Nitration and Inactivation of IDO by Peroxynitrite

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Nitration and Inactivation of IDO by Peroxynitrite

Hidetsugu Fujigaki,*† Kuniaki Saito,2*† Felix Lin,† Suwako Fujigaki,† Kanako Takahashi,* Brian M. Martin,† Cai Y. Chen,† Junichi Masuda,*† Jeffrey Kowalak,† Osamu Takikawa,‡ Mitsuru Seishima,* and Sanford P. Markey†

IDO induction can deplete l-tryptophan in target cells, an effect partially responsible for the antimicrobial activities and antiallogeneic T cell responses of IFN-γ in human macrophages, dendritic cells, and bone marrow cells. l-Tryptophan depletion and NO production are both known to have an antimicrobial effect in macrophages, and the interaction of these two mechanisms is unclear. In this study we found that IDO activity was inhibited by the peroxynitrite generator, 3-(4-morpholinyl)sydnonimine, in PMA-differentiated cytokine-induced THP-1 (acute monocytic leukemia) cells and IFN-γ-stimulated PBMCs, whereas IDO protein expression was unaffected compared with that in untreated cells. Nitrotyrosine was detected in immunoprecipitated (IP)-IDO from PMA-differentiated cytokine-induced THP-1 cells treated with 3-(4-morpholinyl)sydnonimine, but not from untreated cells. Treatment of IP-IDO and recombinant IDO (rIDO) with peroxynitrite significantly decreased enzyme activity. Nitrotyrosine was detected in both peroxynitrite-treated IP-IDO and rIDO, but not in either untreated IP-IDO or rIDO. Peptide analysis by liquid chromatography/electrospray ionization and tandem mass spectrometry demonstrated that Tyr15, Tyr345, and Tyr353 in rIDO were nitrated by peroxynitrite. The levels of Tyr nitration and the inhibitory effect of peroxynitrite on IDO activity were significantly reduced in the Tyr16→Phe mutant. These results indicate that IDO is nitrated and inactivated by peroxynitrite and that nitration of Tyr15 in IDO protein is the most important factor in the inactivation of IDO.


L-tryptophan (L-Trp)3-kynurenine pathway that converts the essential amino acid L-Trp to N-formylkynurenine in mammalian extrahepatic tissues. IDO is induced by IFN-γ in the course of an inflammatory response in many human cell types, including macrophages, astrocytes, fibroblasts, and epithelial cells. IDO induction can deplete l-Trp in target cells, and this effect is partially responsible for the antimicrobial, antiviral, and antiproliferative activities of IFN-γ (1–4). These IDO-sensitive microorganisms are eukaryotic pathogens such as Toxoplasma gondii, prokaryotic pathogens such as Chlamydia psittaci, and bacteria such as group B streptococci and enterococci (5–8). L-Trp depletion is also involved in the inhibition of T cell proliferation by IFN-γ-treated human monocyte-derived macrophages and dendritic cells (9–11). T cells are unable to proliferate in a Trp-depleted environment, and in vivo IDO activity in the mouse placenta protects allogeneic conceptus from being rejected by a T cell-driven mechanism (12, 13). It has been suggested that first-time activation of T cells in the absence of L-Trp may even result in the development of tolerance to the Ag presented (14–16). Furthermore, a recent study has suggested that the suppression of collagen-induced arthritis is caused by IDO-dependent mechanisms that suppress the Ag-specific CD4+ T cells responsible for the development of rheumatoid arthritis (17). In that disease, specific cell populations (CD11c+CD8+ T cells) produce large amounts of IFN-γ, which, in turn, induces IDO and inducible NO synthase (iNOS) expression in CD11b+ monocytes and CD11c+ dendritic cells.

Several studies report that activation of L-arginine metabolism through iNOS induction leads to the formation of NO, which, in turn, down-regulates L-Trp metabolism by both directly inhibiting IDO activity as well as interfering with the induction of the enzyme (18–20). However, the inhibition of IDO by NO varies among species and tissues (21–23). The role of NO production by iNOS in human cells is complex, because NO produces cytostatic and cytotoxic effects. NO may react with superoxide, a product of the respiratory burst in macrophages, to form peroxynitrite, which itself is cytotoxic (24). NO and NO-derived reactive species produce chemical modifications that alter the structure and function of biomolecules. The NO-dependent nitration of protein Tyr residues to 3-nitrotyrosine increases during oxidative inflammatory conditions, resulting in a post-translational modification that reflects the extent of oxidant production under both physiological and pathological conditions (24). There has been increasing interest in the impact of Tyr nitration of protein and enzyme structure-function relationships in diverse clinical pathologies (25). These include changes in the catalytic activity of enzymes, cytoskeletal organization, and cell signal transduction.

Because of the importance of IDO to antimicrobial effects and inflammatory responses, we explored whether IDO is affected by peroxynitrite. We report for the first time the structural and functional consequences of the interaction between IDO and peroxynitrite.

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3 Abbreviations used in this paper: l-Trp, l-tryptophan; DTPA, diethylthetriaminepenta-acetic acid; iNOS, inducible NO synthase; IP, immunoprecipitated; LC-MS/MS, liquid chromatography/electrospray ionization and tandem mass spectrometry; l-Kyn, l-kynurenine; SIN-1, 3-(4-morpholinyl)sydnonimine; SNP, sodium nitroprusside dehydرو; Y15F, Tyr15→Phe mutant; Y345F, Tyr345→Phe mutant; Y353F, Tyr353→Phe mutant; rIDO, recombinant IDO.

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Materials and Methods

**Materials**

Peroxynitrite and anti-nitrotyrosine Ab were purchased from Upstate Biotechnology. 3-[(4-Morpholinyl)sydnonimine (SIN-1) was obtained from Dojindo Laboratories. The pGEX plasmid vector, PreScission protease, glutathione-Sepharose 4B, PD-10 column, membrane-blocking agent, peroxidase-labeled anti-mouse Ab, and ECL Plus Western Blotting Detection System were obtained from Amersham Biosciences. Anti-GAPDH mAb was purchased from Chemicon International. Diethylthiolenineperacetate acid (DTPA), formic acid, perchloric acid, sodium nitroprusside dehydrate (SNP, NO donor), PMA, and acetaminophen were purchased from Sigma-Aldrich. THP-1 cells were obtained from Health Science Research Resources Bank. Spin Concentrators 5000 Molecular Weight Cut-Off was purchased from Agilent Technologies. Endoproteinas Lys-C (sequencing grade) and trypsin (sequencing grade) were purchased from Promega. HPLC grade acetone was purchased from Burdick & Jackson. Human rIFN-γ (sp. act., 2.0 × 10^6 U/mg), and TNF-α (sp. act., 1.0 × 10^8 U/mg) were purchased from BD Biosciences. LPS from *Salmonella abortus* was obtained from Sigma-Aldrich. All other chemicals of analytical grade were purchased from Sigma-Aldrich.

**Culture conditions**

THP-1 cells were regularly cultivated in RPMI 1640 medium (Nikken Bio Medical Laboratory) supplemented with 10% (v/v) heat-inactivated FBS (Roche) and 100 U/ml penicillin-streptomycin (Invitrogen Life Technologies) and were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2, as described previously (26). Human PBMCs from human volunteers were obtained from Advanced Biotechnologies. PBMCs were cultivated in DMEM supplemented with 10% FBS and harvested with trypsin-EDTA as described previously (26). For the SIN-1 treatment experiment, PBMCs were treated with 5 ng/ml IFN-γ for 24 h, and the medium was replaced with IFN-γ alone or IFN-γ plus 500 μM SIN-1, then incubated for an additional 24 h. Also, THP-1 cells were treated with 16 nM PMA for 48 h. At this time, the cells adhered to the bottom and exhibited macrophase-like morphology, and the cytokine mixture (5 ng/ml PMA for 48 h) was incubated for an additional 24 h. Also, THP-1 cells were treated with 16 nM PMA for 48 h. At this time, the cells adhered to the bottom and exhibited macrophase-like morphology, and the cytokine mixture (5 ng/ml IFN-γ, 10 ng/ml TNF-α, and 100 ng/ml LPS) was added to induce IDO. After 24 h, the medium was replaced with fresh medium containing cytokine mixture with 500 μM SIN-1. For the SNP treatment experiment, PBMCs were treated with 5 mg/ml IFN-γ alone or IFN-γ plus 500 mM SNP for 24 h. After treatment, cells were washed with PBS and harvested with Cell Scraper in RIPA buffer containing 10 mM Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM PMSF. Cell lysates were collected by centrifugation and used for immunoprecipitation, Western blotting, and IDO activity assay.

**Measurement of L-kynurenine (l-Kyn) in culture medium**

L-Kyn concentrations in culture medium were measured by HPLC as described previously (27). First, culture medium was mixed with 2 vol of 3% perchloric acid. After centrifugation, the concentrations of l-Kyn in the supernatants were measured using HPLC with a 5-mm octadecylsilane column (Eicom) and a spectrophotometric detector. UV signals were monitored at 355 nm. The mobile phase consisted of 1% of acetonitrile in 0.1 M ammonium bicarbonate (pH 8.2), and the flow rate was maintained at 0.75 ml/min throughout the chromatographic run.

**Measurement of nitrite and nitrate in culture medium**

NO release in culture medium was determined spectrophotometrically by measuring the accumulation of nitrite and nitrate using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical).

**Immunoprecipitation of IDO**

Immunoprecipitation was performed using Dynabeads Protein G (Dyna Biotech) according to the manufacturer’s instructions. Anti-human IDO Ab was cross-linked to Dynabeads Protein G with 20 mM dimethylpimelimidate in 0.2 M triethanolamine (pH 8.2). The cell lysate was added to the cross-linked beads and incubated overnight at 4°C. The protein-IG Dynabeads Protein G complex was washed with 0.05% Tween 20 (v/v) and 20% membrane blocking agent (w/v), and then blots were transferred to nitrocellulose membrane, blocked in PBS containing 0.05% Tween 20 (v/v) and 20% membrane blocking agent (w/v), and probed with anti-nitrotyrosine Ab (0.5 μg/ml), anti-IDO Ab (0.5 μg/ml), or anti-GAPDH Ab (1 μg/ml). After overnight incubations with the primary Abs at 4°C, blots were washed and incubated with peroxidase-labeled anti-mouse Ab for 2 h at room temperature, and immunoreactive protein bands were visualized by the ECL system.

**Identification of nitrotyrosine residues in rIDO**

After treatment with peroxynitrite, 50 μg of rIDO was concentrated, and the buffer was exchanged with 0.1 M ammonium bicarbonate by 4-ml spin concentrators according to the manufacturer’s instructions and evaporated to dryness using a centrifugal rotary evaporator. The protein was dissolved in the denaturation buffer containing 2 M urea and 2.25 mM DTT in ammonium bicarbonate and incubated for 15 min at 50°C to denature the protein. After denaturation, the protein was alkylated by addition of 100 mM iodoacetamide and incubated for 15 min at room temperature in the dark. After alkylation, 0.5 μg of endoproteinas Lys-C was added to the protein, which was incubated for 15 h at 37°C, then 1 μg of trypsin was added to the protein, which was incubated for 8 h at 37°C. The digested samples were evaporated in a centrifugal rotary evaporator, and dried digest samples were dissolved in 50 μl of 5% acetic acid and 50 μl of 1% formic acid (v/v). Two-dimensional liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis was conducted after tryptic digestion. The HPLC used was the LC-VP Series system consisting of a five-solvent delivery pump, an autosampler, and switching valves (all from Shimadzu) combined with a two-position low dead volume valve (Cheminert CN2; VICT Valco Instruments). Chromatographic separation was accomplished by loading peptide samples onto a Poly LC PolySulfoethyl A column (Poly LC) connected to a PicoFrit column (Thermo Hypersil-Keystone BetaBasic 18; New Objective). MS analysis was performed using PicoView nanospray ion source (New Objective) mounted on an LCQ (Thermo Electron) ion trap mass spectrometer (big-five scanning sequence data-dependant mode; mass range, 400-1800). Data analysis to identify nitrated peptides was performed using the Mascot sequence database-searching software (MatrixSciences). The MS/MS spectra of nitrated peptides were also examined by manual inspection to verify sites of nitration.

**Site-directed mutagenesis**

Point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). Oligonucleotides for formation of Tyr15 (Y15F), Tyr255 (Y345F), and Tyr255 (Y353F) to Phe mutants (mutated residues are underlined) were as follows: 5'-CTTGGCAACATCTGAACTGAACTCTTCTTCTGTTGCGCTT-3' for Y15F; 5'-CTTGGCAACATCTGAACTGAACTCTTCTTCTGTTGCGCTT-3' for Y255F; 5'-CTTGGCAACATCTGAACTGAACTCTTCTTCTGTTGCGCTT-3' for Y345F; and 5'-CTTGGCAACATCTGAACTGAACTCTTCTTCTGTTGCGCTT-3' for Y353F. The PCR products were gel-purified on a 1% agarose gel and inserted into a pGEX plasmid vector by conventional methods. The recombinant fusion protein was purified from bacterial lysates by affinity chromatography using glutathione-Sepharose 4B, and a GST fusion tag was removed by cleavage of PreScission Protease. Before use, the purified rIDO was gel-filtered through a PD-10 column eluted with 100 mM potassium phosphate buffer (pH 7.4), and protein concentration was determined by the bicinchoninic acid protein assay (Pierce).

**Treatment of rIDO with peroxynitrite**

rIDO was diluted in 50 μl of potassium phosphate buffer containing 0.1 M DTPA (pH 7.4), then treated with various concentrations of peroxynitrite for 15 min at room temperature while stirring. For decomposition of peroxynitrite, peroxynitrite was added to the buffer and incubated for 15 min before rIDO addition.

**Activity assay of IDO**

IDO activity was determined by the methylene blue/ascorbate assay as previously described (27). The reaction mixture contained 50 μl of rIDO or homogeneate of THP-1 cells and 50 μl of substrate solution. The composition of the substrate solution was 100 mM potassium phosphate buffer (pH 6.5), 50 μM methylene blue, 20 μM catalase, 50 mM ascorbate, and 0.4 mM l-Trp. After incubation of the reaction mixture at 37°C, samples were acidified with 5% perchloric acid and centrifuged at 7000 × g for 10 min at 4°C. The concentrations of the enzymatic products were measured using HPLC. Enzyme activity was expressed as the product content per hour per milligram of protein.

**Western blot analysis**

The samples were separated using NuPAGE 4–12% bis-Tris gels as recommended by the manufacturer (Invitrogen Life Technologies). Proteins were transferred to nitrocellulose membrane, blocked in PBS containing 0.05% Tween 20 (v/v) and 20% membrane blocking agent (w/v), and probed with anti-nitrotyrosine Ab (2 μg/ml), anti-IDO Ab (0.5 μg/ml), or anti-GAPDH Ab (1 μg/ml). After overnight incubations with the primary Abs at 4°C, blots were washed and incubated with peroxidase-labeled anti-mouse Ab for 2 h at room temperature, and immunoreactive protein bands were visualized by the ECL system.
FIGURE 1. Effects of SIN-1 on IDO protein content and IDO activity in cytokine-treated THP-1 or PBMCs. PBMCs were treated with 5 ng/ml IFN-γ for 24 h, and the medium was replaced with fresh medium containing IFN-γ (SIN-1-) or IFN-γ plus SIN-1 (SIN-1+) and incubated for 24 h. After the treatment, culture media and cell extracts were collected. IDO protein and GAPDH were detected by Western blotting (A, upper panel), and L-Kyn concentrations were determined in the culture medium (A, lower panel). Measurement of nitrate and nitrite concentration in culture medium was performed using an enzymatic colorimetric assay kit (Applied Biosystems). A, Control; B, SIN-1-; C, SIN-1+. IDO activity in the THP-1 cell lysate (C) and total concentration of nitrate plus nitrite in culture medium (D) were measured. Western blotting was repeated five times and produced the same results. The results of IDO activity, L-Kyn, and nitrate are the mean ± SD of five independent experiments conducted in duplicate. *p < 0.001 compared with SIN-1-; **p < 0.001 compared with control.

ATCAGGATGAAGTTAGTCAGATTGTGA-3’ for Y353F. The double- and triple-Tyr mutants (Y15/345F, Y15/353F, Y345/353F, and Y15/345/353F) were also created. Each mutagenesis of rIDO was confirmed by DNA sequence analysis with an ABI PRISM 3100 automatic sequencer (Applied Biosystems). All rIDO mutants were also purified as described above.

Statistics
Intrasample difference was assessed using paired Student’s t test. Differences were considered significant at p < 0.05. All results are shown as the mean ± SD.

Results
Inactivation of IDO activity and nitration of IDO protein by SIN-1 in cytokine-treated cells

Human PBMCs stimulated with IFN-γ produced significantly higher amounts of IDO protein and L-Kyn concentrations in culture medium compared with the controls (Fig. 1A). Although IDO protein levels were unchanged between IFN-γ-treated and IFN-γ plus SIN-1-treated cells, the L-Kyn concentration was significantly decreased in IFN-γ plus 500 µM SIN-1-treated human PBMCs compared with IFN-γ-treated cells. Similarly, differentiated THP-1 cells stimulated with PMA and cytokines (IFN-γ, TNF-α, and LPS) produced significantly higher amounts of IDO protein and L-Kyn concentration in culture medium compared with controls. The L-Kyn concentration in culture medium and IDO activity in cell lysate were significantly decreased in SIN-1-treated THP-1 cells, although the IDO protein expression level was not different between SIN-1-treated and untreated cells (Fig. 1, B and C). In contrast, the nitrate concentration was significantly increased in SIN-1-treated THP-1 cells (Fig. 1D). In addition, we found that the L-Kyn concentration was significantly decreased in IFN-γ plus 500 nM SNP (NO donor)-treated PBMCs compared with IFN-γ-treated cells, although IDO protein levels were unchanged between IFN-γ-treated and IFN-γ plus SNP-treated cells (data not shown).

To determine whether IDO is nitrated in SIN-1-treated cells, IDO from PMA-differentiated cytokine-induced THP-1 cells with or without SIN-1 was purified by immunoprecipitation. Western blot analysis using anti-nitrotyrosine Ab was used for the detection of nitrotyrosine in immunoprecipitated (IP)-IDO. Nitrotyrosine was detected in IP-IDO from PMA-differentiated cytokine-induced THP-1 cells with SIN-1, but not in untreated cells (Fig. 2). These results indicate that SIN-1 decreased IDO activity without decreasing IDO protein expression levels, and that IDO protein is nitrated in SIN-1-treated cells.

FIGURE 2. Nitration of IDO in PMA-differentiated, cytokine-induced THP-1 cells treated with SIN-1. PMA-differentiated, cytokine-induced THP-1 cells were treated for 24 h with cytokine mixture alone (SIN-1-) or cytokine mixture plus SIN-1 (SIN-1+) as described in Materials and Methods. IDO was purified from the control and SIN-1-treated THP-1 cells using immunoprecipitation with anti-IDO Ab. Purified IDO was probed with anti-nitrotyrosine (A) and anti-IDO (B) Abs. These experiments were repeated three times and produced the same results.
Inactivation and nitration of IP-IDO and rIDO by peroxynitrite

We analyzed IP-IDO from PMA-differentiated cytokine-induced THP-1 cells and rIDO by IDO activity assay and Western blot analysis using anti-nitrotyrosine Ab and anti-IDO Ab after exposure to peroxynitrite. Enzyme activity in IP-IDO and rIDO was significantly decreased by the addition of peroxynitrite (Fig. 3A). rIDO protein levels were unaffected (Fig. 3B), and nitrotyrosine was detected in both peroxynitrite-treated IP-IDO and rIDO, but was not detected in either untreated IP-IDO or rIDO (Fig. 3C). The addition of increasing amounts of peroxynitrite to rIDO resulted in increased nitrotyrosine levels and inhibition of IDO activity in a dose-dependent manner, with the IDO protein concentration remaining constant (Fig. 4). It is of note that decomposed peroxynitrite had no effect on IDO activity or levels of nitrotyrosine in rIDO (Fig. 4, lane d). These results indicate that IDO is nitrated and inactivated by peroxynitrite.

Identification of nitrated residues

To determine which Tyr residues in the rIDO were nitrated, peroxynitrite-treated or untreated rIDO was digested with endoproteinase Lys-C and trypsin, and peptides were separated by LC-MS/MS. The amino acid sequence coverage obtained by LC-MS/MS analysis of the LysC and tryptic digests of peroxynitrite-treated rIDO is indicated in Fig. 5A (81.14%). All peptides, including Tyr residues, were covered. Assignments of nitration sites were verified by manual inspection of the tandem mass spectra. The results indicated the presence of three nitrated peptides in the peroxynitrite-treated rIDO digests and established the nitration of Tyr15, Tyr345, and Tyr353 residues (Fig. 5, B–D). None of nitration-related peptides was seen in untreated rIDO.

Site-directed mutagenesis of Tyr residues

To investigate which Tyr residue has the most important role in nitration and inactivation of IDO, Tyr15, Tyr345, and Tyr353 residues in rIDO were mutated to Phe, and the effects of peroxynitrite on Tyr nitration and enzyme activity expressed by each mutant were measured. The effect of Tyr mutation on basal IDO activity is shown in Table I. All Tyr-to-Phe mutants of rIDO retained catalytic activity, but some were sensitive to substitution. The conservative substitution of Tyr15, Tyr345, and Tyr353 with Phe had only minor impact on IDO activity (~79 to ~109%), but double and triple mutants were significantly less effective than the wild type (~15 to ~38%). These data suggest that the double and triple mutants are not comparable for the effect of peroxynitrite with wild-type IDO. Thus, we determined the effect of peroxynitrite on Tyr nitration and enzyme activity in the single mutants. Fig. 6B shows the levels of nitrotyrosine in each rIDO as assessed by antisense nitrotyrosine immunoreactivity. The levels of nitrotyrosine in peroxynitrite-treated Y15F mutant were significantly reduced compared with those of wild-type rIDO. In addition, inhibition of enzyme activity by 10 μM peroxynitrite in the wild type was ~68%, although inhibition of enzyme activity in the Y15F mutant was only ~35% (Fig. 6C). These results indicate that nitration of Tyr15 in IDO protein is most responsible for the inactivation of IDO.

Discussion

This study clearly demonstrates for the first time that IDO is nitrated by peroxynitrite, resulting in inhibition of enzyme activity. It is thought that peroxynitrite is produced by inflammatory cells to defend against cancer cells or infection caused by parasites, viruses, and bacteria. At the same time, peroxynitrate can damage...
host cells and tissues (28). Nitration of Tyr residues in proteins is a post-translational modification associated with oxidative stress and activation of NO. Activated macrophages, for example, can simultaneously generate large fluxes of NO and superoxide, neither of which is unusually reactive (25). These two substances, however, rapidly combine to produce the far more reactive peroxynitrite anion with an extensive range of physiological consequences, including lipid oxidation, DNA damage, and protein modification via formation of 3-nitrotyrosine (24). Protein nitration has been observed in connection with >60 human disorders,

FIGURE 5. Amino acid sequence coverage and sites of nitration obtained by LC-MS/MS analysis of peroxynitrite-treated rIDO. IDO was nitratred with peroxynitrite as described in Materials and Methods and subjected to endoproteinase Lys-C and trypsin digestion, and peptides were separated on a reverse phase HPLC column on-line with an electrospray ionization, ion trap mass spectrometer. The amino acid sequence coverage obtained by LC-MS/MS is indicated in bold. The verified nitrated peptide regions are underlined, and asterisks designate nitrotyrosine residues (A). B–D, Annotated mass spectra of peptides containing nitrotyrosine observed after the reaction of peroxynitrate with rIDO. Collisionally induced fragmentation spectrum of m/z 1263.2 (MH\(^+\)) in B corresponds to the amino acid sequence EY(NO\(_2\))HIDEEVGALPNPQENLPDFYNDFWMFIAK; that of m/z 1302 (MH\(^+\)) in C corresponds to the amino acid sequence Y(NO\(_2\))ILIPASQQPK; that of m/z 567 (MH\(^2+\)) in D corresponds to the amino acid sequence SY(NO\(_2\))HLQIVTK. Type b ions contain the N-terminal portion of the peptide; type y ions contain the C-terminal portion.
and there has been increasing interest in the impact of Tyr nitration on protein and enzyme structure-function relationships in diverse clinical pathologies, including neurodegenerative diseases, acute lung injury, atherosclerosis, bacterial and viral infection, and chronic inflammation (29–32). Thus, an understanding of both the mechanisms underlying protein nitration and the impact of this post-translational protein modification on cell and organ functions will provide insight into the pathogenetic mechanisms of inflammatory diseases and novel therapeutic strategies for limiting tissue inflammatory injury (25, 33).

L-Trp depletion and NO production are both known to have an antimicrobial effect, and the interaction of these two events is still under investigation. It was previously thought that these two events were species specific, with the microbialicidal activity of NO restricted to rodent cells and the IDO-mediated Trp depletion occurring only in humans (18, 21). Recently, it has become clear that both events occur in humans, resulting in a more complex interaction (5, 23, 34). It has been suggested that NO donors inhibit IDO activity in IFN-γ-treated human PBMCs (18). A recent study suggests that endogenous and exogenous NOs strongly reduce the IDO protein content in IFN-γ- and IL-1β-treated RT4 cells, which expresses both IDO activity and strong iNOS activity, and that this effect depends not on transcriptional, but on post-translational, regulation resulting from accelerated proteasomal degradation of IDO (20).

However, we demonstrated that IDO activity was significantly reduced in SIN-1-treated PBMCs and THP-1 cells, although IDO protein levels were not different between cytokine-treated and cytokine- plus SIN-1-treated PMA-differentiated THP-1 cells. It is known that SIN-1 can easily penetrate cells and generate peroxynitrite in cell culture systems (35). A recent study demonstrated that the concentration of peroxynitrite in cell culture systems was linearly dependent upon the SIN-1 concentration, and that the maximum concentration of peroxynitrite was very low, ranging from 1.2 to 3.6% of added SIN-1 (36). These studies support the concept that peroxynitrite affects intracellular IDO in SIN-1-treated cells, and in this study we have demonstrated that IP-IDO from SIN-1-treated THP-1 cells is nitrated (Fig. 2). NO adducts of IDO are believed to be involved in modulation of the catalytic activity of this heme protein (37), but no such evidence has been indicated in cell culture systems or in vivo. Much of NO-mediated pathogenicity depends on the formation of peroxynitrite, which is typically more reactive and toxic than NO (24). We also found that the L-Kyn concentration was significantly decreased in IFN-γ- plus 500 nM SNP-treated PBMCs compared with that in IFN-γ-treated cells, although IDO protein levels were unchanged between IFN-γ-treated and IFN-γ- plus SNP-treated cells. It is possible that peroxynitrite derived from SNP affects intracellular IDO in SNP-treated PBMCs, because NO released from SNP is able to react with superoxide anions, resulting in the formation of peroxynitrite in cell culture systems (38, 39). However, the present results demonstrate that a 1000 times lower concentration of SNP (500 nM) inhibited IDO activity to the same extent as SIN-1 (500 μM) in IFN-γ-stimulated PBMCs. It is likely that NO is more efficient than peroxynitrite in inhibiting IDO activity in SNP-treated PBMCs. Additional studies are required to clarify this issue.

A schematic diagram illustrating the interaction between IDO and peroxynitrite is shown in Fig. 7. Enzymatically, IDO initiates the degradation of tryptophan. Functionally, IDO has been demonstrated to be very important in maintaining maternal tolerance (40), and it has been proposed that NO may also play a role in maternal tolerance toward the fetus (41). It is known that peroxynitrite, which is produced by monocyte/macrophage lineage or

### Table I. Effect of tyrosine mutation on basal IDO activity

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<td>20.95 ± 3.56</td>
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</tbody>
</table>

* IDO activity represents the mean ± SD for three to five experiments performed in duplicate.

* IDO activity is expressed as a percentage of the wild type and represents the mean ± SD.

c Significantly different from the wild-type group; p < 0.001.
Gr-1+ dendritic cells, is a powerful oxidant that can inhibit T cell activation and proliferation by impairment of Tyr phosphorylation and apoptotic death (42) or through a CD3/CD28 costimulation mechanism (43). There is a possible mechanism of interaction between IDO and peroxynitrite for the inhibition of T cell proliferation. NO inhibits IDO activity by binding to the heme of IDO (18), and peroxynitrite also inhibits IDO activity by nitration of IDO. Metabolites derived from L-Trp via the IDO pathway can interfere with NO synthase (see Discussion). Arg, arginine; Cit, citrulline; 3HAAG, 3-hydroxyantranilic acid.

The present results demonstrate that IDO is nitrated and inactivated by peroxynitrite, and identify Tyr15, Tyr345, and Tyr553 in IDO as the sites of nitration. Furthermore, nitration of Tyr15 is the most responsible for inactivation of IDO by peroxynitrite.

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Disclosures

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References


