

Cytochrome b_5 , Not Superoxide Anion Radical, Is a Major Reductant of Indoleamine 2,3-Dioxygenase in Human Cells*

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The heme protein indoleamine 2,3-dioxygenase (IDO) initiates oxidative metabolism of tryptophan along the kynurenine pathway, and this requires reductive activation of Fe^{3+} -IDO. The current dogma is that superoxide anion radical (O_2^-) is responsible for this activation, based largely on previous work employing purified rabbit IDO and rabbit enterocytes. We have re-investigated this role of O_2^- using purified recombinant human IDO (rhIDO), rabbit enterocytes that constitutively express IDO, human endothelial cells, and monocyte-derived macrophages treated with interferon- γ to induce IDO expression, and two cell lines transfected with the human IDO gene. Both potassium superoxide and O_2^- generated by xanthine oxidase modestly activated rhIDO, in reactions that were prevented completely by superoxide dismutase (SOD). In contrast, SOD mimetics had no effect on IDO activity in enterocytes and interferon- γ -treated human cells, despite significantly decreasing cellular O_2^- . Similarly, cellular IDO activity was unaffected by increasing SOD activity via co-expression of Cu,Zn-SOD or by increasing cellular O_2^- via treatment of cells with menadione. Other reductants, such as tetrahydrobiopterin, ascorbate, and cytochrome P450 reductase, were ineffective in activating cellular IDO. However, recombinant human cytochrome b_5 plus cytochrome P450 reductase and NADPH reduced Fe^{3+} -IDO to Fe^{2+} -IDO and activated rhIDO in a reconstituted system, a reaction inhibited marginally by SOD. Additionally, short interfering RNA-mediated knockdown of microsomal cytochrome b_5 significantly decreased IDO activity in IDO-transfected cells. Together, our data show that cytochrome b_5 rather than O_2^- plays a major role in the activation of IDO in human cells.

Human indoleamine 2,3-dioxygenase (IDO)³ is a cellular enzyme that catalyzes the initial step of the oxidative metabo-

lism of L-tryptophan (L-Trp) along the kynurenine pathway (1, 2). IDO cleaves the pyrrole ring of L-Trp to N-formyl-kynurenine by incorporating molecular oxygen. Although L-Trp is preferred, the enzyme can use other indoleamines such as tryptamine and serotonin as substrates. IDO is expressed constitutively in a limited number of human tissues (3) and also in rabbit enterocytes (4), but in most tissues and cells, expression of the enzyme requires induction, with the pro-inflammatory cytokine interferon- γ (IFN γ) playing a major role (2). The true physiological function of IDO is becoming clearer, with its induction and the formation of kynurenine pathway metabolites implicated in various physiological and pathological processes, such as in the defense against microbes and tumors, immune regulation, neuropathology, and antioxidant activity (2).

IDO is a monomeric protein of 42-kDa molecular mass and contains protoporphyrin IX as its sole prosthetic group (4). Activation of IDO requires reduction of its ferric (Fe^{3+}) heme to ferrous (Fe^{2+}) heme; Fe^{2+} -IDO rapidly autoxidizes to the inactive Fe^{3+} -IDO (5). Over the last 30 years, a number of electron donors have been suggested as biological reductants for IDO. Of those, superoxide anion radical (O_2^-) has been the most widely studied. In a series of pioneering studies employing purified rabbit enzyme and tissue, Hayaishi and co-workers (6–8) demonstrated that IDO requires O_2^- and O_2 for activity, which was inhibited by superoxide dismutase (SOD). In addition, they reported O_2^- to reductively activate purified rabbit IDO such that L-Trp and O_2 can bind tightly (9). Furthermore, using ¹⁸O₂-labeled potassium superoxide, these authors showed O_2^- to be incorporated into L-Trp during its metabolism to kynurenine by isolated rabbit IDO *in vitro* (7). These results were interpreted as O_2^- acting as co-factor and substrate for IDO, a view that remains commonly accepted. Notwithstanding this, however, later studies by Sono (10) have questioned the ability of O_2^- to maintain maximal steady-state activity of IDO. Indeed, the redox dye methylene blue is required and commonly used for *in vitro* IDO activity assays, together with ascorbate. Also,

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³ The abbreviations used are: IDO, indoleamine 2,3-dioxygenase; CHO, Chinese hamster ovary; FBS, fetal bovine serum; HAEC, human aortic endothelial cells;

HE, hydroethidine; HEK, human embryonic kidney; IFN γ , interferon- γ ; rhIFN γ , recombinant human IFN γ ; Mn-TBAP, Mn(III)tetrakis(4-benzoic acid) porphyrin chloride; MPEG, methoxy-polyethylene glycol; rhIDO, recombinant human IDO; PBS, phosphate-buffered saline; PEG-SOD, bovine superoxide dismutase conjugated to polyethyleneglycol; SOD, superoxide dismutase; 2-OH-E⁺, 2-hydroxyethidium; O_2^- , superoxide anion radical; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; PBS, phosphate-buffered saline; siRNA, short interfering RNA; HPLC, high pressure liquid chromatography; RNAi, RNA interference; L-Trp, L-tryptophan; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

tissue and cellular levels of O_2^- may not reach the levels required to activate ferric IDO, especially because cellular SOD effectively competes for O_2^- as it reacts with O_2^- with a rate constant of $\sim 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, whereas the corresponding rate constant for IDO is $\sim 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (10).

Reduced flavin mononucleotide and biopterin (L-5,6,7,8-tetrahydrobiopterin) have also been suggested as possible reductants for IDO. Indeed, these electron donors can activate purified murine IDO (11, 12), although such a role has not been confirmed for cellular IDO. A recent study using a yeast growth model suggested a role for cytochrome b_5 and cytochrome b_5 reductase in maintaining human IDO activity (13).

Therefore, we re-examined the importance of O_2^- in the activation of IDO, using recombinant human enzyme and different human cells as models. Our results show that in the absence of methylene blue, O_2^- is a poor activator of IDO and that, instead, cytochrome b_5 acts as the likely electron donor for human IDO.

EXPERIMENTAL PROCEDURES

Materials—Human aortic endothelial cells (HAEC) and endothelial cell growth media were purchased from Cell Applications, Inc. Recombinant human IFN γ (rhIFN γ) was from R & D Systems, Inc. Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (Mn-TBAP) was purchased from Cayman Chemical Co. Complete protease inhibitor tablets (Roche Diagnostics) were used as recommended by the manufacturer. Hydroethidine (HE), dispase (from *Bacillus polymyxa*), glucose-6-phosphate dehydrogenase, FMN:NADPH oxidoreductase from *Photobacterium fischeri*, and human NADPH-P450 reductase were purchased from Invitrogen. PD10 (Sephadex G-25) gel filtration columns were obtained from Amersham Biosciences. The mammalian expression vector pcDNA3 carrying the full-length human Cu,Zn-superoxide dismutase cDNA was donated generously by Professor Larry Oberley (Department of Radiation Oncology, University of Iowa). All other chemicals and reagents were purchased from Sigma, unless otherwise stated.

Recombinant Human IDO—rhIDO encoded by the pQE9-IDO plasmid vector was expressed in *Escherichia coli* as a fusion protein to a hexahistidyl tag and purified as described in detail (14, 15). 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 to 280 nm absorption ratios of 1.5–1.8. Before use rhIDO was gel-filtered through a Sephadex G-25 column eluted with 100 mM phosphate buffer, pH 7.4.

Cell Culture Studies—Chinese hamster ovary (CHO) and human embryonic kidney cells (HEK 293) were cultured in RPMI 1640 medium and low glucose DMEM, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. HAEC were maintained in endothelial growth media containing antibiotics (as above), recombinant human epidermal growth factor (10 ng/ml), bovine fibroblast growth factor (6 ng/ml), heparin (6 $\mu\text{g}/\text{ml}$), 1 $\mu\text{g}/\text{ml}$ hydrocortisone, and 5% FBS. HAEC were used from passage 5 to 11 and seeded at 50% confluence before stimulation with rhIFN γ . All cells were cultured in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C, and the medium was replaced every 3–4 days. At 90% confluence, cells

were disrupted with 0.15% trypsin and 1 mM EDTA for 5 min, washed, and plated in new culture plates and flasks for experiments.

Monocytes were isolated from human blood buffy coat (Australian Red Cross Blood Bank) and matured into monocyte-derived macrophages by 8–12 days of culture in RPMI 1640 medium supplemented with 10% pooled human serum (16). Upon maturation, cells were treated with rhIFN γ (500 units/ml) to induce IDO expression and activity.

Isolation of Enterocytes—Rabbit enterocytes were isolated from the small intestine of New Zealand White rabbits as described (17). The distal one-sixth part of the small intestine was removed and the luminal content flushed with 0.9% NaCl. The inner surface was rinsed with Tyrode's balanced salt solution containing 1 mg/ml streptomycin. One end of the intestine was tied, and Tyrode's solution containing 1,000 protease units/ml dispase was poured into the intestinal lumen. After tying the other end, the intestine was immersed into 0.9% NaCl and incubated at 37 °C for 30 min with constant shaking (80 rpm). The resulting cell suspension was collected and passed through a 70- μm nylon cell strainer (BD Biosciences), and the cells were then centrifuged at $500 \times g$ for 5 min. Cells were washed twice with Tyrode's solution and resuspended uniformly in the same solution. Any remaining clustered cells were removed by filtration through 70- μm cell strainers, and the resulting filtrate was used immediately for experiments.

Overexpression of Human IDO and Cu,Zn-SOD—For transient overexpression of human IDO, CHO cells were seeded in 6-well tissue culture plates and grown to 50% confluence. Cells were then transfected with pcDNA3 encoding full-length human IDO cDNA (1 $\mu\text{g}/\text{well}$) or pcDNA3 (empty vector control, 1 $\mu\text{g}/\text{well}$) using FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. After overnight transfection, cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 μM L-Trp and then used for experiments.

For co-expression of IDO and human Cu,Zn-SOD, HEK 293 cells were seeded in 6-well tissue culture plates and grown to 40% confluence in DMEM containing 10% FBS but no antibiotics. Cells were transfected with pcDNA3 (empty vector control, 1 $\mu\text{g}/\text{well}$), pcDNA3 encoding human IDO cDNA (1 $\mu\text{g}/\text{well}$), pcDNA3 encoding full-length human Cu,Zn-SOD cDNA (1 $\mu\text{g}/\text{well}$), or both IDO and Cu,Zn-SOD vectors together using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h of incubation at 37 °C, the medium was replaced with DMEM supplemented with 10% FBS and 100 μM L-Trp and then used for experiments.

Knockdown of Cytochrome b_5 by Short Interfering RNA (siRNA)—HEK 293 cells cultured to 25% confluence in 12-well plates were co-transfected with pcDNA3 encoding human IDO cDNA (0.5 $\mu\text{g}/\text{well}$) and 60 pmol of siRNA targeted against the microsomal form of human cytochrome b_5 (DNA sequence, 5'-TCGCCTTGATGTATCGCCTATACAT-3', siRNA sense 5'-UCGCCUUGAUGUAUCGCUAUACA-3'), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were incubated for 72 h in DMEM supplemented with 10% FBS, and the medium was then replaced with DMEM supplemented with 10% FBS and 200 μM L-Trp, and cells were then incubated for the time indicated. As a control,

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cells were co-transfected with human IDO vector as before but using 60 pmol of scrambled siRNA with similar GC content (44%) to the cytochrome b_5 -targeted siRNA. The scrambled siRNA (sense 5'-CCAAACAAUAGCUUGCCCUCAGAUAA-3') was not a match to any sequence in the human BLAST data base.

IDO Activity Assay—IDO activity was measured as the amount of kynurenine formed from L-Trp, as described previously (18), with modifications. For studies with rhIDO, the standard assay mixture consisted of 100 mM potassium phosphate buffer, pH 7.4, containing 50 or 500 nM rhIDO, 150 units of catalase, and 100 μ M diethylenetriaminepentaacetic acid. The reaction was started by the addition of 400 μ M L-Trp in the presence of 25 μ M methylene blue and 0–10 mM ascorbic acid, and the mixture was incubated for 30 min at 37 °C before cold trichloroacetic acid (4% final concentration, w/v) was added to stop the reaction. Mixtures were then stored at 4 °C overnight to ensure complete conversion of *N*-formyl-kynurenine to kynurenine before analysis by HPLC of L-Trp and kynurenine in the supernatant. For isolated rabbit enterocytes, trichloroacetic acid was added to cells and media to 4% final concentration, w/v. For other cellular studies, medium (~200 μ l) from cultures previously supplemented with 100–200 μ M L-Trp and incubated for the time indicated was deproteinized with trichloroacetic acid (4% w/v).

Prior to HPLC analyses, samples were centrifuged at 12,000 \times *g* for 10 min, and the resulting supernatant was subjected to HPLC (Agilent 1100 HPLC Systems, Agilent) equipped with a Hypersil 3- μ m ODS C18 (1) column (Phenomenex) eluted with 100 mM chloroacetic acid, 9% acetonitrile, pH 2.4, at 0.6 ml/min. Tryptophan and kynurenine were detected by UV absorbance at 280 and 364 nm, respectively. Enzyme activity was estimated according to the amount of kynurenine detected and adjusted for total cellular protein. For rhIDO experiments, IDO activity was estimated as the amounts of kynurenine produced.

Reduction of rhIDO by Cytochrome b_5 —The reduction of rhIDO by recombinant human cytochrome b_5 was investigated by adding 8 μ M Fe³⁺-rhIDO to a reaction mixture purged with argon gas and containing 0.2 μ M human recombinant cytochrome b_5 , 2 μ M purified human NADPH:cytochrome P450 reductase, 56 units of glucose-6-phosphate dehydrogenase, 375 units of bovine catalase, 20 mM glucose-6-phosphate, 4 mM NADP⁺, and 200 μ M diethylenetriaminepentaacetic acid in 100 mM phosphate buffer, pH 7.4. The reaction was carried out at 25 °C in a septum sealed cuvette thoroughly purged with argon gas. Difference spectra were recorded every 30 s from 380 to 680 nm against a reference sample containing the reaction mixture without rhIDO, using a Beckman-Coulter DU800A spectrophotometer. Experiments were performed in the absence and presence of CO (~1 mM), by thoroughly purging the reaction mixture and the cuvette with CO gas. Solutions of glucose-6-phosphate dehydrogenase and NADPH:cytochrome P450 reductase were gel-filtered prior to use to remove any interfering substances such as ammonium sulfate and dithiothreitol.

Determination of Intracellular O₂⁻—Intracellular concentrations of O₂⁻ were assessed by the ratio of 2-hydroxyethidium (2-OH-E⁺) to hydroethidine (HE), determined by HPLC with

electrochemical detection, as described recently (19). Briefly, after treatment with SOD mimetics, HAEC were washed twice with phosphate-buffered saline (PBS) and then incubated for 30 min at 37 °C in 2 ml of endothelial growth media supplemented with 10 μ M HE. The medium was removed, and cells washed twice with PBS before being stored at -80 °C. On the day of analysis, cells were thawed to ambient temperature; any remaining adherent cells were scraped and then lysed in 250 μ l of PBS containing 0.1% Triton X-100 and protease inhibitors. Cell lysates were then centrifuged at 12,000 \times *g* for 10 min, and a 10- μ l sample was used for protein determination. 1-Butanol (0.5 ml) was added to the remaining cell lysate, the suspension mixed vigorously for 1 min, and then centrifuged. The butanol phase was removed, dried under vacuum, and the dried sample dissolved in 100 μ l of 1 mM HCl and subjected immediately to HPLC. HE and 2-OH-E⁺ were separated on an ether-linked phenol column (250 \times 4.6 mm, 4 μ m, Synergy Polar-RP®, Phenomenex) and detected by electrochemistry (19). Standards of 2-OH-E⁺ were prepared by reacting HE with Fremy's salt as reported previously (20), with minor modification. After purification, the silica gel column was replaced by the SPE cartridge C18 Prevail (Alltech). The cartridge was first conditioned with 5 ml of methanol before loading of the extracted reaction mixture. The cartridge was then rinsed with 5 ml of water followed by 5 ml of 50% methanol, before 2-OH-E⁺ was eluted with 80% methanol. The eluate was dried, and the purity of 2-OH-E⁺ was verified by mass spectrometry and HPLC electrochemical detection, respectively.

Western Blot Analysis—Cells were lysed in PBS + 0.1% Triton X-100 supplemented with protease inhibitors or by subjecting cells in PBS supplemented with protease inhibitors to three cycles of freezing and thawing. Western blotting was performed as described (16), with minor modifications. Briefly, equal amounts of protein (8–15 μ g) were loaded onto 4–12% polyacrylamide gels (NU-PAGE, Invitrogen) using SeeBlue® Plus2 (Invitrogen) as molecular mass standards. Electrophoresis was performed at 200 V for 1 h using a MiniProtean II electrophoresis system (Bio-Rad), and separated proteins were transferred onto nitrocellulose membranes (Amersham Biosciences) at 30 V for 90 min using a mini-blot module (NOVEX, San Diego). The blotted membranes were blocked in 5% (w/v) skim milk powder with 0.1% (v/v) Tween 20 in Tris-buffered saline for 3–4 h at room temperature. Blocked membranes were probed overnight at 4 °C with the following primary antibodies at 1:1000 to 1:2000 dilutions in 5% (w/v) skim milk and Tween 20-containing Tris-buffered saline: sheep polyclonal antibody against human IDO (Invitrogen); rabbit polyclonal antibody against human Cu,Zn-SOD (Santa Cruz Biotechnology); rabbit polyclonal antibody against human cytochrome b_5 (Santa Cruz Biotechnology); and mouse monoclonal antibody against α -tubulin (Sigma). Membranes were then probed for 1 h at room temperature with the appropriate anti-horseradish peroxidase-conjugated IgG (1:10,000 dilution) in 1% skim milk and 0.1% Tween 20 in Tris-buffered saline. Protein bands were detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

SOD Activity and Measurement of O₂⁻—Cellular SOD activity was determined using the oxidation of pyrogallol by superoxide

(21). Briefly, solutions containing 100 mM Tris-HCl, pH 7.5, 125 units of catalase, 25 μ M diethylenetriaminepentaacetic acid, and various amounts of cell lysates were incubated at 25 °C for 5 min. Reactions were started by adding 200 μ M pyrogallol and followed as the increase in absorbance at 420 nm every 10 s for 30 min. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol autoxidation by 50%.

The rate of O_2^- generation by xanthine oxidase was determined by the rate of reduction of ferric cytochrome *c* (22). In 50 mM phosphate buffer, pH 7.4, 240 μ g of horse heart cytochrome *c* was mixed with 615 units of bovine catalase, 50 μ M diethylenetriaminepentaacetic acid, and varying concentrations of xanthine oxidase in the absence or presence of 250 units of SOD. Reaction mixtures were incubated at 37 °C for 10 min before addition of 100 μ M hypoxanthine, and $A_{550\text{ nm}}$ was then monitored for 5 min at 37 °C. The rate of O_2^- production was calculated using an extinction coefficient of $21.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Statistical Analysis—Statistical differences between treatments and controls were examined using the Wilcoxon-Mann-Whitney rank sum test. Where appropriate, data were analyzed using a two-factor repeated measures analysis of variance with post-hoc Bonferroni test. Significance was accepted at $p < 0.05$.

RESULTS

Role of O_2^- in Activation of rhIDO—To date, studies examining the ability of O_2^- to activate isolated IDO protein have been limited to the rabbit and mouse enzyme. We therefore first investigated the role of O_2^- in the activation of rhIDO, incubating the purified enzyme (500 nM) with L-Trp in the presence of methylene blue and ascorbate, a system commonly used for *in vitro* activation of IDO. IDO activity, measured by the accumulation of kynurenine, increased with increasing concentrations of ascorbate (Fig. 1A). Activity was not affected by the presence of SOD (Fig. 1A), consistent with previous findings demonstrating that ascorbate reduces methylene blue, and reduced methylene blue, rather than O_2^- , then activates IDO (10). We next replaced ascorbate/methylene blue with hypoxanthine and xanthine oxidase, a well established enzymatic system to generate O_2^- . We chose the concentrations of hypoxanthine and xanthine oxidase such that the amounts of O_2^- generated (see Fig. 1B) were comparable with the concentrations of ascorbate employed in Fig. 1A. Similar to the situation with purified rabbit IDO (6, 10), hypoxanthine and xanthine oxidase activated purified rhIDO in a reaction that was prevented completely by SOD (Fig. 1C), demonstrating that under these conditions O_2^- activated rhIDO. However, the extent of this activation was substantially less than that seen with ascorbate and methylene blue (compare Fig. 1, A with C), indicating that O_2^- was less efficient than ascorbate/methylene blue in activating rhIDO. Consistent with this interpretation, reagent O_2^- , added to rhIDO in the form of potassium superoxide, activated the enzyme only modestly; this activation again was prevented completely by SOD (Fig. 1D). Previous studies with rabbit IDO reported SOD to inhibit O_2^- -mediated activity by 70–95% (6, 23). Using rhIDO at 10 times lower concentration yielded measurable enzyme activity with ascorbate and methylene blue but not with enzymatically generated O_2^- or potassium superoxide (data not shown).

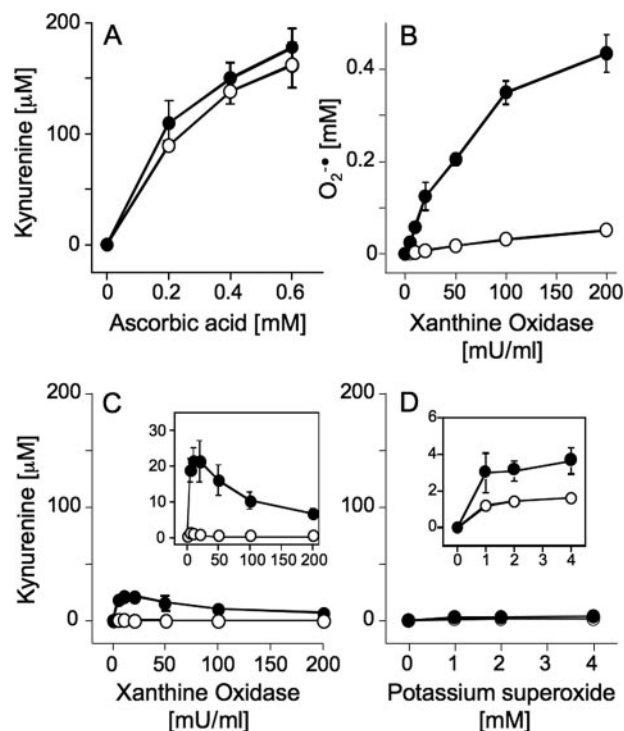


FIGURE 1. Activation of recombinant human IDO activity by methylene blue/ascorbic acid, xanthine oxidase/hypoxanthine, or potassium superoxide. A, rhIDO (500 nM heme) was incubated for 30 min at 37 °C with 400 μ M L-Trp, 25 μ M methylene blue, and varying concentrations of ascorbic acid in the absence (●) or presence (○) of 250 units of Cu,Zn-SOD. B, superoxide anion radical formation was measured following reduction of cytochrome *c* at 550 nm as described under "Experimental Procedures," in the absence (●) or presence (○) of 250 units of Cu,Zn-SOD. C, rhIDO (500 nM heme) in standard assay mixture was incubated for 30 min at 37 °C with 400 μ M L-Trp, 100 μ M hypoxanthine, and varying concentrations of xanthine oxidase in the absence (●) or presence (○) of 250 units of Cu,Zn-SOD from bovine erythrocytes. D, rhIDO (500 nM heme) was incubated for 30 min at 37 °C with 400 μ M L-Trp, and varying concentrations of potassium superoxide in DMSO + 1% crown ether were infused into the standard assay mixture at 10 μ l/min. The final DMSO concentration was 10% (v/v). All reactions were performed in 100 mM potassium phosphate, pH 7.4, and were stopped by adding ice-cold trichloroacetic acid (4% final concentration, w/v). Kynurenine content was measured in the supernatants as described under "Experimental Procedures." Results are mean \pm S.E. of three independent experiments.

These data show that although O_2^- can activate isolated rhIDO, the extent of this activation is modest, at least when compared with the ascorbate/methylene blue system.

Role of O_2^- in Activation of Cellular IDO Activity—The present dogma that O_2^- is the biological activator of cellular IDO is based in part on previous work carried out in rabbit enterocytes, which constitutively express IDO activity (17). Hayaishi and co-workers (17) proposed that, by providing O_2^- , cellular xanthine oxidase contributes to IDO activation, because addition of the xanthine oxidase substrate inosine increased, whereas xanthine oxidase inhibitors abolished, IDO activity. Indeed, we confirmed that inosine increased IDO activity 4-fold compared with untreated rabbit enterocytes (Fig. 2A). However, this enhancement was entirely dependent on the presence of methylene blue, and it was not affected by the xanthine oxidase inhibitor oxypurinol (Fig. 2A). These results are inconsistent with the notion that xanthine oxidase-derived O_2^- is involved in the activation of IDO in rabbit enterocytes.

We next investigated the effect of modulating cellular SOD on IDO activity in rabbit enterocytes. As methylene blue is not

Reduction of Human IDO by Cytochrome b_5

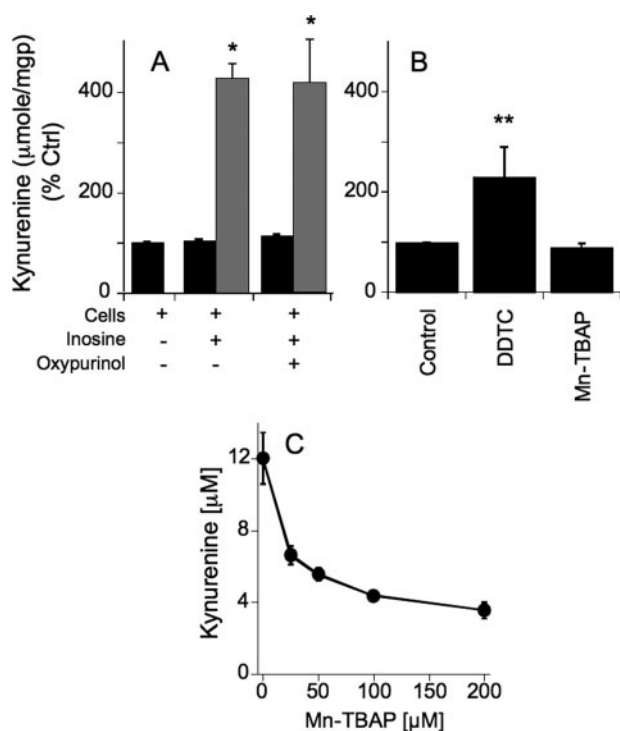


FIGURE 2. Effect of inosine, oxypurinol, methylene blue, and SOD mimetics on IDO activity in enterocytes. *A*, rabbit enterocytes (5×10^5) were pretreated with inosine ($400 \mu\text{M}$) \pm oxypurinol ($200 \mu\text{M}$) for 15 min at 37°C . L-Trp ($100 \mu\text{M}$) was then added, and cells were incubated for 1 h at 37°C in the absence (black columns) or presence (gray columns) of $25 \mu\text{M}$ methylene blue. *B*, rabbit enterocytes (5×10^5) were pretreated with diethyldithiocarbamate (DDTC, 5 mM) or Mn-TBAP ($100 \mu\text{M}$) for 15 min at 37°C , before addition of L-Trp ($100 \mu\text{M}$) and further incubation of the cells for 1 h at 37°C in the absence of methylene blue. *C*, rhIDO (500 nm heme) was incubated for 30 min at 37°C with $400 \mu\text{M}$ L-Trp, $100 \mu\text{M}$ hypoxanthine, and 10 milliunits/ml xanthine oxidase in the presence of various concentrations of Mn-TBAP in a final volume of $400 \mu\text{l}$. Following incubation, the concentration of kynurenine in the cell supernatant (*A + B*) or the reaction mixture (*C*) was determined as described under "Experimental Procedures" as a measure of IDO activity. Results represent the mean \pm S.E. of at least three independent experiments. *, $p < 0.05$; **, $p < 0.01$ (Wilcoxon Mann-Whitney rank test).

a biological molecule, we performed these and all subsequent experiments in the absence of this redox active compound. We pretreated enterocytes with diethyldithiocarbamate, a copper chelator and inhibitor of Cu,Zn-SOD previously shown to increase IDO activity in rabbit enterocytes (17). We confirmed that diethyldithiocarbamate increased IDO activity (Fig. 2*B*). As the copper chelator is known to have cellular effects in addition to inhibiting Cu,Zn-SOD (24, 25), we also modulated cellular SOD activity employing the SOD mimetic Mn-TBAP (26). Addition of Mn-TBAP to rabbit enterocytes did not alter IDO activity (Fig. 2*B*), despite concentration-dependently inhibiting the activation of isolated rhIDO by hypoxanthine and xanthine oxidase (Fig. 2*C*).

To investigate the role of O_2^- in the activation of IDO in human cells, we first primed HAEC with rhIFN γ to induce expression of active IDO, and we then incubated these cells, expressing active IDO enzyme, in the absence and presence of either diethyldithiocarbamate, Mn-TBAP, or bovine SOD conjugated to polyethyleneglycol (PEG-SOD) in fresh medium supplemented with L-Trp. In contrast to the situation with rabbit enterocytes, incubation of IDO-expressing HAEC with diethyldithiocarbamate decreased kynurenine accumulation in

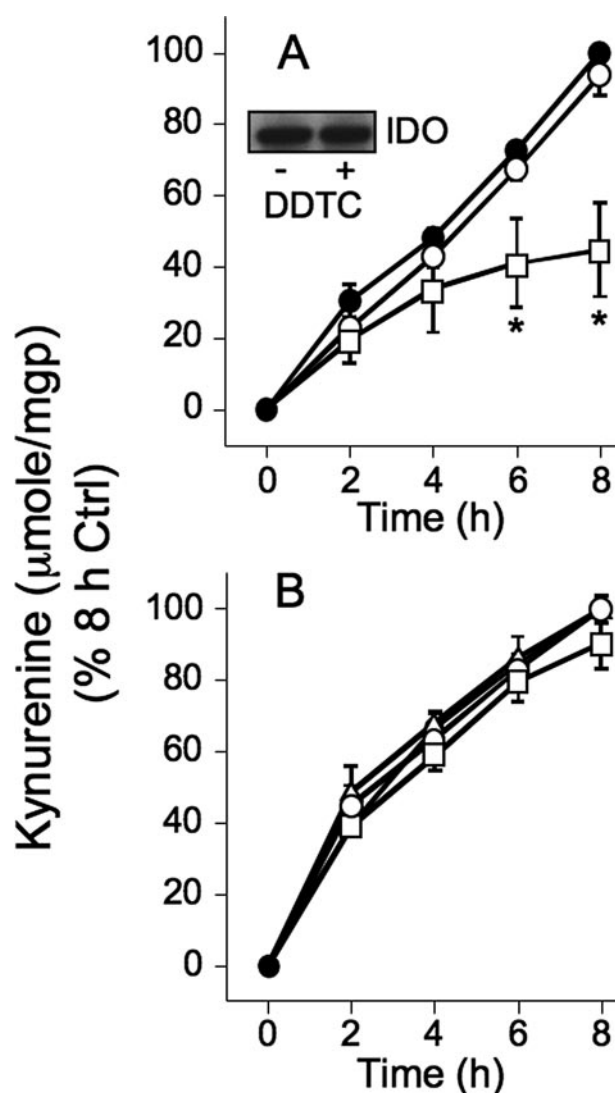


FIGURE 3. Mn-TBAP or PEG-SOD does not decrease IDO activity in rhIFN- γ primed s. *A*, confluent HAEC, stimulated with rhIFN γ (500 units/ml) for 72 h, were incubated for 8 h in endothelial growth medium in the presence of $100 \mu\text{M}$ L-Trp only (●), L-Trp + Mn-TBAP ($100 \mu\text{M}$) (○), or L-Trp + diethyldithiocarbamate (DDTC, 1 mM) (□). The medium was analyzed for kynurenine at specified times as described under "Experimental Procedures." Values are the mean \pm S.E. of 3–5 independent experiments. *B*, confluent HAEC, stimulated with rhIFN γ (500 units/ml) for 72 h, were incubated in endothelial growth medium only (●), medium + methoxy-PEG ($1 \mu\text{mol}$) (◇), medium + PEG-SOD (215 units) (□), or medium + PEG-SOD (430 units) (△). After 18 h, $100 \mu\text{M}$ L-Trp was added to the cells, and the medium was analyzed for kynurenine at specified times as described under "Experimental Procedures." IDO activity is expressed as a percentage of the amount of kynurenine accumulated in the medium after 8 h of incubation (% 8 h Ctrl). Values represent the mean \pm S.E. of four independent experiments, with data standardized to total soluble protein. *, $p < 0.001$ between diethyldithiocarbamate and control using two-factor analysis of variance with post-hoc Bonferroni test.

the medium (Fig. 3*A*). Importantly, this decrease in IDO activity was not because of the copper chelator decreasing IDO protein (Fig. 3*A*, inset) or intracellular O_2^- . In fact, diethyldithiocarbamate increased cellular O_2^- , as assessed by measuring the ratio of 2-OH- E^+ to HE (19, 27) (Fig. 4*A*). In addition, Mn-TBAP (Fig. 3*A*) and PEG-SOD (Fig. 3*B*) had no material effect on IDO activity in rhIFN γ -primed HAEC, although both SOD mimetics decreased cellular ratios of 2-OH- E^+ to HE by up to 50% (Fig. 4, *A* and *B*). In the case of PEG-SOD, this was because of

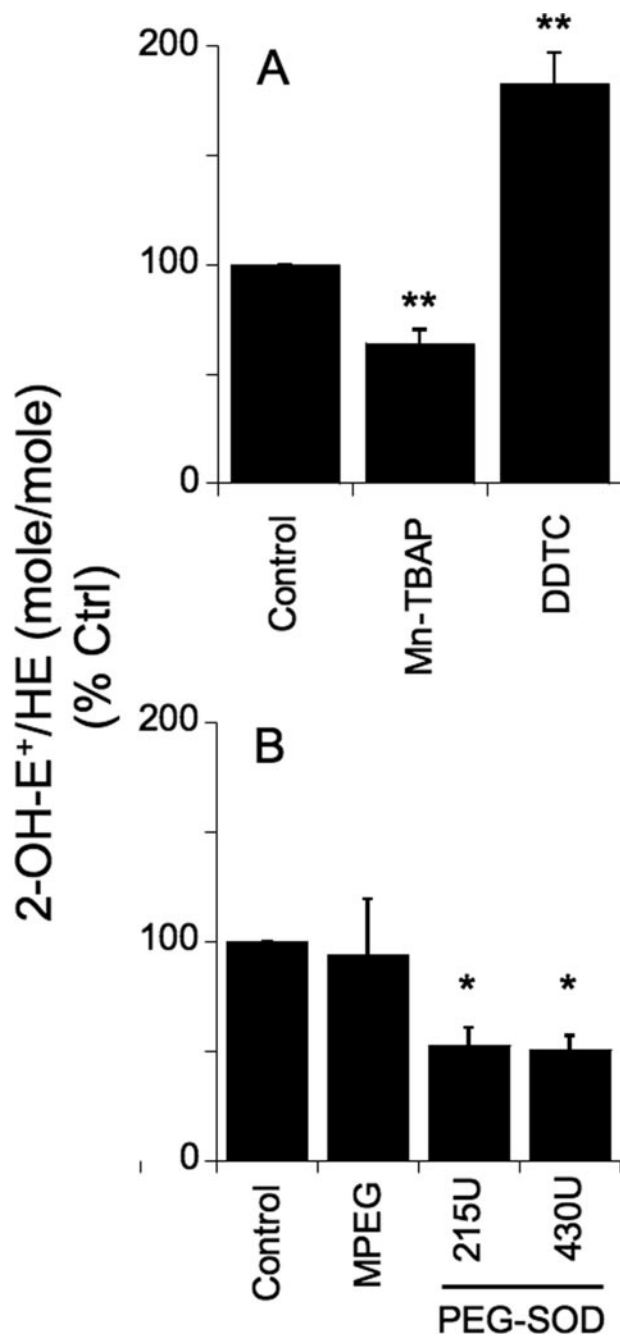


FIGURE 4. Mn-TBAP and PEG-SOD decrease cellular O₂⁻ in rhIFN- γ -primed HAEC. *A*, confluent HAEC, stimulated with rhIFN- γ (500 units/ml) for 72 h, were incubated for 8 h in endothelial growth medium in the presence of 100 μ M L-Trp only (control), L-Trp + Mn-TBAP (100 μ M), or L-Trp + diethyldithiocarbamate (DDTC, 1 mM) as described in the legend to Fig. 3*A*. *B*, confluent HAEC, stimulated with rhIFN- γ (500 units/ml) for 72 h, were incubated for 18 h in endothelial growth medium only (control), medium + 1 μ M of MPEG, medium + PEG-SOD (215 units), or medium + PEG-SOD (430 units) as described in the legend to Fig. 3*B*. After 8 h of incubation with 100 μ M L-Trp for IDO activity measurements, cells were washed twice with PBS and then incubated further for 30 min at 37 °C in 2 ml of medium containing 10 μ M HE, washed twice in PBS, and then finally lysed in 300 μ l of PBS + 0.5% Triton X-100. HE and 2-OH-E⁺ were then extracted into 500 μ l of 1-butanol, dried, and re-dissolved in 100 μ l of 1 mM HCl. HE and 2-OH-E⁺ were analyzed by HPLC with electrochemical detection as described under "Experimental Procedures." Data represent the mean \pm S.E. of 3–5 independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with control (Wilcoxon Mann-Whitney rank test).

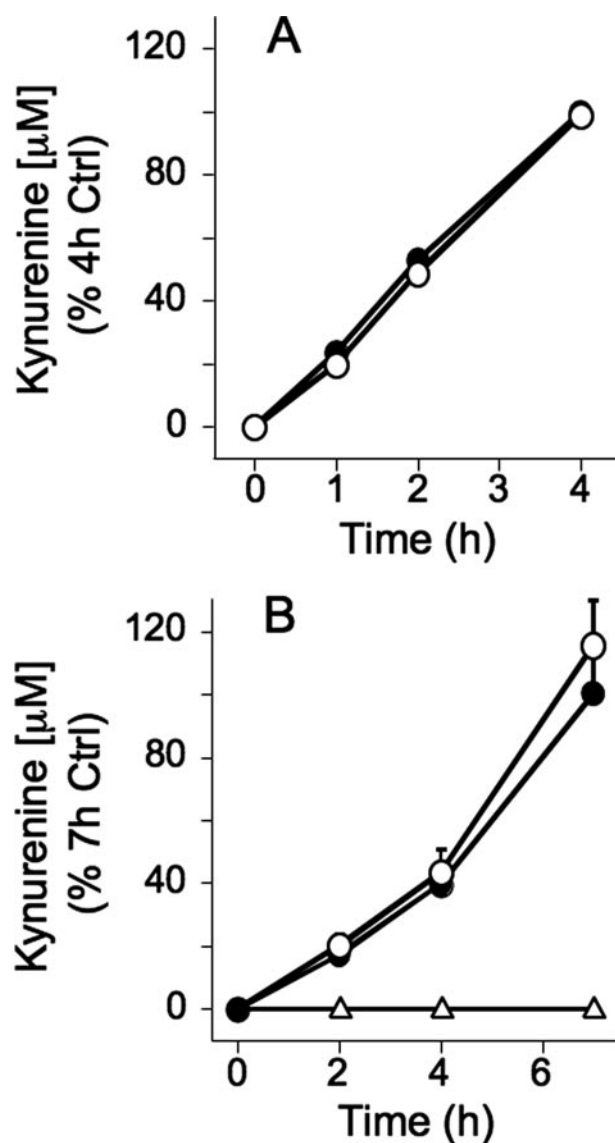


FIGURE 5. Mn-TBAP does not decrease IDO activity in rhIFN- γ -primed MDM or CHO cells transfected with human IDO. *A*, MDM stimulated with rhIFN- γ (500 units/ml) for 32 h were incubated for 4 h at 37 °C in RPMI 1640 medium in the presence of 100 μ M L-Trp only (●) or L-Trp + Mn-TBAP (100 μ M) (○). The medium was then analyzed for kynurenine at specified times as described under "Experimental Procedures." Results are the mean \pm S.E. of three independent experiments. *B*, CHO cells, transfected for 18 h with pcDNA3 vector containing the human IDO transcript, were treated with 100 μ M L-Trp (●) or L-Trp + Mn-TBAP (100 μ M) (○) for 7 h at 37 °C as described under "Experimental Procedures." Δ indicates CHO cells transfected with pcDNA3 vector only for 18 h and then incubated for 7 h with 100 μ M L-Trp only. The medium was analyzed for kynurenine at specified times as described under "Experimental Procedures." IDO activity is expressed as a percentage of the amount of kynurenine accumulated in the medium after 7 h of incubation (% 7 h Ctrl). Data represent the mean \pm S.E. of at least five independent experiments.

enzymatic activity, as methoxy-polyethyleneglycol alone did not affect the 2-OH-E⁺ to HE ratio. This inability of the SOD mimetics to decrease IDO activity was also seen in different cells. Thus, Mn-TBAP had no effect on IDO activity in rhIFN- γ -primed human monocyte-derived macrophages (Fig. 5*A*) and CHO cells overexpressing human IDO (Fig. 5*B*).

Next, we investigated whether increasing intracellular O₂⁻ affected IDO activity. We incubated IDO-expressing CHO cells with menadione, a quinone known to undergo intracellular

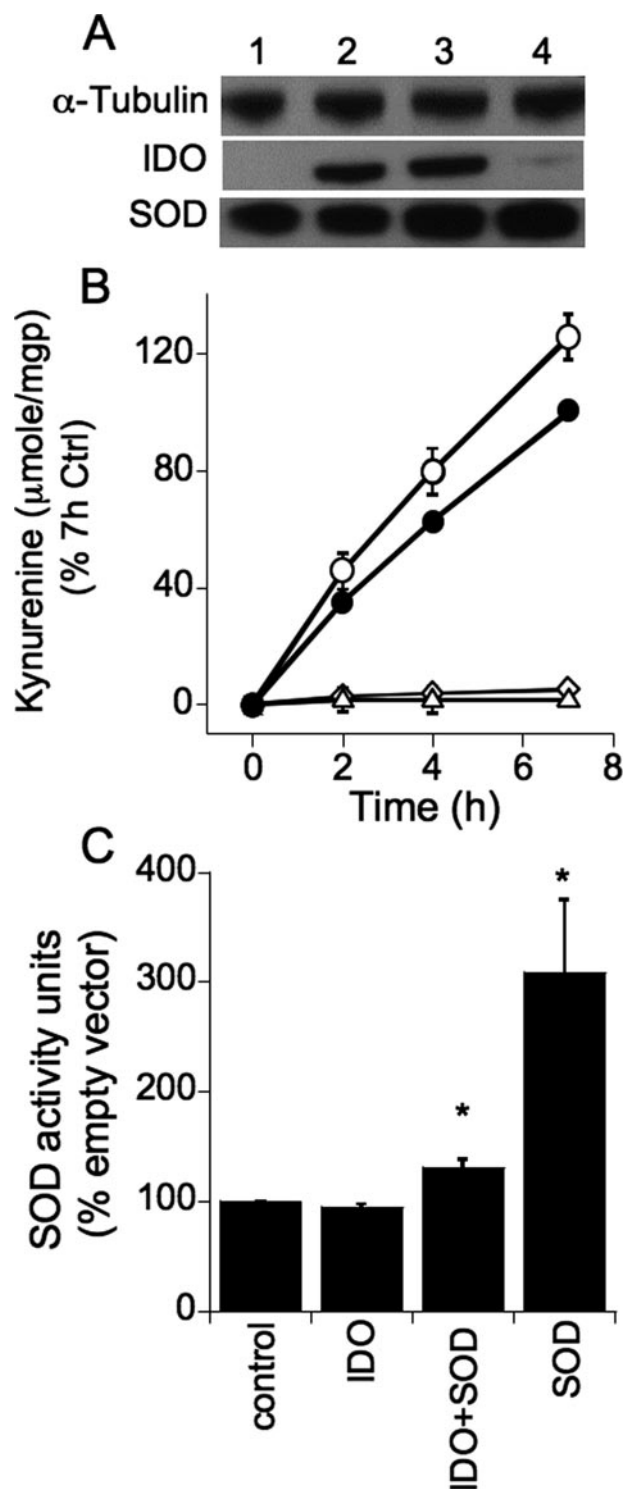


FIGURE 6. Co-expression of human Cu,Zn-SOD does not decrease IDO activity in IDO-expressing HEK 293 cells. *A*, HEK 293 cells (40% confluent), transfected for 48 h with empty vector (pcDNA3) (Δ and lane 1), vector encoding human IDO (\bullet and lane 2), vector encoding human Cu,Zn-SOD (\diamond and lane 4), or with human IDO and Cu,Zn-SOD vectors (\circ and lane 3), were incubated in DMEM containing 100 μ M L-Trp for 7 h at 37 $^{\circ}$ C as described under "Experimental Procedures." Cellular IDO, Cu,Zn-SOD, and α -tubulin protein were analyzed by Western blotting of cell lysates as described under "Experimental Procedures." The Western blot shown is representative of four independent experiments. *B*, at specified times, 200 μ l of medium was removed, trichloroacetic acid added, and kynurenine in the supernatants determined as described under "Experimental Procedures." IDO activity is expressed as a percentage of the amount of kynurenine formed per total soluble protein of IDO-transfected cells after 7 h of incubation (% 7 h Ctrl). *C*, SOD activity was

redox cycling, resulting in the generation of $O_2^{\cdot -}$ (28). Addition of menadione at nontoxic micromolar concentrations did not increase IDO activity (data not shown), despite the fact that it increased $O_2^{\cdot -}$ in these cells (19).

In the next series of experiments, we examined whether co-expression of human IDO with Cu,Zn-SOD affected metabolism of L-Trp to kynurenine in model cells. As expected, expression of human IDO in HEK 293 cells increased IDO protein (Fig. 6A) and activity (Fig. 6B) compared with cells transfected with the empty vector. This activity slightly increased in cells co-expressing Cu,Zn-SOD, without affecting the cellular content of IDO (Fig. 6A), and despite cellular SOD activity increasing by \sim 50% (Fig. 6C). Together, these data show that changes in cellular $O_2^{\cdot -}$ do not translate to respective changes in cellular IDO activity, inconsistent with the notion that $O_2^{\cdot -}$ is required as an important activator of IDO in various human cells.

Activation of Human IDO Independent of $O_2^{\cdot -}$ —We next investigated possible activator(s) of human IDO other than $O_2^{\cdot -}$. As reduced flavin mononucleotide (FMN), FADH₂, and biopterin have been reported previously to activate purified murine IDO (11, 12), we tested these agents for their ability to activate rhIDO. Tetrahydrobiopterin was inactive, even in the presence of ascorbate or NADPH plus NAD(P)H: dihydropteridine oxidoreductase to maintain the pterin in the reduced form (data not shown). In contrast, the reduced forms of both flavins, generated by the addition of NADPH plus NAD(P)H:FMN oxidoreductase to FMN and FAD, activated rhIDO; FMN and FAD were comparatively less active in the presence of NADPH alone and inactive in the presence of ascorbate alone (Fig. 7A).

To assess whether flavin-mediated processes may be involved in the activation of cellular IDO, we exposed IDO-expressing HAEC to low micromolar concentrations of diphenyleneiodonium, a flavin analog and known inhibitor of flavin-dependent enzymes (29). As can be seen in Fig. 7B, such treatment did not decrease IDO activity. Similar results were observed with rhIFN γ -primed human monocyte-derived macrophages (data not shown) and peripheral blood mononuclear cells (18), where diphenyleneiodonium at concentrations up to 20 μ M did not modulate IDO activity.

Previous phylogenetic (30) and structural analyses (8) have revealed similarities between IDO and myoglobin (31). As ferric myoglobin is reduced by cytochrome b_5 , we therefore examined whether cytochrome b_5 participated in the reductive activation of IDO. To do this, we first carried out reconstitution experiments. Recombinant human cytochrome b_5 effectively activated rhIDO in the presence of purified human cytochrome P450 reductase and an NADPH-regenerating system (Fig. 8). Cytochrome P450 reductase is a physiological electron donor of cytochrome b_5 (32). The presence of both cytochrome b_5 and cytochrome P450 reductase was required for this activity, as either pro-

analyzed in cell lysates after incubation for 7 h as described under "Experimental Procedures." Results are expressed as the percentage of the values obtained with empty vector transfected cells (control) and represent the mean \pm S.E. of five independent experiments. *, $p < 0.01$ compared with cells transfected with IDO alone (Wilcoxon Mann-Whitney rank test).

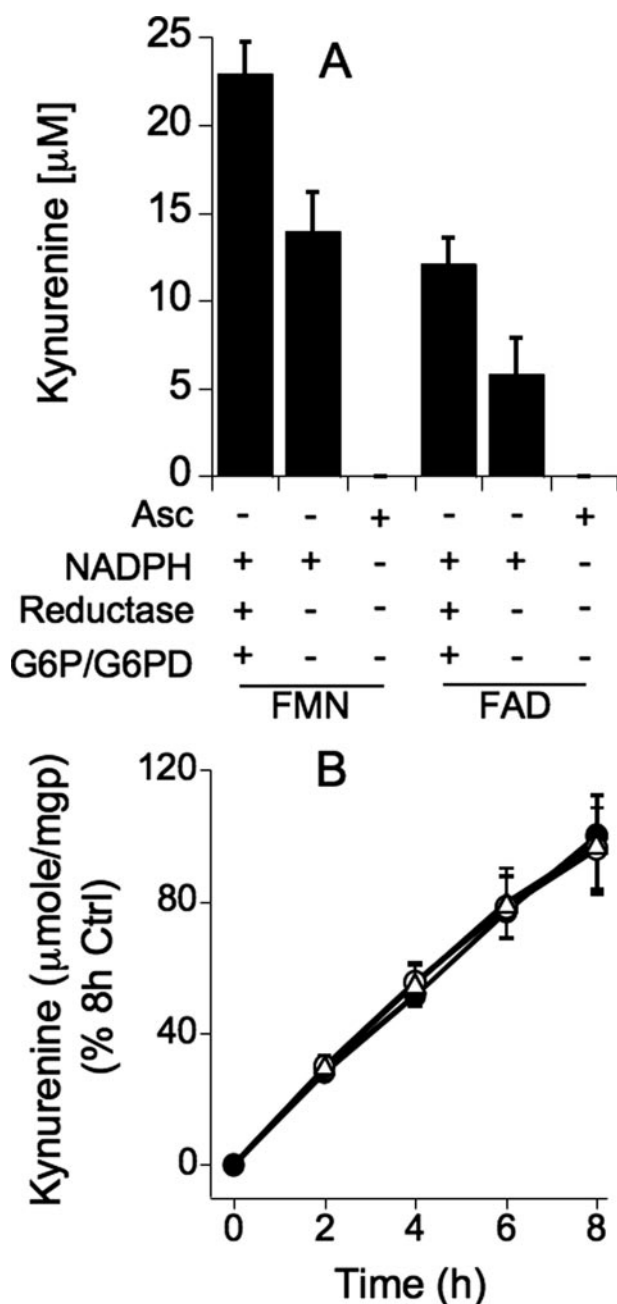


FIGURE 7. FMN activates rhIDO. *A*, in the standard assay mixture, 50 nM rhIDO was incubated with 400 μ M L-Trp for 30 min at 37 °C in the presence of various concentrations of enzymatically and nonenzymatically reduced electron donors: 25 μ M FMN, 25 μ M FAD, 0.1 units of NADPH:FMN oxidoreductase (reductase), 5 mM glucose 6-phosphate (G6P), 2.8 units of glucose-6-phosphate dehydrogenase (G6PDH) and NADPH (5 mM alone or 200 μ M in the presence of reductase and G6P/G6PDH). All reactions were performed in 100 mM phosphate buffer, pH 7.4, stopped by adding trichloroacetic acid, and kynurenine was measured in the supernatants as described under "Experimental Procedures." *B*, confluent HAEC, stimulated with IFN γ (500 units/ml) for 72 h, were incubated for 8 h in endothelial growth medium in the presence of 100 μ M L-Trp only (●), L-Trp + 1 μ M diphenyleneiodonium (○), or L-Trp + 2 μ M diphenyleneiodonium (Δ). The medium was analyzed for kynurenine at specified times as described under "Experimental Procedures." Values are the mean \pm S.E. of three independent experiments. Asc, ascorbate.

tein alone failed to activate IDO, even in the presence of NADPH (Fig. 8). Activation of rhIDO by cytochrome b_5 plus cytochrome P450 reductase increased with increasing concentrations of cytochrome b_5 . However, this reaction was

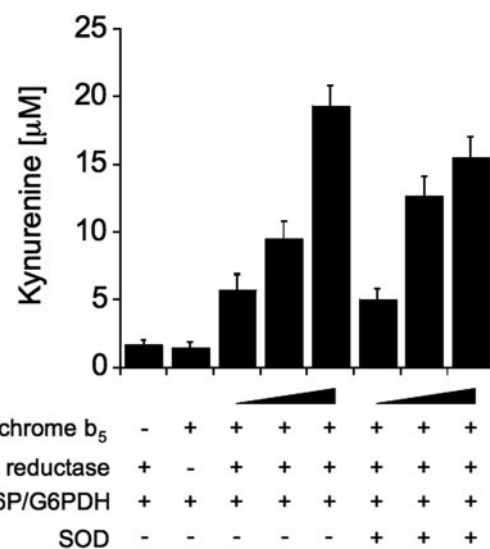


FIGURE 8. Reconstituted recombinant human cytochrome b_5 activates rhIDO activity. *A*, in the standard assay mixture, 50 nM rhIDO was incubated with 400 μ M L-Trp for 30 min at 37 °C in the presence of 0, 50, 150, and 300 nM recombinant human cytochrome b_5 and 50 nM NADPH cytochrome P450 reductase. Concentrations of other compounds were as follows: 5 mM glucose 6-phosphate (G6P), 2.8 units of glucose-6-phosphate dehydrogenase (G6PDH), 2 mM NADPH, and 250 units Cu,Zn-SOD from bovine erythrocytes. All reactions were performed in the standard assay mixture in 100 mM phosphate buffer, pH 7.4, stopped by adding trichloroacetic acid, and kynurenine was measured in the supernatants as described under "Experimental Procedures." Values are the mean \pm S.E. of three independent experiments.

inhibited only modestly by SOD (Fig. 8), indicating that O_2^- plays a minor role in this activation pathway.

To test whether cytochrome b_5 can reduce Fe^{3+} -IDO, we monitored the spectral changes Fe^{3+} -rhIDO undergoes when added to recombinant human cytochrome b_5 in the presence of cytochrome P450 reductase and an NADPH-regenerating system under anaerobic conditions. Using UV-visible absorption spectroscopy, difference spectra (Fig. 9A) showed a time-dependent decrease in absorbance at \sim 402 nm (γ -Soret band) and the appearance of a peak at \sim 420 nm. In addition, the characteristic α/β bands of Fe^{3+} -IDO at 502/632 nm (33, 34) decreased, and this was accompanied by an increase in absorbance maxima at 542 and 577 nm (Fig. 9A, inset). These changes resemble the difference spectra reported to occur during the reduction of methemoglobin by cytochrome b_5 plus methemoglobin reductase and NADH (35). To confirm the ability of cytochrome b_5 to reduce Fe^{3+} -IDO, we carried out experiments in the presence of excess CO, which binds to Fe^{2+} -IDO but not to cytochrome b_5 (36). In this case, absorbance at 404 nm also decreased time-dependently (Fig. 9B), indicative of a decrease in Fe^{3+} -IDO. Concomitantly, absorbance at 419 nm increased, indicative of formation of Fe^{2+} -CO IDO (33). This interpretation is supported by the observed spectral changes in the α/β bands (Fig. 9B, inset). Together, these results provide direct evidence that in the presence of cytochrome P450 reductase and a NADPH-regenerating system, human cytochrome b_5 can reduce Fe^{3+} -rhIDO.

We next assessed the potential role of cytochrome b_5 in the activation of cellular IDO, using RNA interference to suppress cytochrome b_5 , known to be located in the endoplasmic reticulum and the mitochondrial outer membrane (37). As can be

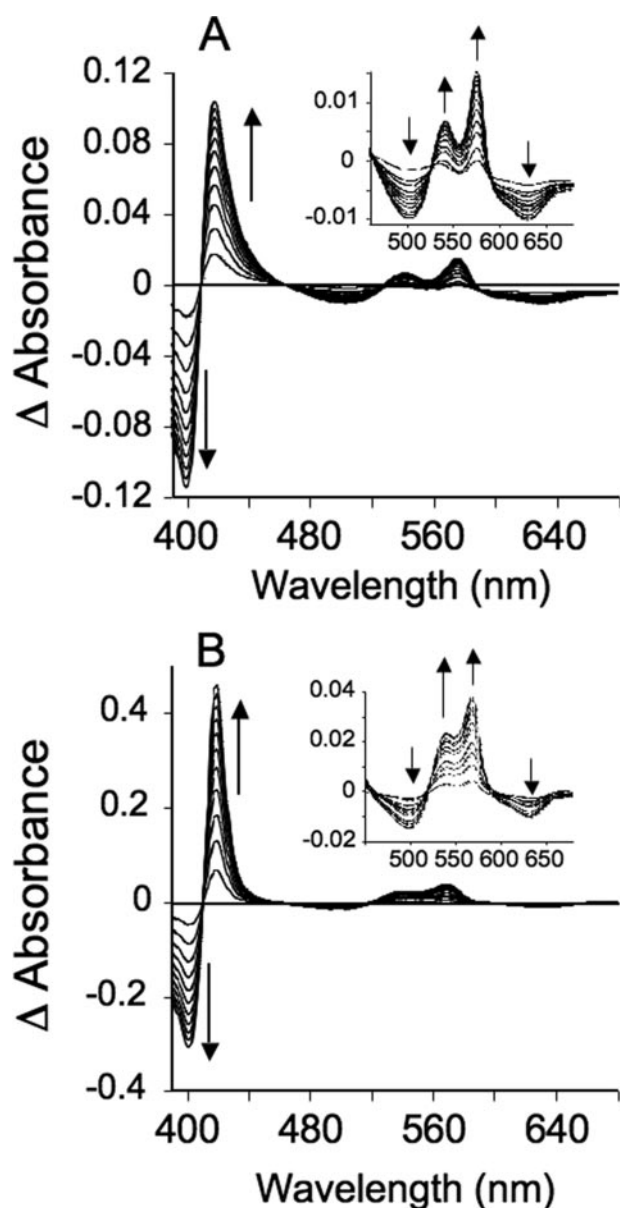


FIGURE 9. Reduction of Fe^{3+} -rhIDO by cytochrome b_5 . Fe^{3+} -rhIDO ($\sim 8 \mu\text{M}$) was added to a reaction mixture equilibrated at 25°C and containing $0.2 \mu\text{M}$ human recombinant cytochrome b_5 , $2 \mu\text{M}$ human NADPH cytochrome P450 reductase, 56 units of glucose-6-phosphate dehydrogenase, 375 units of bovine catalase, 20 mM glucose 6-phosphate, 4 mM NADP^+ , and $200 \mu\text{M}$ diethylenetriaminepentaacetic acid in 100 mM phosphate buffer, pH 7.4, bubbled with argon (A) or CO (B). Difference spectra were recorded every 30 s from 380 to 680 nm against a reference containing the reaction mixture without Fe^{3+} -rhIDO. Insets show spectral changes in the α/β region. \uparrow and \downarrow indicate increases and decreases in absorbance over time, respectively.

seen in Fig. 10, compared with control (scrambled) RNAi, RNAi specific for microsomal cytochrome b_5 significantly decreased both the content of cytochrome b_5 and IDO activity in HEK 293 cells. Such inhibition was obtained without changes to the extent of expression of IDO protein (Fig. 10).

DISCUSSION

Since the pioneering work of Hayaishi and co-workers some 30 years ago (6–8), O_2^- is thought to be responsible for the reductive activation of IDO and xanthine oxidase to provide O_2^- for this activity. Using several different experimental

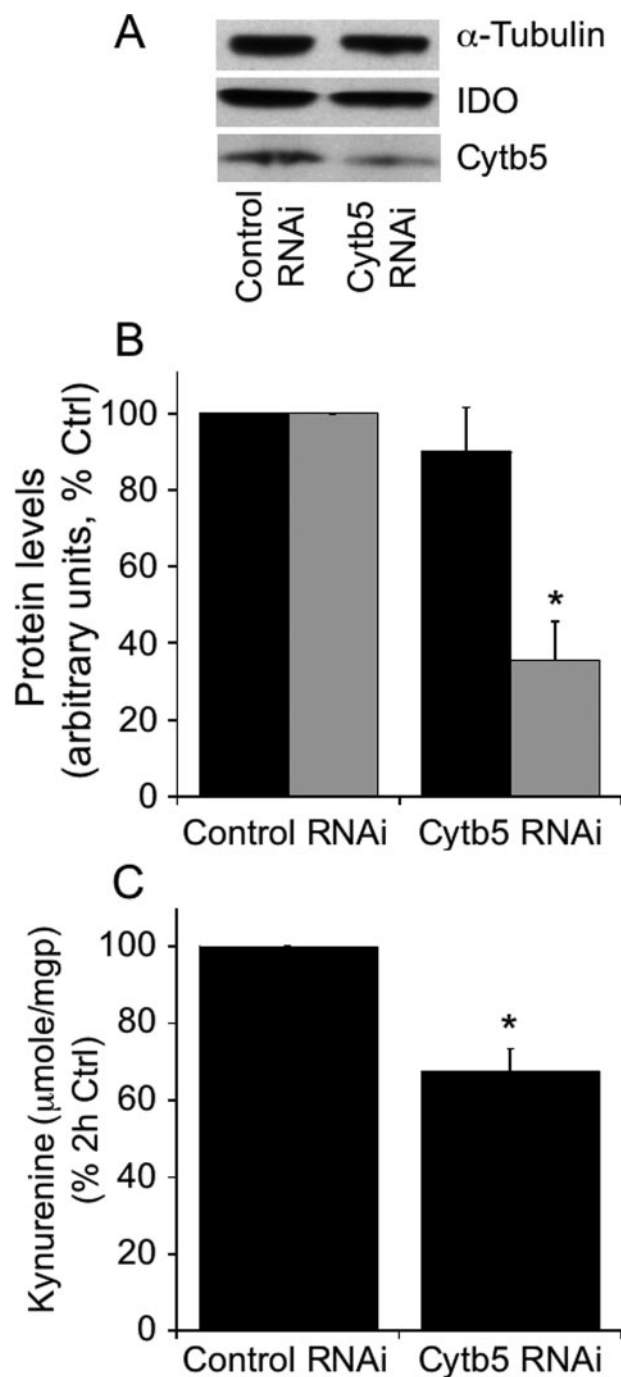


FIGURE 10. Knockdown of cytochrome b_5 in IDO-expressing HEK 293 decreases IDO activity. A, HEK 293 cells (25% confluent) were transfected for 72 h with pcDNA3 vector encoding human IDO and scrambled siRNA (Control RNAi) or siRNA targeted to microsomal cytochrome b_5 (Cytb5 RNAi) as described under "Experimental Procedures." Cells were then incubated in medium supplemented with $200 \mu\text{M}$ L-Trp for 2 h. Cellular IDO, cytochrome b_5 , and α -tubulin protein were analyzed by Western blotting of cell lysates as described under "Experimental Procedures." The Western blot shown is a representative of four independent experiments. B, IDO (black columns) and cytochrome b_5 (gray columns) protein levels were determined by densitometry of the above Western blots and standardized to loading controls (α -tubulin protein levels). C, after 2 h of incubation, $100 \mu\text{l}$ of medium was removed and trichloroacetic acid added, and the supernatants were assayed for kynurenine as described under "Experimental Procedures." IDO activity is expressed as a percentage of the amount of kynurenine formed per protein of IDO-transfected cells after 2 h of incubation (% 2 h Ctrl). Values are the mean \pm S.E. of four independent experiments. *, $p < 0.05$ compared with control siRNA-transfected cells (Wilcoxon Mann-Whitney rank test).

approaches, the results presented here challenge this dogma and instead show that cytochrome b_5 effectively activates isolated human IDO and is likely involved in the activation of IDO in human cells.

The evidence against the importance of O_2^- in the activation of IDO is based on studies with isolated recombinant human IDO, and four different mammalian cells in which IDO expression is either constitutive or was induced by treatment with IFN γ or transfection of naive cells with the human IDO gene. Thus, we show that although O_2^- can activate rhIDO, it does so only with modest efficacy, and a similar role for O_2^- in the activation of cellular IDO appears unimportant. The latter is supported by our findings that two widely used SOD mimetics, Mn-TBAP (26, 38) and PEG-SOD (39), failed to alter IDO activity in rabbit enterocytes, CHO cells expressing IDO, and rhIFN γ -primed human endothelial cells and macrophages. Importantly, both SOD mimetics diminished cellular concentrations of O_2^- significantly, demonstrating that the O_2^- pool available for the proposed reaction with IDO was decreased. This would have been expected to decrease IDO activity if O_2^- were an important activator of the enzyme. A recent study adding SOD to lysates of IFN γ -stimulated T24 cells also showed no effect on IDO activity (40). In addition, we show here that expression of human Cu,Zn-SOD in IDO-transfected HEK 293 cells increased cellular SOD activity without materially affecting IDO protein and activity. Conversely, increasing cellular concentrations of O_2^- by menadione exposure failed to increase cellular IDO activity (data not shown). Although the results obtained with IFN γ -primed cells do not support a major role for O_2^- in cellular IDO activation, the interpretation of the data is complicated, given that IFN γ has multiple cellular effects (41). However, the results obtained with CHO and HEK 293 cells are compelling and do not support the notion that O_2^- is important for cellular IDO activation.

In apparent contrast to the above findings, we observed the Cu,Zn-SOD inhibitor, diethyldithiocarbamate, to increase IDO activity in rabbit enterocytes (Fig. 2B), similar to an earlier finding reported by Tanaguchi *et al.* (17). These authors interpreted this result as indirect evidence for O_2^- participating in IDO activation. However, the results in Fig. 3A show that diethyldithiocarbamate has the opposite effect on rhIFN γ -primed HAEC, *i.e.* where the inhibitor decreased IDO activity significantly, as was reported recently for IFN γ -stimulated T24 cells (40). Importantly, and not assessed in previous studies by others, inhibition of IDO by diethyldithiocarbamate was associated with increased, not decreased, O_2^- . Therefore, our studies, for the first time, dissociate IDO activity from cellular concentrations of O_2^- . Dithiocarbamates like diethyldithiocarbamate were designed as copper chelators, and it is well known that they are not specific inhibitors of Cu,Zn-SOD (24, 25). Therefore, differences in the extent of inhibition of transition metal-dependent processes unrelated to Cu,Zn-SOD may explain the difference in IDO activity in rabbit enterocytes *versus* HAEC. For example, we previously showed that in human monocyte-derived macrophages, dithiocarbamate inhibits IDO activity by interfering with the incorporation of heme into the enzyme (16).

Kinetic and biochemical considerations also support our contention that O_2^- is not important for the activation of cellular

IDO. First, O_2^- reacts with IDO at a rate that is at least 3 orders of magnitude slower than it reacts with SOD (*i.e.* $\sim 1 \times 10^6$ *versus* $\sim 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (10). Thus, SOD would be expected to out-compete IDO for any available O_2^- , particularly as SOD is an abundant protein, occurring in most cells at a concentration of $\sim 10^{-5} \text{ M}$ (42). Furthermore, previous studies by Sono *et al.* (9) have established that O_2^- alone is not sufficient to maintain maximal steady-state activity of IDO and that another cofactor is required (see below).

The proposed role of xanthine oxidase in the activation of cellular IDO is based on the ability of the xanthine oxidase substrate inosine to enhance, and its inhibitor allopurinol to decrease, L-Trp metabolism to kynurenine in rabbit enterocytes (17). However, we demonstrate here that inosine itself did not activate IDO. Rather, an increase in cellular IDO activity by inosine was dependent entirely on the presence of methylene blue (Fig. 2A), thereby making it impossible to draw conclusions from these experiments on an involvement of xanthine oxidase in the activation of IDO. Previous studies have established that reduced methylene blue can effectively activate IDO (10). Importantly, the early observations of Hayaishi and co-workers (17) with inosine and rabbit enterocytes are fully consistent with our results. Indeed, these authors added methylene blue to the cells prior to IDO activity being determined but, unfortunately, did not report respective enzyme activities in the absence of methylene blue. Also consistent with this, inosine had no effect on IDO activity in homogenates of IFN γ -treated T24 cells (40). Additional evidence against a role for xanthine oxidase in IDO activation comes from our experiments with oxypurinol. This xanthine oxidase inhibitor did not affect IDO activity in rabbit enterocytes (Fig. 2A), a finding corroborated by the recently reported inability of allopurinol to alter IDO activity in homogenates of IFN γ -treated T24 cells (40). Hayaishi and co-workers (17) evoked an involvement of xanthine oxidase because allopurinol inhibited IDO activity in rabbit enterocytes. However, all of these earlier experiments (17) were carried out in the presence of methylene blue, thereby invalidating conclusions on the direct impact of allopurinol on IDO activity. The totality of available data therefore suggests that xanthine oxidase is unlikely to be important in modulating cellular IDO activity.

Our arguments against an important role of O_2^- in the activation of cellular IDO in part hinges on the ability to assess changes in cellular O_2^- . Although quantification of O_2^- inside cells remains a challenge (43), there is increasing consensus that HE is useful for this purpose, because this probe is cell-permeable and the major product of its reaction with O_2^- has been identified as 2-OH-E $^+$ (44). Furthermore, cellular concentrations of HE and 2-OH-E $^+$ can be quantified readily and robustly by HPLC-EC detection, thereby allowing the use of the 2-OH-E $^+$ /HE ratio as a simple and reliable indirect measure of cellular O_2^- , as done in this study.

As our results ruled out an important role for O_2^- in the activation of human IDO, we investigated other cellular reductants as potential activators of IDO. Of these, tetrahydrobiopterin has been shown to activate isolated murine IDO (11, 12), and in human cells its synthesis is induced in parallel to that of IDO (45). Contrary to the situation with the murine enzyme, how-

Reduction of Human IDO by Cytochrome b_5

ever, tetrahydrobiopterin failed to activate rhIDO even in the presence of regenerating systems that maintained the pterin in its reduced form. Previous studies by others employing cytokine-treated human fibroblasts, macrophages, and glioblastoma cells also reported cellular IDO activity to be independent of pterin (46). In contrast to tetrahydrobiopterin, reduced FMN and FAD effectively activated rhIDO (Fig. 8A), as was reported for the murine enzyme (11, 12). In the case of murine IDO, activation was inhibited only partially by SOD, and the authors suggested that reduced FMN may directly reduce ferric to ferrous IDO (11, 12). However, this notion is not supported by the recently published crystal structure of human IDO (47), which does not reveal putative flavin-binding sites.

Human IDO shares amino acid homology with certain myoglobins (30), and it forms a stable dioxygen adduct with electronic spectrum identical to that of oxymyoglobin (8). Also, reduced IDO autoxidizes similarly to oxymyoglobin and oxyhemoglobin. In the case of myoglobin and hemoglobin, the resulting oxidized proteins are reduced by cytochrome b_5 , which in turn is maintained in the reduced state by electrons derived from NAD(P)H through the respective reductases (48, 49). Interestingly, the activity of methemoglobin reductase is stimulated by methylene blue (50). This raised the possibility that, by analogy, cytochrome b_5 could similarly be involved in the reduction of ferric IDO. Indeed, our studies show for the first time that cytochrome b_5 in the presence of NADPH cytochrome P450 reductase plus NADPH effectively activated purified human IDO, in a manner largely independent of O_2^- (Fig. 8). Also, we provide direct spectral evidence for the ability of cytochrome b_5 to reduce Fe^{3+} -rhIDO (Fig. 9). Furthermore, knockdown of microsomal cytochrome b_5 significantly decreased IDO activity in HEK 293 cells (Fig. 10), providing direct support for the notion that cytochrome b_5 acts as a reductant and activator of cellular IDO. Interestingly, the observed slight decrease in cytochrome b_5 -mediated activation of rhIDO by SOD suggests that O_2^- might play a minor, perhaps accessory, role in this reaction.

Using a tryptophan auxotroph yeast strain transduced with human IDO, Vottero and co-workers (13) recently observed that the deficiency in cytochrome b_5 and, to a lesser extent, cytochrome b_5 reductase, led to increased cell growth. These findings were interpreted as evidence for an involvement of cytochrome b_5 in the activation of IDO and, hence, degradation of L-Trp (13). Although consistent with our present findings, the experimental approach used by Vottero and co-workers (13) is indirect, as it did not measure IDO activity. That study also did not consider the effect of the yeast homolog of tryptophan dioxygenase, BNA2 (51), that also catabolizes tryptophan. In addition, cytochrome b_5 is involved in a diverse array of biological processes, including the anabolic metabolism of fats and steroids, and the catabolism of xenobiotics (32, 37), all of which could have interfered with the biological readout used by Vottero *et al.* (13).

Judged by their respective redox potentials, electron transfer from cytochrome b_5 ($E^0 = -10$ to $+10$ mV (52)) to ferric IDO ($E^0 = 16$ mV) is feasible (33). Indeed, our spectroscopic studies (Fig. 9) provide direct evidence for the feasibility of such electron transfer from cytochrome b_5 to Fe^{3+} -IDO. As indicated, cytochrome b_5 itself is reduced by reductases, *i.e.* NADH cyto-

chrome b_5 reductase and NADPH cytochrome P450 reductase (32). We successfully reconstituted IDO activity *in vitro* using recombinant human cytochrome b_5 and human cytochrome P450 reductase in the presence of an NADPH-regenerating system (Fig. 8). Based on these results, and the fact that knocking down cytochrome b_5 decreased cellular IDO activity (Fig. 10), we propose that NADPH cytochrome P450 reductase participates in the cytochrome b_5 -mediated reductive activation of IDO in human cells. This is despite the fact that the reductase requires flavins, yet low micromolar concentrations of the inhibitor of flavin-containing enzymes, diphenyleneiodonium, did not impair IDO activity in HAEC (Fig. 8C). The likely reason for this is that the K_i value for inhibition of NADPH cytochrome P450 reductase by diphenyleneiodonium is 2.8 μM (53), whereas we employed the inhibitor at low micromolar concentrations (*i.e.* <20 μM). Higher concentrations of the flavin analog could not be used because they were toxic to cells (data not shown). A potential contribution of NADH cytochrome b_5 reductase, in addition to NADPH cytochrome P450 reductase, requires investigation.

This is the first study reporting IDO activity induced by rhIFN γ in human arterial endothelial cells. We employed endothelial cells for two reasons. First, previous studies have shown IDO to be induced predominantly in the vascular endothelium in a murine model of cerebral malaria (54). Second, we observed recently that in this model of systemic inflammation, kynurenine derived from IDO-mediated metabolism of L-Trp is a novel endothelium-derived vascular relaxing factor.⁴ In this context, IDO could conceivably attenuate O_2^- -mediated endothelial dysfunction, implicated in conditions of oxidative stress such as inflammation (55), if the protein reacted with or even required O_2^- for activity. However, the findings reported in this study do not support such a role for IDO.

In summary, our studies show that cytochrome b_5 reduces and activates human IDO *in vitro* and that cytochrome b_5 , rather than O_2^- , plays a major role in maintaining IDO activity in human cells. However, we cannot totally exclude that O_2^- generated by the cytochrome P450 reductase/cytochrome b_5 system has an indirect and minor role in IDO activation. Clearly, further studies are required to elucidate the interaction and electron transfer between cytochrome b_5 and IDO, as well as the relative contribution of NADPH cytochrome P450 *versus* NADH cytochrome b_5 reductase in this process, and whether the findings reported here for IDO extend to tryptophan 2,3-dioxygenase.

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