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Nitration and Inactivation of IDO by Peroxynitrite

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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-morpholino)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-morpholino)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 Tyr15-to-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. The Journal of Immunology, 2006, 176: 372–379.

Indoleamine 2,3-dioxygenase is the rate-limiting and first enzyme of the l-tryptophan (l-Trp)→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 macrocyte-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 concepti 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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Materials and Methods

Materials

Peroxynitrite and anti-nitrotyrosine Ab were purchased from Upstate Biotechnology. 3-(4-Morpholino)sydoniumine (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. Diethylstilbestrolpentacetic acid (DTPA), formic acid, perchloric acid, sodium nitroprusside dehydrate (SNP; NO donor), PMA, and actinomycin 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. Endoproteasines Lys-C (sequencing grade) and Asp-N (sequencing grade) were purchased from Promega. HPLC grade acetonitrile was obtained from Roche. Trypsin (sequencing grade) was purchased from Sigma-Aldrich. The pGEX plasmid vector, PreScission protease, and a GST fusion tag were removed by cleavage of rIDO activity assay.

Purification of recombinant IDO (rIDO)

IDO activity was determined by the methylene blue/ascorbate assay as previously described (27). The reaction mixture contained 50 μl of rIDO or homogenate 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 μg of 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

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 DTPA (pH 7.4), and protein concentration was determined by the bicinchoninic acid protein assay (Pierce).

Activity assay of IDO

IDO activity was determined by measuring the accumulation of nitrite and nitrate using the Nitrate/Nitrite assay kit (Cayman Chemical). The reaction mixture contained 10 ml 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-kyurenine (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 octadecyl silane 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 sodium phosphate (pH 3.9), 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 (DynaBiotech) according to the manufacturer’s instructions. Anti-human IDO Ab was cross-linked to Dynabeads Protein G with 20 mM dimethylpylamidine 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 containing PBS, and protein was eluted with 0.1 M citrate (pH 2.0).

Purification of recombinant IDO (rIDO)

A 1.2-kb full-length human IDO cDNA was ligated into a pGEX plasmid vector, and rIDO was expressed as a GST fusion protein. E. coli was grown in the presence of 7 μM hemin. 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 mM DTPA (pH7.4), 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 homogenate 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 μg of 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.

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 endoproteasines 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 digests of 100 μl of 5% acetic acid (v/v). Two-dimensional liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis was conducted after trypptic digestion. The HPLC used was the LC-VP Series system consisting of a five-solute delivery pump, an autosampler, and switching valves (all from Shimadzu) combined with a two-position low dead volume valve (Cheminert CN2; VICI Valco Instruments). Chromatographic separation was accomplished by loading peptide samples onto a Poly LC PolySulfoethyl A column (Poly LC) connected to a FicorFrit 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-dependent 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 generation of Tyr15 (Y15F), Tyr455 (Y345F), and Tyr553 (Y353F) to Phe mutants (mutated residues are underlined) were as follows: 5’-CTGGACAATCTGAAAGTGGG-3’ and 5’-CCACTTCTGTTACCTGCTGCG-3’; 5’-CACCATCTGTTACCTGCTGCG-3’ and 5’-CCACTTCTGTTACCTGCTGCG-3’; 5’-ACGATGGAGCTTCCACCGG-3’ for Y345F; and 5’-TGCAGAGGATG11032-3’ for Y353F. The MS/MS spectra of nitrated peptides were also examined by manual inspection to verify sites of nitration.
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) were measured. THP-1 cells were stimulated for 48 h with PMA (16 nM), and the medium was replaced with fresh medium containing a cytokine mixture. After 24 h, the medium was replaced with fresh medium containing cytokine mixture alone (SIN-1⁻) or cytokine mixture plus SIN-1 (SIN-1⁺) and incubated for 24 h. IDO protein and GAPDH were detected by Western blotting (B, upper panel), and L-Kyn concentrations were determined in the culture medium (B, lower panel) were measured. At the same time, IDO activity in the THP-1 cell lysate (C) and the 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.

ATCAGGTGAACCTTAGTCAGATTGCA-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 nitrination 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 lysates 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). IDO 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 antinitrotyrosine 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. rIDO was nitratet 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 peroxynitrite with rIDO. Collisionally induced fragmentation spectrum of m/z 1263.2 (MH+<sup>+</sup>) in B corresponds to the amino acid sequence EY(NO<sub>2</sub>)HIDEEVGALPNPQENLPDFYNDFWMFIK; that of m/z 1302 (MH<sup>+</sup>) in C corresponds to the amino acid sequence Y(NO<sub>2</sub>)ILIPASQQPK; that of m/z 567 (MH<sup>2+</sup>) in D corresponds to the amino acid sequence SY(NO<sub>2</sub>)HLQIVTK. Type b ions contain the N-terminal portion of the peptide; type y ions contain the C-terminal portion.
Effects of peroxynitrite on nitrotyrosine levels and IDO activity in each mutant IDO. Wild-type IDO and Y15F, Y345F, or Y353F mutants were treated with the indicated concentrations of peroxynitrite as described in Materials and Methods and subjected to immunoblotting with an Ab against IDO (A) and nitrotyrosine (B). Peroxynitrite (100 μM) that had been decomposed before use was ineffective on levels of IDO and nitrotyrosine in each rIDO (lane d). These experiments were repeated four times and produced the same results. Wild-type and each mutant IDO were treated with 10 μM peroxynitrite and subjected to IDO activity assay (C). IDO activity is expressed as a percentage of the untreated control value for each mutant. Results represent the mean ± SD of four to six independent experiments conducted in duplicate. * Significantly different from the wild-type group at p < 0.001.
IDO. Metabolites derived from L-Trp degradation via the IDO pathway can interfere with IDO and peroxynitrite for the inhibition of T cell proliferation (43). There is a possible mechanism of interaction between IDO and peroxynitrite for the inhibition of T cell proliferation and apoptotic death (42) or through a CD3/CD28 costimulation and iNOS may play a role in tolerance, but elaborating these mechanisms requires additional experimental evidence.

Fig. 6 shows that nitration of Tyr15 in IDO protein is most responsible for the inactivation of IDO by peroxynitrite. The IDO activity of Y15F mutant treated with 10 μM peroxynitrite was ~30% higher than that of the wild type treated with the same concentration of peroxynitrite. Table I shows that Y15F mutant expresses wild-type levels of basal IDO activity, and the activities of Y345F and Y353F are ~10 and ~20% lower than those in the wild type, respectively. These data indicate that substitution of Tyr15, Tyr345, and Tyr353 with Phe had only a minor impact on IDO activity in the single mutants. However, the double and triple mutants expressed much lower levels of basal IDO activity (15–38% of wild type). Although all double and triple mutants were much less inhibited by 10 μM peroxynitrite than the wild type cells treated with the same concentration of peroxynitrite (10–20% higher than wild type), the inhibition ratio was higher than that for Y15F mutant. Furthermore, even though the basal levels of activity and the inhibition ratio were different between mutants, all Tyr mutants of rIDO were almost completely inhibited by 1 mM peroxynitrite (data not shown). Taken together, these data indicate that Tyr nitration is not exclusively the mode of IDO inhibition by peroxynitrite. Several studies have suggested that the inhibition of enzyme activity (e.g., Tyr hydroxylase and tryptophan hydroxylase) by peroxynitrite is caused by oxidation of cysteine residues or sulfhydryl oxidation instead of Tyr nitration (44, 45). There is a possibility that IDO may be affected by such modifications. However, the concentration of peroxynitrite in the physiological state is very low, and peroxynitrite is estimated to have a very short half-life (~20 ms) (25). Our study suggests that the inhibition of IDO activity by low concentrations of peroxynitrite may be caused mainly by nitration of Tyr15. Furthermore, considering the basal activity of each mutant, the possibility exists that the tertiary structure of each double- and triple-mutant IDO may be altered.

The present results demonstrate that IDO is nitrated and inactivated by peroxynitrite, and identify Tyr15, Tyr345, and Tyr353 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
The authors have no financial conflict of interest.

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