The heme protein indoleamine 2,3-dioxygenase (IDO) is induced by the proinflammatory cytokine interferon-γ (IFNγ) and plays an important role in the immune response by catalyzing the oxidative degradation of L-tryptophan (Trp) that contributes to immune suppression and tolerance. Here we examined the mechanism by which nitric oxide (NO) inhibits human IDO activity. Exposure of IFNγ-stimulated human monocyte-derived macrophages (MDM) to NO donors had no material impact on IDO mRNA or protein expression, yet exposure of MDM or transfected COS-7 cells expressing active human IDO to NO donors resulted in reversible inhibition of IDO activity. NO also inhibited the activity of purified recombinant human IDO (rhIDO) in a reversible manner and this correlated with NO binding to the heme of rhIDO. Optical absorption and resonance Raman spectroscopy identified NO-inactivated rhIDO as a ferrous heme of rhIDO. Reversible inhibition by NO in this manner and this correlated with NO binding to the heme of rhIDO. Optical absorption and resonance Raman spectroscopy identified NO-inactivated rhIDO as a ferrous iron (FeII)-NO-Trp adduct. Stopped-flow kinetic studies revealed that NO reacted most rapidly with FeII rhIDO in the presence of Trp. These findings demonstrate that NO inhibits rhIDO activity reversibly by binding to the active site heme to trap the enzyme as an inactive nitrosyl-FeII enzyme adduct with Trp bound and O2 displaced. Reversible inhibition by NO may represent an important mechanism in controlling the immune regulatory actions of IDO.

4 Supported by a NHMRC senior principal research fellowship, a University of New South Wales 2052, Australia, 6Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, Florida 33431, 7National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi 474-8522, Japan, and 8School of Chemistry, University of Sydney, Sydney 2006, Australia

The JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 33, pp. 23778 –23787, AUGUST 17, 2007

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kines (11, 12). These studies suggest that post-translational control is important for regulating IDO activity in antigen-presenting cells, although the mechanisms involved remain largely unknown.

Similar to the situation with IDO, proinflammatory cytokines, including IFNγ, also up-regulate the expression of inducible nitric-oxide synthase (NOS2), which produces large amounts of nitric oxide (NO) (13). Also similar to IDO, NOS2 represents an important component of the innate immune response (13), and there is evidence that the NOS2 and IDO pathways are interrelated (2). One aspect of this proposed “interaction” is that the product(s) of one pathway regulates the enzyme of the other pathway. Specifically, several studies have reported NO to inhibit IDO activity (14–16), although the underlying mechanism of this regulation remains unclear. The present study set out to understand how NO inhibits human IDO activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagent peroxynitrite and the NO donors S-nitroso-N-acetylpenicillamine (SNAP), glutathione nitric oxide adduct (GSNO), diethylamine NONOate (DEANO), and spermine NONOate were obtained from Cayman Chemicals. Chemically pure grade NO gas was purchased from Airgas. Solutions of authentic NO were prepared as described previously (14). Recombinant human IFNγ was from R&D Systems. PD10 gel-filtration columns were obtained from Amersham Biosciences. Unless indicated otherwise, all other materials were purchased from Sigma-Aldrich and were of the highest purity available.

**Cell Culture and IDO Transfection**—Monocytes were isolated from human blood buffy coats (Australian Red Cross Blood Bank) and matured into monocyte-derived macrophages (MDM) by 8–12 days of culture in RPMI 1640 medium supplemented with 10% pooled human serum (10). Upon maturation, MDM were treated with recombinant human IFNγ (500 units/ml) to induce IDO expression and activity. COS-7 cells (from ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus penicillin and streptomycin. For transient transfection, COS-7 cells were seeded in antibiotic-free medium in 6-well tissue culture plates and grown to 90% confluence. Cells were then cultured in serum-free Opti-MEM medium and transfected with pcDNA3 encoding full-length human IDO cDNA (1 μg/well) or pcDNA3 (empty vector control, 1 μg/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After overnight transfection, COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and used for experiments. For all experiments, MDM or COS-7 cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 and in culture media supplemented with 200 μM l-Trp.

**RT-PCR**—RNA was extracted from cultured MDM with TRIzol reagent (Invitrogen). Reverse transcription of 1 μg of RNA was performed with a commercial kit (Invitrogen) according to the manufacturer’s instructions. Conventional PCR conditions included a 10-min hot start at 95 °C followed by 35 cycles of denaturation (95 °C, 40 s), annealing (63 °C, 30 s), and extension (72 °C, 30 s). Real-time PCR reactions were performed in a total volume of 20 μl containing 4 μl of cDNA sample and 16 μl of SYBR Green PCR Master Mix (Quantace SensiMix) and specific primers (final concentration of 250 nm). Cycling conditions were 10 min hot start at 95 °C followed by 40 cycles of a denaturation step at 95 °C for 40 s and an annealing step at 60 °C for 60 s. Human IDO-specific sequences were amplified using a primer set described previously (17): fwd 5’-CACAAGGTCATGGAGATGTC-3’, rev 5’-CCACCAATGAGAGACCAGG-3’. This primer set produced a 240-base pair PCR product. Human β-actin was used as a housekeeping gene to normalize IDO transcript abundance. PCR primers for β-actin were: fwd 5’-CCTGGACGGTGAAAGGAT-3’, rev 5’-CCGCACATTGTGAACCTTG-3’. Real-time PCR was performed using a Corbett RotoGene 3000, and data were analyzed using Rotor Gene version 6.0 software (Corbett Research).

**Western Blotting and Cellular IDO Activity**—Western blotting was performed as described previously using a mouse monoclonal antibody directed against human IDO (10). The presence of 3-nitrotyrosine in IDO was assessed by Western blotting using a mouse monoclonal antibody (Clone 1A6, Upstate Biotechnology). Cellular IDO activity was assessed by measuring the extent to which L-Trp in the culture medium was converted to kynurenine. l-Trp, kynurenine and 3-hydroxyanthranilic acid were measured by HPLC as described previously (18, 19).

**Recombinant Human IDO**—Recombinant human IDO (rhIDO) encoded by the pQE9-IDO plasmid vector was
Mechanism by Which Nitric Oxide Inhibits IDO Activity

expressed in *Escherichia coli* as a fusion protein to a hexahistidyl tag and purified as described in detail (20, 21). Different batches of purified rhIDO used for the present studies appeared as a single major protein band at ~42 kDa following SDS-PAGE and Coomassie Blue staining (not shown) and exhibited 404–700 nm absorption ratios of 1.5–1.8, with specific activities of ~70–100 mol kynurenine formed/min/mol enzyme. IDO activity was determined routinely by the ascorbate/methylene blue assay (10, 14, 22).

UV-visible Spectroscopy—Optical absorption spectra of rhIDO (5–10 μM in heme) were recorded under anaerobic or aerobic conditions in 100 mM potassium phosphate buffer (pH 7.0) with 500 mM EDTA using a PerkinElmer Lambda EZ210 spectrophotometer. When added, the final concentration of L-Trp was 4 mM. When necessary, solutions were made anaerobic by purging with argon in septum-sealed quartz cuvettes (1-cm path length). Ferrous iron (FeII) rhIDO was formed by the addition of a molar excess of a buffered sodium dithionite solution. NO was added by direct addition of the purified gas using a gas-tight syringe.

All experiments examining catalytically active rhIDO and its inhibition by NO were performed under normal atmospheric aerobic conditions using a Varian-Cary 300 spectrophotometer in quartz cuvettes (1-cm path length) containing rhIDO dissolved in the ascorbate/methylene blue assay buffer (500 mM KPO4 buffer (pH 7.0) with 500 μM EDTA, 50 μM of catalase, 400–800 μM L-Trp, 25 μM methylene blue, 10 mM ascorbate). For these experiments, NO was added either as a bolus from a 2 mM saturated NO gas solution or delivered by DEANO.

Stopped-flow Kinetic Studies—Kinetic data for the reactions of NO with rhIDO were obtained using an Applied Photophysics SX-17 MV stopped-flow spectrophotometer at 22 °C. Solutions of dithionite-reduced FeII rhIDO (10 μM in heme) in the absence or presence of L-Trp (2 mM final concentration) were mixed rapidly with an anaerobic solution of NO gas (1 mM final concentration), and the initial rate of decay of the γ-heme Soret absorption intensity of FeII rhIDO was monitored at 428 nm. The concentration of NO was in large molar excess of rhIDO to ensure pseudo first-order conditions. The observed initial rate of reaction of NO with rhIDO (kobs) was calculated by fitting the stopped-flow kinetic data to a single exponential function.

Resonance Raman Spectroscopy—Samples of rhIDO (~100 μl, 20–30 μM in heme) were prepared in a septum-sealed, cylindrical quartz cell that was rotated at ~1000 rpm. The sample cell was irradiated at 413.1 nm (~3–5 milliwatts) using a mixed krypton/argon ion laser (Spectra Physics, Beamlok 2060). The spectral acquisition time was typically 1–5 min. The scattered light was collected at right angles to the incident beam and focused on the entrance slit (125 μm) of a 0.8-m spectrometer where it was dispersed by a 600 groove/mm grating and then detected by a liquid-N2-cooled charge-coupled device camera (Horiba-JY). Spectral calibration was performed against the lines of mercury and indene.

RESULTS

NO Inhibits IDO Activity in Intact Cells—We reported previously that cultured human MDM do not contain measurable IDO enzyme activity, although treatment of these cells with IFNγ induces such activity in a time-dependent manner (10). Consistent with this finding, nonactivated human MDM did not contain detectable IDO mRNA or protein (supplemental Fig. S1A), whereas treatment of these cells with IFNγ resulted in the time-dependent induction of IDO as indicated by the parallel increases in IDO mRNA, protein, and activity, the latter reflected by increased L-Trp consumption and accumulation of kynurenine and 3-hydroxyanthranilic acid in the culture medium (supplemental Fig. S1B).

We also reported previously that nontoxic concentrations of NO-generating compounds inhibit IDO activity in IFNγ-stimulated MDM (14). Inhibition of IDO activity by NO could result from inhibition at the level of transcription, activity, or promotion of IDO protein degradation (14–16). To determine whether NO affected IDO expression in IFNγ-stimulated MDM, we tested the effect of adding the NO donor SNAP or GSNO concomitant with IFNγ and examining IDO mRNA and protein expression 18 h later. SNAP and GSNO when added to cells cultured in serum-supplemented medium produce NO...
with a half-life of 4–5 and 1–2 h, respectively (23–25). We observed that neither SNAP (Fig. 2, A, B, and D) nor GSNO (Fig. 2, C and E) significantly impacted on IDO mRNA or protein expression when used at concentrations up to 500 μM.

To investigate whether NO directly inhibited IDO activity, we first stimulated human MDM with IFNγ for 32 h to express functional IDO protein and then exposed the cells to increasing concentrations of NO donors for 4 h in fresh medium supplemented with L-Trp prior to assessing IDO protein and enzyme activity. The NO donors we employed were GSNO or Spermine NONOate, the latter donor releasing 2 mol of NO/mol of parent compound with a half-life of 40 min at pH 7 and 37 °C (26).

Fig. 3 shows that GSNO and spermine NONOate dose-dependently inhibited IDO activity, as indicated by the decrease in the extent to which MDM metabolized L-Trp to kynurenine. This inhibition was not due to altered IDO protein expression (Fig. 3). Similar findings were made with SNAP, which at 50 μM inhibited enzyme activity exhibited by IDO-expressing MDM by 75 ± 5% (n = 4) compared with control MDM after 4 h of incubation.

We next tested whether inhibition of cellular IDO activity by NO donors is reversible. For this, we exposed IFNγ-primed MDM to GSNO and examined the capacity of these cells to metabolize L-Trp to kynurenine over the ensuing 8 h. We employed GSNO, which decays under cell culture conditions with a half-life of ~1–2 h (24, 25), so that under the experimental conditions employed the majority of NO was anticipated to be liberated within the first 2–4 h. Indeed, measurement of the increase in nitrite afforded by GSNO as an index of NO generation showed that the donor produced NO for the initial 3 h only under the current experimental conditions (not shown). As shown in Fig. 4A, GSNO inhibited IDO activity relative to control cells over the initial 4 h of incubation. However, at later time points (4–8 h), when GSNO ceased releasing NO, the rate at which MDM metabolized L-Trp to kynurenine returned essentially to the corresponding control cell values. Further support for the implied reversible inhibition of IDO enzyme activity by NO was obtained with transfected COS-7 cells.
expressing active human IDO and exposed to GSNO for increasing periods of time. Thus, although GSNO substantially (i.e. by >75%) inhibited IDO activity over the initial 4 h, the extent of this inhibition decreased with increasing duration of incubation such that after 18 h IDO activity had returned to control cell levels (Fig. 4B). These changes in enzyme activity occurred independently of any significant changes in IDO protein expression by the transfected COS-7 cells (Fig. 4B).

**NO Inhibits rhIDO Activity**—Results from the cellular experiments described above indicated that NO inhibited cellular IDO activity at the post-translational level. We next asked whether the observed NO-mediated inhibition of cellular IDO activity could be recapitulated with the purified protein in the presence of co-factors known to activate the isolated enzyme. For this, we exposed rhIDO to NO donors in the presence of ascorbate and methylene blue co-factors, which reduce the inactive ferric iron (FeIII) to the active FeII form of the enzyme. The NO donors employed were DEANO and spermine NONOate, which at pH 7 and 37 °C liberate 1.5 or 2.0 mol of NO/mol of parent molecule with half-lives of 2 and 40 min, respectively (26). Both NO donors dose-dependently inhibited the activity of rhIDO, with DEANO the more efficacious, consistent with its shorter half-life of NO release (Fig. 5). For example, based on half-life after 15 min of incubation at 37 °C, 1 µM DEANO and Spermine NONOate produce 1.5 and 0.5 µM NO, which inhibited the activity of rhIDO (0.1 µM in heme) by ~60 and ~35%, respectively (Fig. 5).

**Spectroscopic Properties of NO-rhIDO Adducts**—Reduction of the IDO heme from the FeIII to the FeII form facilitates binding of L-Trp and O2 to the active site (27). As NO binding to heme can regulate the activity of various enzymes (28), we next characterized the spectral properties of FeIII and FeII rhIDO ± L-Trp and NO using UV-visible absorption spectroscopy. In the absence of L-Trp, native FeIII rhIDO exhibited a Soret band maximum at 404.5 nm, as well as α/β absorption band maxima at 501, 535, ~570, and 632 nm (Table 1, supplemental Fig. S2, A and B). Addition of NO resulted in a large shift of the Soret band maximum from 404.5 to 416 nm and the appearance of two distinct bands in the α/β region at 532 and 566 nm (Table 1, supplemental Fig. S2, A and B). The subsequent addition of L-Trp induced a slight shift of the Soret band back to 415.5 nm but no changes to the α/β absorption band maxima. Reduction of FeIII to FeII rhIDO shifted the Soret band from 404.5 to 428 nm and resulted in the appearance of new peaks in the α/β region at ~530 (shoulder) and 558 nm (Table 1, supplemental Fig. S2, C and D). The subsequent addition of L-Trp produced a slight blue-shift in the Soret band to 426 nm and in the α/β region to 557 nm. In contrast, the addition of NO to FeIII-IDO caused a large blue-shift in the Soret band to 418 nm and the formation of new peaks at 545 and 574 nm in the α/β region. The addition of L-Trp further shifted the Soret band to 415 nm and altered the relative intensities of the absorption peaks at 546 and ~573 nm (Table 1, supplemental Fig. S2, C and D). The results of this spectral analysis show that each individual rhIDO NO-heme adduct (i.e. FeIII or FeII ± L-Trp) has a unique, distinguishable, spectroscopic signature that allows for positive identification.

![FIGURE 5. NO donors inhibit rhIDO activity.](http://www.jbc.org/)

**TABLE 1**

<table>
<thead>
<tr>
<th>Optical properties of ferric and ferrous NO complexes of rhIDO</th>
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</thead>
<tbody>
<tr>
<td>IDO adduct</td>
<td>γ-Soret banda</td>
<td>α/β bandsb</td>
</tr>
<tr>
<td>FeIII</td>
<td>404.5</td>
<td>501, ~535, ~570, 632</td>
</tr>
<tr>
<td>FeIII-Trp</td>
<td>406</td>
<td>538, ~570, ~632</td>
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<tr>
<td>FeIII-NO</td>
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<td>532, 566</td>
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<tr>
<td>FeII-Trp-NO</td>
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<td>532, 566</td>
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<tr>
<td>FeII-Trp</td>
<td>428</td>
<td>~530, 557</td>
</tr>
<tr>
<td>FeII-NO</td>
<td>426</td>
<td>~530, 557</td>
</tr>
<tr>
<td>FeII-Trp-NO</td>
<td>418</td>
<td>545, 574</td>
</tr>
<tr>
<td>FeII-Trp-NO-NO</td>
<td>415</td>
<td>546, ~573</td>
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* Spectra were obtained under anaerobic conditions at pH 7.0. The uncertainty in absorption band maxima is ±0.2 nm.

**Spectroscopic Properties of Active rhIDO**—To examine how NO inhibits rhIDO, we first assessed the utility of optical absorption spectroscopy to study changes in the heme prosthetic group of rhIDO following activation of the enzyme by ascorbate plus methylene blue under aerobic conditions in the presence of L-Trp. Activation of rhIDO decreased the intensity and shifted the γ-Soret maximum to a longer wavelength (supplemental Fig. S3, A and B). In addition, a shoulder appeared that, upon subtraction of the resting rhIDO spectrum, exhibited a maximum of ~426–429 nm (supplemental Fig. S3B, inset). We postulated that this maximum is characteristic for the active quaternary FeII species formed from the deoxy precursor, i.e. FeII-O2-Trp, which is likely to occur in resonance with the FeIII-superoxo-Trp adduct (FeIII-O2-Trp) (29). Also coinciding with activation of IDO was the time-dependent increase in absorbance at ~325 nm, resulting from formation of N-formyl-kynurenine, the immediate reaction product of the IDO-catalyzed oxidation of L-Trp (supplemental Fig. S4, arrow). This increase in absorbance at 325 nm correlated with the decrease in L-Trp and the accumulation of kynurenine as determined by HPLC (not shown). Together, these results indicate that the optical spectral changes observed as a
result of activation of rhIDO directly reflect the changes occurring to both the heme environment and activity of the enzyme.

Raman Characterization of Active rhIDO—Resonance Raman spectroscopy was employed to monitor changes to the structure, coordination, spin, and redox state of the heme active site as the result of activation by the addition of ascorbate and methylene blue. The top spectrum in Fig. 6 represents “resting” rhIDO under anaerobic conditions in the presence of methylene blue and L-Trp but the absence of ascorbate. As observed, this spectrum exhibited features almost identical to those of Fe$^{III}$-IDO with L-Trp, which we reported previously (21). The core-size marker bands $v_3$ (1481/1500 cm$^{-1}$) and $v_5$ (1561/1573 cm$^{-1}$) are indicative of a six-coordinate, predominantly low-spin, heme iron.

The addition of ascorbate under anaerobic conditions led to the partial reduction of heme, as evidenced by the shoulder appearing at $\sim$1353 cm$^{-1}$ on the low frequency edge of the $v_3$ band and the appearance of a peak at 1468 cm$^{-1}$ (Fig. 6, +AH$^-$), both of which are characteristic of a deoxy heme species (21). The subsequent addition of oxygen caused activation of the enzyme and reversion of the spectrum to a pattern very similar to that of the resting enzyme (Fig. 6, +O$_2^+$). Under the conditions employed, rhIDO was active for $\sim$90 s (as assessed by parallel UV-visible spectroscopy), and the active rhIDO resonance Raman spectrum was recorded in the first 60 s of the reaction. The bottom spectrum in Fig. 6 was recorded after the addition of authentic NO gas to the active enzyme (+NO), discussed in more detail below.

Characterization of NO-inactivated rhIDO—The addition of the NO donor DEANO to catalytically active rhIDO resulted in the immediate inhibition of enzyme activity (Fig. 7A). This was associated with a concomitant shift of the $\gamma$-Soret band to 415 nm (Fig. 7B) and the appearance of peaks in the $\alpha/\beta$ region at $\sim$546 and $\sim$573 nm (Fig. 7C). Of note, the optical spectrum of the NO-inactivated rhIDO adduct was identical to the spectrum of Fe$^{III}$-Trp-NO IDO (cf. Fig. 7, B and C, and supplemental Fig. S2, C and D).

The experiments in Fig. 7 using the NO donor DEANO reflect a situation where active rhIDO is exposed to a continuous source of NO over the time period examined. We next studied the time-dependent changes in enzyme activity and heme spectrum that occurred upon addition of rhIDO to a single bolus addition of authentic NO gas. We found that at the enzyme concentration employed (i.e. 6 $\mu$M in heme), activated rhIDO rapidly converted L-Trp into N-formyl-kynurenine over the initial 3 min at a rate of $\sim$225 $\mu$M/min (Fig. 8A). After 4 min, rhIDO had consumed all available L-Trp (800 $\mu$M), and this represented 100% of L-Trp converted into N-formyl-kynurenine (Fig. 8A). Co-addition of 20 $\mu$M NO gas resulted in the immediate inhibition of rhIDO activity over the initial 2-min period (Fig. 8A). This coincided with the formation of an NO-IDO adduct exhibiting a $\gamma$-Soret band maximum of 415 nm (Fig. 8B). The degree of inhibition of enzyme activity decreased with increasing incubation time post-NO addition (Fig. 8A). That is, the rate of L-Trp converted into N-formyl-kynurenine over the first 2 min post-NO addition was 2 $\mu$M/min compared...
Mechanism by Which Nitric Oxide Inhibits IDO Activity

Inhibition of rhIDO by NO is reversible. A, rhIDO (6 μM in heme) under normal air atmosphere in enzyme assay buffer (pH 7.0) at 22 °C was activated by the addition of ascorbate (10 mM) in the absence (control (CTL)) or presence of NO gas (20 μM, +NO) followed by assessment of the time-dependent formation of N-formyl-kynurenine expressed as the percentage of L-Trp converted to N-formyl-kynurenine. B, the γ-Soret optical absorption spectrum of rhIDO after 2 or 10 min of exposure to 20 μM NO. C, curves i + ii, optical spectrum obtained upon addition of the spectrum of catalytically active rhIDO (i) with the spectrum of NO-trapped rhIDO in assay buffer under normal air atmosphere at 22 °C (ii), D, active rhIDO (8 μM in heme) under normal air atmosphere in enzyme assay buffer (pH 7.0) at 22 °C was incubated in the absence (control (CTL)) or presence (+NO) of NO and incubated for 1 min and the optical absorption spectra recorded. Both rhIDO species were subsequently gel-filtered (GF) into assay buffer (pH 7.0) under normal air atmosphere, and optical absorption spectra of the diluted enzyme solutions were recorded before (CTL GF, +NO GF) and after (CTL GF Active, +NO GF Active) activation of the enzyme by the readdition of ascorbate, methylene blue, and L-Trp. The arrow indicates the increase in N-formyl-kynurenine and hence rhIDO activity over the initial 2-min period after reactivation of the enzyme. Results shown are representative of three independent experiments.

with an average of 22 μM/min between the 10–20-min time period. By comparing rates, rhIDO activity 20 min post-NO addition had recovered to ~10% of control enzyme activity (i.e., 225 μM/min) indicating that inhibition of rhIDO activity by NO is at least partially reversible under the aerobic enzyme assay conditions employed. This partial restoration of enzyme activity coincided with both a shift in the γ-Soret band spectrum from 415 nm to 414 nm and an overall broadening of the spectrum to the left (Fig. 8B). We interpreted this as reflecting a mixed population of NO-inactivated rhIDO and a minor pool of cycling rhIDO formed by dissociation of NO from the heme of rhIDO. Consistent with this interpretation, the addition of the optical spectra of active cycling rhIDO (Fig. 8C, Spectrum i) with that of NO-inactivated rhIDO (Fig. 8C, Spectrum ii) gave rise to a rhIDO species exhibiting a γ-Soret maximum at 414 nm (Fig. 8C, Spectrum i + ii).

To further investigate the reversible nature of NO-mediated inhibition of rhIDO activity we performed studies with gel-filtered enzyme. We found that gel-filtration of NO-inactivated rhIDO (Fig. 8D, +NO) under aerobic conditions to remove enzyme substrate and co-factors resulted in loss of the FeII-NO-Trp complex and regeneration of native FeIII-IDO (Fig. 8D, +NO GF). This previously NO-inactivated rhIDO exhibited full enzyme activity (Fig. 8D, +NO GF Active), i.e., upon read-ascorbate/methylene blue and L-Trp and immediately recorded resonance Raman spectra of the trapped (inactive) enzyme (Fig. 6A, +NO). In the low frequency region (Fig. 6A, +NO), the NO-trapped species displayed a characteristic pattern of bands between 289–423 cm−1, indicative of a FeIII-NO-Trp or a FeII-NO-Trp complex. In particular, a peak around 326 cm−1 is observed only for heme-NO adducts with L-Trp bound (30). This observation alone is not sufficient to distinguish the FeIII-NO-Trp from the FeIII-NO-Trp species. However, the spectral pattern observed in the high frequency region (Fig. 6B, +NO) further indicated that the NO-trapped species was that of the FeII-NO-Trp complex. This conclusion is based on the single peak observed at 1503 cm−1 and the strong vinyl stretching mode νvinyl appearing at 1626 cm−1, which is observed for authentic FeII-NO-Trp rhIDO, and contrasts with the FeIII-NO-Trp species that exhibits a double peak in the 1490–1510 cm−1 region and a strong peak at 1642 cm−1 in the νvinyl region (30).

Stopped-flow Analysis of NO Binding to rhIDO—The data given above indicate that NO most preferentially inhibits active rhIDO by trapping the enzyme as FeII rhIDO with L-Trp bound. We next obtained information on the initial rate of NO binding to FeII rhIDO in the absence or presence of substrate using stopped-flow analyses. We found that NO binds ~6-fold more...
Mechanism by Which Nitric Oxide Inhibits IDO Activity

FIGURE 9. NO-mediated inhibition of rhIDO occurs independently of formation of 3-nitrotyrosine. rhIDO (1 μM in heme) under normal air atmosphere, present as native (FeIII), dithionite-reduced (FeII), or dithionite-reduced plus 400 μM L-Trp (FeII-Trp) or incubated in ascorbate plus methylene blue enzyme assay buffer (NO-Trapped rhIDO), was exposed to 100 μM DEANO for 5 min prior to the addition of SDS loading buffer and analysis of the level of 3-nitrotyrosine or total rhIDO by Western blotting as outlined under “Experimental Procedures.” Native rhIDO (1 μM) exposed to 100 μM peroxynitrite (ONOO−) under normal air atmosphere for 5 min acted as a positive control for formation of 3-nitrotyrosine on rhIDO. The Western blot is representative of three independent experiments.

rapidly to FeII-Trp rhIDO (kobs = 208 ± 4 s−1, n = 4) than to FeIII rhIDO (kobs = 33 ± 11 s−1, n = 4).

NO and rhIDO Amino Acid Modification—The above data support that NO inhibits rhIDO by direct binding to the heme group. However, NO and the reactive nitrogen species derived from it in the presence of O2 could also conceivably affect enzyme activity through amino acid modification. For example, a recent study reports that peroxynitrite inhibits IDO activity, in part, via the formation of 3-nitrotyrosine (31). We, therefore, examined whether NO donors also induced formation of 3-nitrotyrosine in rhIDO. The results in Fig. 9 indicate that addition of NO to rhIDO under aerobic conditions did not cause formation of 3-nitrotyrosine, irrespective of the heme redox or activation state of the enzyme. In contrast, addition of reagent peroxynitrite to rhIDO caused the formation of 3-nitrotyrosine on the enzyme (Fig. 9). These findings indicate that NO inhibits rhIDO activity through heme binding rather than via formation of 3-nitrotyrosine.

DISCUSSION

Growing evidence indicates that the IDO and NOS2 pathways are interrelated (reviewed in Ref. 2). We were the first to report that NO inhibits IDO activity in macrophages (14). Subsequent studies confirmed that NO inhibits IDO activity, although various underlying mechanisms were proposed, including inhibition at the level of transcription (15) and activity (14) or promotion of IDO degradation via the proteasome (16). The current study, therefore, set out to define more precisely how NO inhibits human IDO activity. The data presented here demonstrate that NO directly and reversibly inhibits IDO activity in cultured cells. Studies with rhIDO further established that NO inhibits enzyme activity by binding to the heme of active enzyme to generate a nitrosyl-FeIII adduct with L-Trp bound and O2 displaced; this is the underlying mechanism by which NO inhibits rhIDO.

Previous studies (30, 32, 33) have shown that NO binds to the heme of purified or recombinant IDO, although how this results in inhibition of enzyme activity is unknown. In the current study we examined the binding of NO to rhIDO by employing optical absorption and resonance Raman spectroscopy to assess changes in the heme environment of catalytically active rhIDO before and after the addition of NO and to determine how these changes relate to enzyme activity. Using this approach we ascertained that rhIDO activity was immediately inhibited upon exposure to NO, and that this coincided with the formation of the catalytically inactive FeII-Trp-NO rhIDO adduct. Thus, although NO binds to rhIDO heme independently of the iron redox state or presence of substrate (30, 32, 33) (Table 1, supplemental Fig. S2), the current study shows that NO inhibits catalytically active FeII-rhIDO by displacing O2, with L-Trp remaining bound.

We have assigned the resonance Raman spectrum obtained for the NO-inactivated rhIDO species in the presence of ascorbate and methylene blue co-factors (Fig. 6, +NO) to that of the FeII-Trp-NO enzyme adduct based on a recent study employing resonance Raman spectroscopy to characterize the structure of the heme moiety in rhIDO-NO adducts (30). As shown in the current study, at neutral pH the catalytically inactive FeII-Trp-NO rhIDO adduct is a six-coordinate species. Other heme enzymes, such as neuronal nitric-oxide synthase (34, 35) and heme oxygenase-1 (36), are reportedly inhibited in a reversible fashion by the binding of NO to the heme to form a six-coordinate ferrous-nitrosyl complex. This contrasts with the five-coordinate complex obtained when NO binds to the heme of soluble guanylate cyclase to cleave the proximal Fe-His bond and activate the enzyme (37), or the five-coordinate ferrous-nitrosyl complex of human cystathionine β-synthase that inactivates the enzyme (38). A five-coordinate heme-NO complex is clearly distinguishable from a six-coordinate species by a blue-shifted Soret band (~394 nm) in the UV-visible absorption spectrum and also by an exceptionally high frequency for the ν3 mode (~1508 cm−1) in the resonance Raman spectrum of the ferrous species (30, 39). Samelson-Jones and Yeh (30) reported that at pH < 6.0, a five-coordinate FeIII-NO species of rhIDO is formed. The cleavage of the proximal Fe-His bond under these lower pH conditions was proposed to lead to a conformational change to the protein that could trigger proteosomal degradation, which would be expected to result in irreversible inhibition of IDO activity by NO. In this study we have demonstrated that inhibition of cellular and rhIDO is reversible, suggesting that at neutral pH, NO binds directly to the distal site of the rhIDO heme iron.

An important observation of this study is the ability of NO to inhibit IDO activity in a reversible manner. This was established for both rhIDO and cellular IDO. With rhIDO, reversion of activity correlated with the dissociation of NO from enzyme heme (Fig. 8). The reversible nature of this inhibition supports our original proposal (14) that NO-mediated regulation of IDO activity represents a physiological mechanism to achieve tonic control of IDO activity.

The preference of NO to trap catalytically active rhIDO as the FeII-Trp-NO adduct likely reflects the rate of reaction of NO with the enzyme. With respect to this, we observed that the initial rate of binding of NO to FeII-rhIDO was ~6 times faster in the presence than the absence of L-Trp, indicating that binding of the substrate may have an allosteric effect that facilitates subsequent binding of NO to FeII-rhIDO. This notion is con-
Mechanism by Which Nitric Oxide Inhibits IDO Activity

consistent with a previous report that the equilibrium binding constant of diatom molecules for IDO is higher when l-Trp is bound (27).

Previous resonance Raman (21) and crystal structure (40) studies of rhIDO support a catalytic reaction mechanism where FeII-IDO is 5-coordinate, and the distal heme iron coordination position is open for diatom ligand binding. l-Trp binds first, close but not directly to the distal heme iron, followed by binding of O2 to the vacant sixth iron coordination position. Our current findings indicating that NO traps the active enzyme as the FeII-Trp-NO rhIDO adduct further supports this proposed catalytic mechanism. Thus, if O2 had bound before l-Trp, we might have expected that the addition of NO would result in trapping a significant portion of rhIDO as the FeII-NO adduct, which was not the case. Therefore, our studies support the notion that NO out-competes O2 for binding to this sixth coordination position to inhibit rhIDO enzyme activity after l-Trp has bound.

A recent study reported peroxynitrite to inhibit IDO activity, in part, via formation of 3-nitrotyrosine (31). It is plausible that the ascorbate/methylene blue reaction conditions lead to the formation of superoxide anion radical, which reacts with NO to form peroxynitrite (22). However, we found that the addition of NO under aerobic conditions to catalytically active rhIDO did not cause the formation of 3-nitrotyrosine (Fig. 9). Also, the addition of peroxynitrite to rhIDO does not induce the spectral changes to the heme of rhIDO achieved with NO (not shown). Moreover, the addition of NO to ferrous rhIDO in the presence of l-Trp under anaerobic conditions gives rise to the same heme spectral species obtained when NO is added to catalytically active rhIDO under aerobic conditions. These observations indicate that NO, and not its oxidation products such as peroxynitrite, is responsible for formation of the inactive rhIDO heme adduct. They also show that NO inhibits rhIDO activity by binding to heme rather than via formation of 3-nitrotyrosine.

Previous studies by others have indicated that NO can inhibit IDO transcription in IFNγ-stimulated mouse macrophages (15). We could not confirm this for human macrophages in the present study. Thus, exposure of MDM to 500 μM SNAP or GSNO over a time period when IDO transcription was occurring in response to IFNγ had no material impact on IDO mRNA or protein expression (Fig. 2). In contrast, when employed at 5–10-fold lower concentrations, SNAP or GSNO inhibited IDO activity by ~75% when the NO donor was added to MDM already expressing active enzyme. These data clearly illustrate the greater propensity of NO to inhibit human IDO at the level of activity rather than transcription. Thus, although IDO transcription is sensitive to NO in murine macrophages (15), this appears not to be the case for human macrophages. The reason(s) for this apparent species difference is not clear, although it may reflect differences in the mechanisms controlling murine and human IDO transcription.

A recent study reported NO to inhibit IDO in a human epithelial cell line by promoting its degradation via the proteasomal pathway (16). We believe this mechanism of post-translational control is not responsible for the findings of the current study. Thus, we observed NO donors to inhibit cellular IDO activity independently of changes in the level of IDO protein expressed both in IFNγ-stimulated MDM and in COS-7 cells transfected with human IDO. This lack of effect on IDO protein is consistent with the observed reversible nature by which NO inhibits IDO activity in cells. The ability of NO to promote IDO degradation may, therefore, depend on the cell type or experimental conditions employed.

No significant differences were observed between the resonance Raman spectrum of the resting and active forms of the enzyme (Fig. 6). This is surprising considering that the resting and active forms are characterized by different heme oxidation states, i.e. the ferric and ferrous states, respectively, and that the resonance Raman spectra of ferric and ferrous rhIDO are very different (21). The similarity of the resting and active spectra may reflect the fact that the active quaternary species is principally a loosely bound FeIII-superoxo (FeIII-O2−) species, thus resembling FeIII rhIDO. Alternatively, the similarity in spectra may suggest that the steady-state concentration of active (i.e. reduced) enzyme is very low compared with that of the resting (i.e. oxidized) form when the enzyme is activated by the methylene blue and ascorbate co-factors. This interpretation is supported by our optical absorption spectroscopy studies, which detected only a weak shoulder on the long wavelength edge of the heme γ-Soret band with a maximum of ~426–429 nm for the active enzyme.

Optical absorption spectroscopy was employed previously to examine the interaction of methylene blue and ascorbate with the heme group of purified rabbit IDO under anaerobic and aerobic conditions (22). That study showed that ascorbate reduces methylene blue, which in turn acts as an electron donor to reduce the heme iron and activate the enzyme. Under anaerobic conditions the ascorbate/methylene blue co-factor system reduced the ferric IDO by only 25–40% (22). This is consistent with the incomplete reduction observed using resonance Raman spectroscopy in the present work (Fig. 6, +AH−). The incomplete reduction was attributed to the relatively low oxidation potential of the IDO heme iron, E0 = 0.011 V at pH 7 (22). The FeII-O2 form of IDO is highly autoxidizable, especially in the presence of methylene blue (29), which is reflective of the low oxidation potential of the heme iron. The reduced form of methylene blue (leuco-methylene blue) is also readily reoxidized by oxygen (22). Together these oxidation reactions ensure that under catalytic reaction conditions the steady-state concentration of reduced methylene blue and hence reduced or active rhIDO remains very low, even though ascorbate provides a continuous source of electrons.

In addition to IDO, the oxidative cleavage of the pyrrole ring of l-Trp can also be catalyzed by tryptophan 2,3-dioxygenase (TDO), an enzyme localized primarily to the liver. IDO and TDO constitute an important yet poorly understood class of heme dioxygenase enzymes. Despite catalyzing identical biochemical reactions, IDO and TDO exist as monomeric and homotetrameric enzymes, respectively, and share only 10% sequence identity (41). However, recent crystal structure studies reveal that rhIDO (42) and TDO (41, 43) exhibit similarity with respect to their heme active sites. Similar to IDO, the sixth coordination site of TDO heme is vacant for O2 binding. As observed in our previous study of IDO, Trp does not bind
directly to the heme in TDO, leaving the sixth coordination position vacant for oxygen binding. As such we would predict that NO would also inhibit TDO activity in a similar fashion to that of IDO by out-competing O₂ for the vacant coordination site.

It is likely that the findings from the current study have important physiological implications. Increasing evidence implicates IDO as an innate immune host defense against infectious agents and an immune control enzyme capable of suppressing T cell activation and promoting immune tolerance under various pathological situations (3). Therefore, in situations where NOS2 and IDO are co-expressed, such as in microbe-infected and inflammatory tissues, it is plausible that NO temporarily attenuates IDO activity. If so, our data suggest a novel way by which NO and IDO interact to contribute to the innate immune response. The extent to which this occurs in vivo, however, requires further investigation.

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Post-translational Regulation of Human Indoleamine 2,3-Dioxygenase Activity by Nitric Oxide

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doi: 10.1074/jbc.M700669200 originally published online May 29, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M700669200

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