a steady decrease in the iNOS dimer/monomer ratio, with the iNOS specific activity approaching a maximum during the culture period. NO, by promoting iron loss, appears to also up-regulate iNOS mRNA and protein expression (36), explaining why we observed more total iNOS protein synthesized in cultures generating NO than in cultures whose NO synthesis was blocked by l-NAME. Thus, NO regulates the expression of active iNOS in different ways at both the transcriptional and post-translational levels.

It is interesting to note that in the absence of NO synthesis, interferon-γ and LPS treatment induced an increase in soluble porphyrin and heme levels in conjunction with induction of iNOS protein synthesis. This represents the first example of cytokine-induced heme synthesis in a highly differentiated myeloid cell line. Co-induction of heme and hemoprotein synthesis was previously observed during chemical stimulation of cytochrome P-450 expression in chick embryo hepatocytes (37). Thus, like hepatocytes, cytokine-activated RAW 264.7 macrophages appear to undergo gene induction to ensure enough heme is made available for insertion into the newly synthesized hemoprotein (iNOS). However, this response can only be observed in RAW 264.7 cells in the absence of NO synthesis.

To conclude, our results bring to three the means by which NO can regulate iNOS expression: NO directly inhibits catalysis by binding to the enzyme's heme iron to form an inactive iron-nitrosoyl complex (32, 33), it increases iNOS mRNA and protein expression through promoting iron loss (36), and it inhibits the post-translational assembly of dimeric iNOS by down-regulating hememe insertion and availability. It is remarkable that all three regulatory mechanisms involve reactions between NO and iron-containing cellular components. The ability of NO to down-regulate its own production by affecting enzyme assembly is novel and represents an additional means by which NO synthesis can be controlled during an inflammatory response.

Acknowledgments—We thank Pam Clark and Margaret Loftus for excellent technical assistance.
REFERENCES

1. Nathan, C. F. (1992) FASEB J. 6, 3051–3064
2. Bredt, D. S., and Snyder, S. H. (1994) Annu. Rev. Biochem. 63, 175–195
3. Marsden, P. A., Heng, H. Q., Duff, C. L., Shi, X.-M., Tsui, L.-C., and Hall, A. V. (1994) Genomics 19, 183–185
4. Marsden, P. A., Heng, H. Q., Schener, S. W., Stewart, R. J., Hall, A. V., Shi, X.-M., Tsui, L.-C., and Schappert, K. T. (1993) J. Biol. Chem. 268, 14778–14888
5. Griffith, O. W. and Stuehr, D. J. (1995) Annu. Rev. Physiol. 57, 707–736
6. Ignarro, L. J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 535–560
7. Ghosh, D. K., and Stuehr, D. J. (1995) Annu. Rev. Physiol. 57, 707–736
8. McMillan, K., and Masters, B. S. S. (1995) Biochemistry 34, 3686–3693
9. Abu-Soud, H. M., Yoho, L., and Stuehr, D. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 10769–10772
10. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991) J. Biol. Chem. 266, 6259–6263
11. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550
12. McMillian, K., Bredt, D. S., Hirsh, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. S. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11141–11145
13. Wang, J., Stuehr, D. J., Ikeda-Saito, M., and Rousseau, D. L. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10769–10772

14. Sono, M., Stuehr, D. J., Ikeda-Saito, M., and Dawson, J. H. (1995) J. Biol. Chem. 270, 19943–19948
15. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
16. Cho, H. J., Xie, Q.-W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C. F. (1992) J. Exp. Med. 176, 699–704
17. Nathan, C. F., and Xie, Q. (1994) Cell 78, 915–918
18. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Billiar, T. R. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3539–3543
19. Baek, K. J., Thiel, B. A., Lucas, S., and Stuehr, D. J. (1993) J. Biol. Chem. 268, 21120–21129
20. Takeshi, F., and Nison, J. C. (1980) Anal. Biochem. 122, 176–188
21. Morrison, G. R. (1965) Anal. Chem. 37, 1124–1126
22. Sassa, S. (1978) J. Exp. Med. 143, 305–315
23. Wang, W. W., Jenkins, C. P., Griscavage, J. M., Park, R. M., Arabolos, N. S., Byrn, R. E., Cederbaum, S. D., and Ignarro, L. J. (1995) Biochem. Biophys. Res. Commun. 210, 1009–1016
24. Sakai, N., Kaufman, S., and Middel, S. (1992) Mol. Pharmacol. 43, 6–10
25. Hibbs, J. B., Jr, Taintor, R. R., Vavrin, Z., Granger, D. L., Drapier, J. C., Amber, I. J., and Lancaster, J. R., Jr. (1990) In Nitric Oxide from L-Arginine A Bioregulatory System (Moncada, S., and Higgs, E. A., eds) pp. 189–223. Elsevier Science Publishers B. V., Amsterdam
26. Lancaster, J. R., and Hibbs J. B., Jr. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 1227–1227
27. Kim, Y. M., Bergonia, H. A., Muller, C., Pitt, B. R., Watkins, W. D., and Lancaster, J. R., Jr. (1995) J. Biol. Chem. 270, 5710–5713
28. Weiss, G., Goossen, B., Doppler, W., Pantopoulos, K., Werner-Felmayer, G., Watchter, H., and Hentze, M. W. (1993) Eur. Mol. Biol. Org. 12, 3651–3656
29. Klausner, R. D., and Rouault, T. A. (1993) Mol. Biol. Cell 1021, 1–5
30. Harteneck, C., Klatt, P., Schmidt, K., and Mayer, B. (1994) Biochem. J. 304, 683–686
31. Hershman, A. R., and Marietta, M. A. (1995) Biochemistry 34, 5627–5634
32. Abu-Soud, H. M., Wang, J., Rousseau, D. L., Fukuto, J., Ignarro, L. J., and Stuehr, D. J. (1995) J. Biol. Chem. 270, 22997–23006
33. Khatsenko, O. G., Groo, S. S., Rifkind, A. B., and Vane, J. R. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 11147–11151
34. Stadler, J., Trockfeld, J., Schmalix, W. A., Brill, T., Siewert, J. R., Greim, H., and Doehmer, J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3559–3563
35. Weiss, G., Werner-Felmayer, G., Werner, E. R., Grunewald, K., Watcher, H., and Hentze, M. W. (1994) J. Exp. Med. 180, 969–976
36. Shedlofsky, S. I., Sinclair, P. R., Bonkovsky, H. L., Healy, J. F., Swim, A. T., and Robinson, J. M. (1987) J. Biol. Chem. 262, 21120–21129
37. Furukawa, T., Kohno, H., Tokunaga, R., and Taketani, S. (1995) Biochem. J. 310, 533–538
38. O'Keefe, D. H., Ebel, R. E., and Peterson, J. A. (1978) J. Biol. Chem. 253, 3509–3514
39. Tsukahara, K., Higashitani, Y., Fujimoto, Y., Ikekawa, N., and Hori, H. (1988) Biochemistry 27, 4878–4882
40. Shiro, Y., Fujii, M., Ishida, S., Iizuka, T., Obayashi, E., Makino, R., Nakahara, K., and Shouk, H. (1995) Biochemistry 34, 9052–9058
41. Gardner, L. C., Smith, S. J., and Cox, T. M. (1991) J. Biol. Chem. 266, 22010–22018
42. Inoue, H., Hoshino, Y., and Hori, H. (1989) Biochemistry 28, 533–538