

DECREASED PROTEIN NITRATION IN MACROPHAGES THAT OVEREXPRESS INDOLEAMINE 2, 3-DIOXYGENASE

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Abstract: The activity of indoleamine 2, 3-dioxygenase (IDO; E.C. 1.13.11.42) catalyzes the oxidative cleavage of tryptophan to form kynurenine. IDO activity consumes superoxide anions; therefore, we postulated that over-expression of IDO might mitigate superoxide-anion dependent, oxidative modification of cellular proteins *in vitro*. We prepared and characterized RAW 264.7 macrophages that were stably transfected with either an IDO expression vector or the control (empty) vector. We detected IDO mRNA, protein, and enzyme activity in the IDO-transfected macrophages, but not in the macrophages transfected with the empty vector. To generate superoxide anions *in situ*, we treated the IDO- and control-transfected cultures with xanthine or hypoxanthine, and then used ELISA methods to quantitate the relative levels of oxidatively modified proteins in total cell lysates. The levels of protein carbonyls were similar in IDO-transfected and vector-transfected macrophages; however, protein nitration was significantly less in IDO-transfected cells compared to control transfectants. In addition, steady-state levels of superoxide anions were significantly lower in the IDO-transfected cultures compared with control transfectants. Our results are consistent with the concept that, besides degrading tryptophan, IDO activity may protect cells from oxidative damage.

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Abbreviations used: CMV – cytomegalovirus; H₂O₂ – hydrogen peroxide; IDO – indoleamine 2,3-dioxygenase; NAD – nicotinamide adenine dinucleotide; NO – nitric oxide; ONOO⁻ – peroxynitrite; O₂^{-•} – superoxide anion; PBS – phosphate buffered saline (pH 7.4); ROS – reactive oxygen species; SOD – superoxide dismutase

Key words: RAW 264.7 macrophages, Indoleamine 2, 3-dioxygenase, Oxidative stress, Protein oxidation, Superoxide anion

INTRODUCTION

Indoleamine 2, 3-dioxygenase (IDO; E.C. 1.13.11.42) catalyzes the oxidative cleavage of the essential amino acid tryptophan; the reaction product is *N*-formylkynurenine, which enzymatically or spontaneously converts to kynurenine [1, 2]. IDO is a cytosolic, heme-containing, interferon- γ -inducible enzyme that is present in various mammalian tissues [1-15]; however, the physiological function of IDO is not entirely known, except for the function of IDO in the context of host response to pathogens. During infection, cytokine-induced IDO activity [5, 16] depletes tryptophan and forms toxic products, which are proposed to limit the proliferation of invading bacterial [16-20] and viral [21, 22] pathogens.

Our research emphasis encompasses the biological roles and potential therapeutic implications of antigen presenting cell (APC) IDO activity on inhibiting T-cell proliferation [23, 24]. In parallel, we became interested in a possible antioxidant function of IDO [11, 25-27]; IDO activity requires and consumes (scavenges) superoxide anions [1, 28-32]. Certainly, enzymes that degrade (remove) superoxide anions are fundamental antioxidant defenses, since superoxide anions are requisite precursors for other reactive oxygen species (ROS) including hydrogen peroxide, peroxynitrite, and hydroxyl radicals (see Fig. 1) [33-35]. ROS may perturb cell function or structure by oxidatively modifying lipids [36], DNA [37], proteins [38, 39]; therefore, preventing

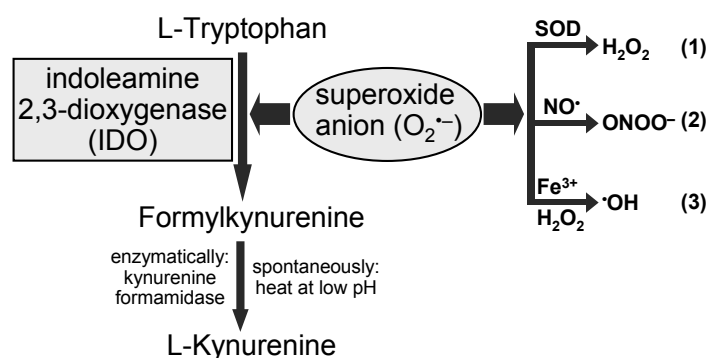


Fig. 1. The activity of the tryptophan-degrading enzyme, indoleamine 2,3-dioxygenase (IDO) requires (consumes) superoxide anion ($O_2^{\bullet-}$), so that $O_2^{\bullet-}$ is unavailable to make (1) hydrogen peroxide (H_2O_2), (2) peroxynitrite ($ONOO^-$), or (3) hydroxyl radical ($\bullet OH$). Superoxide anion is a precursor to three other reactive oxygen species (ROS): superoxide dismutase (SOD) decomposes $O_2^{\bullet-}$ to H_2O_2 (1); nitric oxide (NO^{\bullet}) reacts with $O_2^{\bullet-}$ to form $ONOO^-$ (2); superoxide anion can reduce an iron III salt (Fe^{3+}) to an iron II salt (Fe^{2+}), which reacts with hydrogen peroxide (Fenton reaction) to form $\bullet OH$ (3).

excessive formation of ROS is essential to maintaining cellular homeostasis. Only two ROS-mediated oxidative modifications were evaluated in our studies: (1) peroxynitrite-mediated nitration of specific amino acid side-chains in proteins [39-41], and (2) ROS-dependent introduction of carbonyl groups into proteins [39, 42-44].

When the formation of ROS and consequent oxidative damage exceeds an organism's antioxidant defenses, the organism is in a state of oxidative stress [35, 45]. Oxidative stress is evident in diverse disorders [46-48] including: cancer [49, 50], diabetes [51], autoimmune disorders [52-54], alcoholism [55, 56], and neurodegenerative diseases [57-59]. Hence, identifying the diverse cellular mechanisms that limit ROS production and decrease oxidative stress is fundamental to designing therapeutic interventions to treat or prevent diseases that exhibit oxidative stress. We postulate that modulation of IDO activity could be a determining factor in the diverse array of diseases that exhibit oxidative stress, if a physiological role of IDO is to scavenge superoxide anions [11, 25-27], thereby decreasing ROS formation and oxidative damage.

We began to investigate that postulate using the experiments presented in this report: (1) generate a macrophage cell line that is stably transfected with a functional IDO expression vector, and (2) determine whether over-expression of IDO mitigates superoxide anion-dependent oxidative damage to cellular proteins *in vitro*.

MATERIALS AND METHODS

Construction of IDO expression vector

RAW 264.7 cells (BALB/c mouse macrophage cell line) were a gift from Dr. David Greaves (Sir William Dunn School of Pathology, University of Oxford, United Kingdom). The cell culture media contained Iscove's Modified Dulbecco's Medium (Cellgro #15-016-CV), 10% fetal calf serum (Sigma, St. Louis, MO), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Full-length murine IDOcDNA was isolated from IFN-γ-stimulated (200 units/ml, 24 hrs) RAW 264.7 cells using standard RT-PCR and DNA cloning procedures as previously described [60]. We cloned the IDOcDNA into the pGEM T-Easy vector (Promega #A1360) and sequenced the IDO insert as previously reported [60]. The full-length (1.2-kb) IDOcDNA was digested with Not-I enzyme, and cloned into the Not-I-cut pcDNA-3 cDNA expression vector (Invitrogen, Carlsbad, CA), which contains CMV promoter elements. We linearized the construct using Pvu I digestion before transfection into RAW 264.7 macrophages.

Transfection of RAW 264.7 macrophages and selection of transfectants

Confluent RAW 264.7 cell cultures were harvested using a cell scraper and washed twice with sterile ice-cold PBS. We resuspended 2×10^7 cells in 500 µl of Opti-MEM[®] media (Gibco Life Technologies #51985-034) and electroporated (300 V, 975 µF, room temperature) in the presence of 20 µg of Pvu I-linearized

pcDNA3-IDO or Pvu 1-linearized pcDNA-3 vector DNA. We immediately seeded the electroporated cells into tissue culture flasks containing culture media (see above). Before beginning clonal dilution, the electroporated cells were allowed to recover for two days at 37°C, 95% relative humidity, in a 5% carbon dioxide atmosphere. The cells were harvested, distributed into 96-well plates, and cloned by limiting dilution in complete media that we supplemented to contain 500 µM tryptophan (102 mg/L) and G418 (Gibco Life Technologies, Rockville, MD). We determined the optimal G418 concentration for selection by titrating G418 (200 to 1500 µg/ml) into log cultures; the lowest concentration of G418 that killed 100% of the cells was used in the selection process. After three weeks of selection in G418 (200 µg/ml), we randomly picked single clones for analyses of IDO expression and enzymatic activity.

RT-PCR for IDO expression in transfected cells

We used an RNA STAT-30™ kit according to the manufacturer's directions (Tel-Test Inc, Friendswood, TX) to isolate total RNA from G418-selected cells. The cDNA for IDO and γ -actin were amplified from total RNA (1 µg) using the reagents and protocol in the RT-PCR kit (Access RT-PCR System™, Promega). The IDO primers (5' GTA CAT CAC ATG GCG TAT G 3' and 5' GCT TTC GTC AAG TCT TCA TTG 3') amplified a 750 bp IDOcDNA, and the actin primers (5' AGA AGA AAT CGC CGC ACT C 3' and 5' GGT CCA GAC GCA AGA TGG 3') gave a 533 bp γ -actin fragment. The cDNA was synthesized at 48°C for 45 minutes in a Biometra thermocycler. The actin and IDOcDNA were amplified using 35 cycles at 58°C T_m . The PCR products were separated on 2% agarose gels containing 2.5 µg/ml ethidium bromide, and then visualized under UV transillumination.

Western blot analysis of IDO protein in transfected cells

The pcDNA-3-IDO-transfected clones and vector (Vo)-transfected clones were cultured to confluency, and then total protein was extracted using RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 ng/ml PMSF, 66 ng/ml aprotinin) and brief (no heating or bubbling) sonication. We measured protein concentration in the samples using the bicinchoninic acid (BCA) reagent system (Pierce Biotechnology, Inc., Rockford, IL). The cell lysate (50 µg protein) was separated on a 12% SDS-PAGE TRIS-glycine gel according to standard methods [60]. The gel was blotted onto a nitrocellulose membrane using a Trans-Blot apparatus according to the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA). After protein transfer, the membrane was blocked (agitated one hour) with 5% (w/v) non-fat dried milk in PBS. We detected the IDO protein using a polyclonal antibody (diluted 1:4,000 in blocking agent) that we prepared from rabbits immunized with a synthetic murine IDO C-terminal peptide [60]. The IDO antibody was detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (diluted 1:10,000, #sc 2054, Santa Cruz Biotechnology, Santa Cruz, CA). We detected

the actin protein (loading control) using a mouse anti-actin monoclonal antibody (1:1,000 dilution, #MAB 1501, Chemicon International, Temecula, CA), followed by an anti-mouse IgG-HRP (diluted 1:10,000 #sc 2055, Santa Cruz Biotechnology). The HRP signal was detected using the Western Lightning Chemiluminescence Kit (#NEL 105, Perkin Elmer Life Science, Boston MA).

Analysis of tryptophan and kynurenine levels by HPLC

To determine whether IDO-expressing clones had enzymatically-active IDO, we determined whether the cells converted tryptophan to kynurenine by measuring tryptophan and kynurenine levels in cell culture supernatants. Theoretically, cells with high IDO activity have low tryptophan/high kynurenine in the culture supernatant, while cells with no IDO activity have high tryptophan/low kynurenine in the culture supernatant.

We plated the transfected RAW 264.7 cells (10^5 per well, 96-well plates) in 200 μ l/well of medium containing G418 (200 μ g/ml). The culture media was harvested after 3 days, centrifuged at 3000 rpm for 10 min at 4°C to pellet the cells, and the supernatant removed. We extracted the cell culture supernatant (75 μ l) with 1.4 ml of HPLC-grade methanol to precipitate proteins. The precipitated proteins were removed by centrifugation (14,000 rpm, 15 min, 0°C), and 1.2 ml of the resulting supernatant was collected and dried using a centrifugal vacuum concentration system (Savant DNA SpeedVac) set on low heat. Standard solutions were prepared that contain a mixture of L-tryptophan and kynurenine at 50, 25, 12.5, 6.25 and 1.25 μ M each. Duplicate standards at each concentration were subjected to the same extraction treatment as described for the experimental samples. The dried extracts were stored at -20°C until time of HPLC analysis. The dried extracts were reconstituted in 100 μ l of deionized-distilled water, and transferred to HPLC sample vials. We performed HPLC using a Beckman HPLC system equipped with an autosampler (Model 508), a pumping station (Model 126A), and a flow cell absorbance detection unit (Model 166). The sample (20 μ l) was injected into a C18 column (Luna™ C18(2), 250 x 4.6 mm, 5 μ m; catalog #00G-4252-E0, Phenomenex, Torrance, CA), eluted with a linear gradient of water:acetonitrile (0 to 80% acetonitrile) over 20 min, and detected at 254 nm. For specific clones, we cultured transfectants for four days in T25 flasks (5×10^6 cells per flask) containing culture media (without supplemental tryptophan), and then measured tryptophan and kynurenine levels in the cell culture supernatant as described above.

Cell culture conditions before purine exposure

IDO (clone 101)- and control (Vo)-transfected RAW 264.7 macrophages were maintained in 80 cm² vent-cap tissue flasks (Nunc #178891) in an incubator at 37°C, 95% relative humidity, and 5% carbon dioxide. The culture media contained 90% (v/v) Iscove's Medium Modified (Cellgro #15-016-CV), 10% (v/v) fetal bovine serum (Biowhittaker #14501-F), 100 i.u. /ml penicillin plus 100 μ g/ml streptomycin (Cellgro # 30-002-CI), and 200 μ g/ml G418

(Cellgro #61-234-RG). The media was replenished every day because the IDO-transfected cells rapidly deplete tryptophan from the media.

We used a cell scraper to detach the cells from the flask, re-suspended the cells in media, and then plated the cell suspension (0.5 ml of suspension per well $\approx 10^5$ cells) into 48-well tissue culture plates (Costar #3548). One day after plating, the cells were exposed to the media alone (no purine), or media containing 100 μ M purine (xanthine or hypoxanthine) as detailed below.

In situ superoxide anion generation

Xanthine oxidase (EC 1.1.3.22) is a ubiquitous intracellular enzyme that catalyzes two steps in purine (e.g., hypoxanthine, xanthine) degradation; in addition to hydrogen peroxide, these reactions generate superoxide anions when the purine concentration is 100 μ M [61, 62]. We dissolved the hypoxanthine (Sigma #H-0377) or xanthine (Sigma #X-0125) in cell culture media, and then control- and IDO transfected RAW 264.7 macrophages (10^5 cells/well) were maintained for 48 hours in media alone or in media that contained purine (100 μ M, 0.5 ml per well). We replenished the media (with or without purine) after 24 hours to maintain the availability of purine (xanthine oxidase substrate) [61] and tryptophan (IDO substrate). In addition, the Iscove's Medium Modified media, which contains 16 mg/L tryptophan, was supplemented with additional tryptophan to 40 mg/L tryptophan to assure that IDO activity was not limited because of insufficient tryptophan (substrate).

Preparation of cell lysates for protein assay and ELISA

After 48 hours in media alone or purine-containing media, the culture media was aspirated and the cells were washed twice with 0.5 ml of phosphate-buffered saline (PBS, pH 7.4). We completely removed the last PBS wash, and added 0.2 ml of Milli-Q water to osmotically lyse the cells. The 48-well plate was kept in a freezer at -80°C for at least an hour before thawing and mixing plate contents at 40°C in a Jitterbug™ microplate incubator-mixer (Boekel Scientific, Feasterville, PA). We repeated the freezing, thawing, and mixing steps one or two more times until the cells were completely lysed as determined by visual examination using light microscopy. The cell lysates from each well of the 48-well plate were stored at -80°C until the lysates were used.

Protein assay on cell lysates

We used the Bio-Rad Protein Assay (Bio-Rad #500-0006) according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA). Briefly, 10 μ l of cell lysate or protein standard (bovine serum albumin, 0-1 mg/ml in water) was mixed with 200 μ l of diluted Bio-Rad dye (1 part dye concentrate: 3 parts Milli-Q water). We prepared the assay in 96-well polystyrene plates (Fisher #12-565-50), and measured the absorbance of each well at 590 nm using a SPECTRAFluor Plus microplate reader (Tecan US, Research Triangle Park, NC). Standard curves and protein concentrations were determined using WinSeLecT Microplate Reader Control & Data Analysis Software (version 2.2, Tecan U.S. Inc., Research Triangle Park, NC).

ELISA for nitrated proteins

RAW 264.7 macrophages express nitric oxide synthase [63], which generates nitric oxide that may subsequently react with superoxide anions to form peroxynitrite, which can nitrate proteins on cysteine, methionine, tyrosine, and tryptophan residues [64]. We developed an ELISA for nitrated proteins by modifying the direct ELISA described in "Current Protocols in Molecular Biology" [65]. An aliquot (5.0 μg protein) of each cell lysate was diluted in 100 μl of coating buffer (50 mM sodium carbonate, pH 9.5), and the mixtures were transferred to a 96-well Nunc Maxisorp[®] plate (Nunc #446469). We sealed the plate with Parafilm[®], and incubated the plate overnight at 4-6°C in a humidified chamber. The next morning we washed the wells five times with 300 μl of PBS. The wells were blocked (1.5 hours at 20-25°C) using 300 μl of PBS containing 1% (w/v) nonfat dry milk plus 0.05% (v/v) Tween 20. We washed the wells five times with 300 μl of PBS, and then incubated with 100 μl /well of rabbit anti-nitrotyrosine antibody (Upstate Biotechnology #06-284; diluted to 1 $\mu\text{g}/\text{ml}$ in blocking buffer, 2 hours room temp). We removed the anti-nitrotyrosine antibody and rinsed the wells five times with 300 μl of PBS. Horseradish peroxidase-conjugated donkey anti-rabbit antibody (Jackson Immunochemicals #711-035-152; diluted 1:20,000 in blocking buffer; 100 μl /well) was added to the rinsed wells, the plate was sealed with Parafilm[®], and kept at room temperature for one hour. After rinsing the wells five times with 300 μl PBS, the HRP-conjugated secondary antibody was detected using 3,3',5,5'-tetramethylbenzidine (TMB) substrate mix (Kirkegaard & Perry #50-76-00; 100 μl /well for 20 minutes); HRP activity converts the TMB to a blue product. We stopped (quenched) the TMB reaction by adding 100 μl of 1 M phosphoric acid, which caused the color to change from blue to yellow. The plate was shaken for 1-2 minutes in a Jitterbug[™] before measuring the absorbance (450 nm) in each well using a SPECTRAFluor Plus plate reader. The absorbance values (corrected for the quantity of protein per well) are reported as mean (N=4) \pm standard error of the mean (s.e.m.).

ELISA for protein carbonyls

Oxidative damage to proteins may introduce carbonyl groups into proteins [44]. To detect protein carbonyls, the carbonyl groups are reacted with 2,4-dinitrophenylhydrazine (DNP), which forms 2,4-dinitrophenylhydrazone (DNPH)-derivatized protein. DNPH-derivatized proteins can be measured spectrophotometrically, by HPLC, or immunochemically with anti-DNP antibodies [66]. We modified the method from Buss *et al.* [67] to quantitate protein carbonyls in RAW 264.7 cell lysates. We prepared a solution containing 10 mM DNP (Sigma #D-2630) in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer (pH 2.5), and then added 5 μl of the DNP solution to 1.5 ml tubes containing 5 μl of cell lysate (~2.5 μg protein). The tubes were gently rocked at room temperature for one hour, before adding 200 μl of coating buffer (10 mM sodium phosphate buffer containing 140 mM NaCl, pH 7.0) to each tube.

Two hundred μl of each mixture were added to the wells of a 96-well Maxisorp[®] ELISA plate (Nunc #446469). We maintained the plates overnight at 4-6°C in a humidified chamber, and then rinsed the wells five times with 300 μl PBS before and after blocking with 300 μl of blocking buffer. We added 100 μl of anti-DNP antibody solution (Sigma #D8406, clone SPE-7, diluted 1:1,000 in blocking buffer) to each well, covered the plate with Parafilm[®], and maintained the plate for two hours at room temperature. After rinsing the wells five times with 300 μl of PBS, we detected the anti-DNP antibody with an HRP-conjugated, donkey anti-mouse, secondary antibody (Jackson #715-035-151, diluted 1:20,000) and TMB substrate (same as above).

Measurement of superoxide anions

Vector only (Vo)- and IDO transfected RAW 264.7 macrophages were plated in 96-well tissue culture plates (Costar #3595) at \sim 20,000 cells per well in 0.1 ml culture media per well. The media contained 90% (v/v) Iscove's Medium Modified (Cellgro #15-016-CV), 10% (v/v) fetal bovine serum (Biowhittaker #14501-F), 100 i.u./ml penicillin plus 100 $\mu\text{g}/\text{ml}$ streptomycin (Cellgro # 30-002-CI), and 200 $\mu\text{g}/\text{ml}$ G418 (Cellgro #61-234-RG). We added fresh media daily, because the IDO-transfectants rapidly depleted tryptophan from the media. Three days after plating, we incubated (48 hours total) the cells in tryptophan-rich (40 mg/L) culture media (100 $\mu\text{l}/\text{well}$), or media (100 $\mu\text{l}/\text{well}$) containing 100 μM hypoxanthine or 100 μM xanthine. The appropriate media was replenished after 24 hours, and the assay for superoxide anions was carried out 24 hours later.

Superoxide anions were measured using the ferricytochrome C reduction assay described by Quick *et al.* [62]; superoxide anions reduce ferricytochrome C, which is measured as an increase in absorbance at 550 nm. Note that we measured steady-state levels of superoxide anions (one time point after 48 hours purine exposure); we did not determine the contribution of superoxide oxide dismutase (SOD) activity on the steady-state superoxide anion measurements. We prepared a 150 μM stock solution of ferricytochrome C (horse heart cytochrome C, Sigma #C-2505) in culture media, and added 50 μl of this stock to cultures that had been exposed to media, hypoxanthine, or xanthine in 96-well plates. The final concentration of ferricytochrome C was 50 μM , since the wells contained 0.1 ml of culture media before adding 50 μl of ferricytochrome C stock (150 μM). The 96-well plates were gently shaken to mix the contents of the wells, and then incubated in a cell culture incubator for 30 minutes. The reduced ferricytochrome C was detected at 550 nm on a SPECTRAFluor Plus plate reader.

Statistical analyses

Statistical analyses (ANOVA and unpaired t-test with Welch correction) were done using GraphPad InStat version 3.02 for Windows 95, GraphPad Software, San Diego, CA (www.graphpad.com).

RESULTS

IDO-transfected cells express IDO mRNA and IDO protein

RAW 264.7 macrophages were transfected with an IDO expression vector or empty (Vo) vector, and then analyzed for IDO RNA, IDO protein, and IDO activity. We isolated and archived several clones (data not shown) that exhibited varying levels of IDO expression and activity. Clone 101, which had the highest IDO activity, was used in the experiments reported here. Fig. 2A shows that IDO mRNA (750 bp IDO band) was detected in clone 101 and that the vector only (Vo)-clone was negative for IDO expression. Furthermore, Western blot analysis (Fig. 2B), using an anti-IDO polyclonal antibody, demonstrated the presence of IDO protein (~42-45 kD band) in lysates of IDO-transfected clone 101, while IDO protein was undetectable in Vo-transfectants.

IDO-transfected cells have measurable IDO activity

IDO activity consumes tryptophan and generates kynurenine; therefore, we used HPLC analyses to measure tryptophan and kynurenine levels in cell culture supernatants from Vo- and IDO-transfectants. Cells with functional (enzymatically-active) IDO should deplete tryptophan from the media, and exhibit commensurate increase in kynurenine (the product of IDO activity) in the media. Cells that lack functional IDO should have measurable tryptophan levels in the cell culture supernatant and trace to no kynurenine. Fig. 2C (trace 1) shows the chromatogram for a standard mixture (authentic compounds) containing tryptophan and kynurenine. The chromatogram in Fig. 2C (trace 2) shows that cell-free culture media contains tryptophan (note the peak alignment with the tryptophan standard), undetectable kynurenine, and a non-specific peak (*). This peak (*) is probably a component of serum and is also present in cell culture supernatants (Fig. 2C, trace 3 and 4). We assessed IDO activity (tryptophan depletion and kynurenine formation) in Vo-transfected cells and IDO-transfected clone 101 by measuring the amount of tryptophan and kynurenine remaining in the cell culture supernatant after four days in culture without supplemental tryptophan (media initially contained tryptophan at 16 mg/L). Fig. 2C (trace 3) shows the chromatogram resulting from the analysis of the cell culture supernatant from Vo-transfected cultures; note the presence of a tryptophan peak and a non-quantifiable kynurenine peak. Cell culture supernatant from Vo-transfected cultures contained tryptophan (Fig. 2D, condition Vo, tryptophan bar) and no measurable kynurenine (Fig. 2D, condition Vo, **). In contrast, HPLC analysis of the IDO-101 supernatant (Fig. 2C, trace 4) shows a prominent kynurenine peak and no tryptophan peak. After four days in culture, IDO-101 cells had depleted all of the tryptophan from the media (see Fig. 2D, condition 101, ***) and formed kynurenine (Fig. 2D, condition 101, kynurenine bar). These results demonstrate that IDO-transfected clone 101 has functional (enzymatically active) IDO, whereas the Vo-transfected cells have undetectable IDO activity.

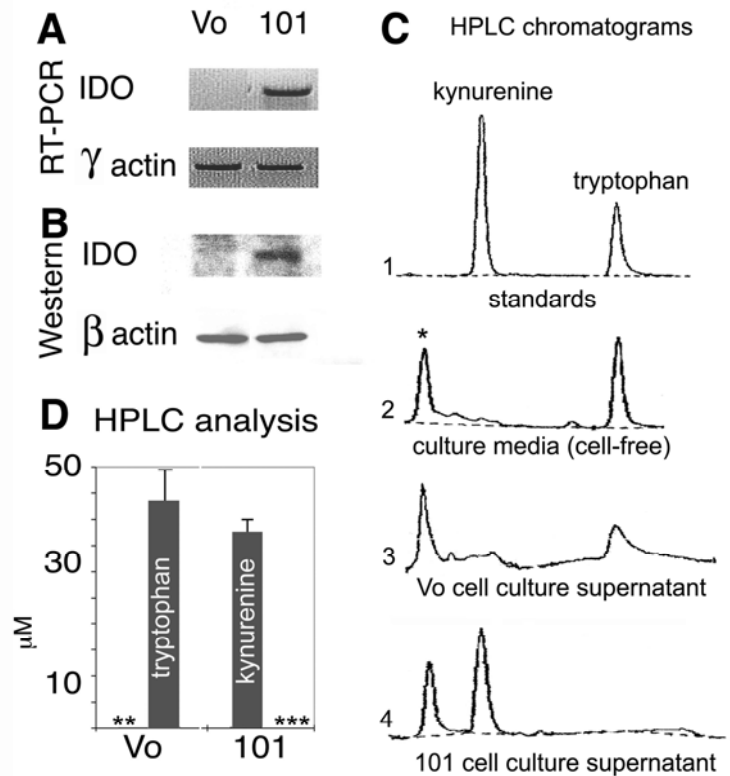


Fig. 2. RAW 264.7 macrophages transfected with an IDO expression vector (clone 101) or empty vector (Vo) were analyzed for (A, B) IDO expression and (C, D) IDO activity (conversion of tryptophan to kynurenine). (A) RT-PCR analysis of total RNA isolated from transfectants detected IDO mRNA in clone 101, but not in Vo-transfectants. Loading control was γ -actin. (B) Western blot analysis detected IDO protein in cell lysates from IDO-transfectant (clone 101), but not in Vo-transfectants. Loading control was β -actin. (C) IDO activity converts tryptophan to kynurenine; HPLC analyses of tryptophan and kynurenine in cell culture supernatants show IDO activity in IDO-101 transfected cells, but not in Vo-transfected cells. HPLC chromatograms from analysis of: (C1) standard containing kynurenine and tryptophan; (C2) cell-free culture media showing presence of tryptophan in the media, the absence of kynurenine, and non-specific peak (*) that is media specific (* also present in C3 and C4); (C3) cell culture media from Vo-transfected cultures shows no kynurenine peak and presence of tryptophan peak; (C4) cell culture supernatant from IDO-expressing clone 101 reveals that tryptophan has been converted to kynurenine. (D) Quantitative data for IDO enzyme activity corresponding to chromatogram 2C and 2D. We cultured transfectants (5×10^6 cells) for four days, before measuring kynurenine and tryptophan in cell culture supernatants. Cell culture supernatant from Vo-transfected cells contained only tryptophan and no detectable kynurenine (**). IDO-clone 101 degraded all available tryptophan (undetectable ***) to kynurenine.

Similar protein carbonyl levels in control- and IDO-transfected cells

We exposed vector only (Vo-) and IDO-transfected cells to purine levels that should elevate superoxide anions (*via* cellular xanthine oxidase activity) [61, 62]. In the presence of media only or hypoxanthine-containing media, RAW 264.7 macrophages that are stably transfected with an IDO expression vector (Fig. 3, black bars) and Vo-transfectants (Fig. 3, white bars) have comparable levels of protein carbonyls. However, after 48 hours in xanthine-containing media, the relative level of protein carbonyls was significantly less ($p = 0.032$) in IDO-transfectants compared to Vo-transfectants (Fig. 3, +xanthine condition, note that black bars are smaller than white bars).

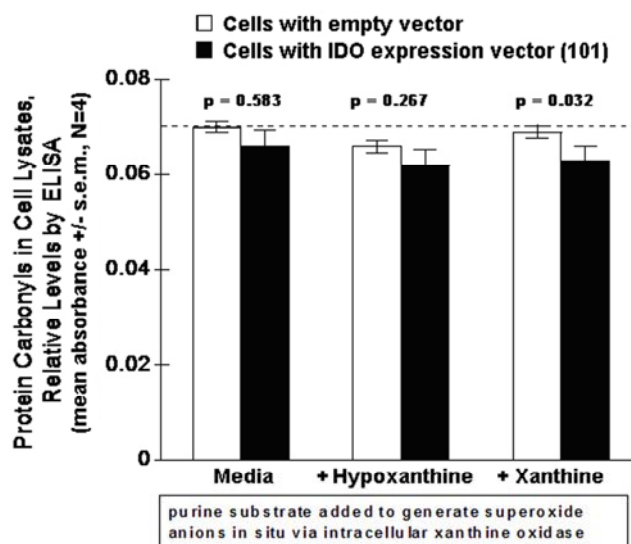


Fig. 3. Comparable levels (p -values above each pair) of protein carbonyls in cell lysates from RAW 264.7 macrophages that overexpress IDO (black bars) compared to cells that do not express IDO (white bars). IDO-transfected cells (clone 101) and vector-transfected (Vo) cells incubated (48 hours) in culture media or media containing 100 μ M purine (hypoxanthine or xanthine). We derivatized protein carbonyls (total cell lysate, ~ 2.5 μ g protein) with dinitrophenylhydrazine (DNP), and applied derivatized lysate to Maxisorp ELISA plate. DNP-derivatized protein detected with: anti-DNP antibody; HRP-conjugated 2^o antibody; TMB (HRP substrate). We acidified the blue product and measured absorbance (450 nm) of yellow product. Higher absorbance \approx more protein carbonyls. For each condition (media, + hypoxanthine, or + xanthine), we used a 2-tailed t-test with Welch correction to compare mean absorbance for IDO-transfectants (black bars) to mean absorbance of Vo-transfectants (white bars).

Decreased nitrated protein levels in IDO-transfected cells

RAW 264.7 macrophages express nitric oxide synthase, which generates nitric oxide [63]. Nitric oxide can react with superoxide anion to form peroxynitrite, which may nitrate (oxidatively modify) protein side-chains [64]. Compared to

Vo-transfectants, cells that were stably transfected with the IDO expression vector had lower levels of nitrated proteins (Fig. 4, see black bars smaller than white bars), particularly after treatment with hypoxanthine ($p = 0.013$) or xanthine ($p = 0.025$). One-way analysis of variance (ANOVA) indicates that protein nitration was similar ($p = 0.529$) in IDO-transfected cells incubated in media, hypoxanthine, or xanthine (Fig. 4, compare the three black bars), consistent with the idea that IDO may protect against superoxide anion-dependent oxidative stress. For Vo-transfected cells, there was a trend toward increased protein nitration after purine treatment; however, this increase was not statistically significant (Fig. 4, compare the three white bars; ANOVA, $p = 0.281$).

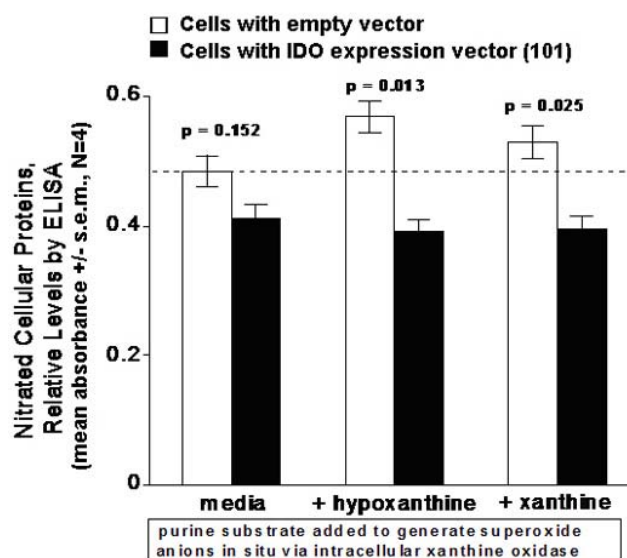


Fig. 4. Cell lysates from RAW 264.7 macrophages that overexpress IDO (black bars) have significantly lower levels (p -values above each pair) of nitrated proteins compared to cells that do not express IDO (white bars). IDO-transfected cells (clone 101) and vector-transfected (Vo) cells incubated (48 hours) in culture media only or media plus 100 μ M purine (hypoxanthine or xanthine). Applied cell lysates (5 μ g protein/well) to Maxisorp ELISA plate. Nitrated proteins detected with: anti-nitrotyrosine antibody; HRP-conjugated 2^o antibody; TMB (HRP substrate). We acidified blue product and measured absorbance (450 nm) of yellow product. Higher absorbance \approx more nitrated proteins. For each condition (media, + hypoxanthine, or + xanthine), we used a 2-tailed t-test with Welch correction to compare mean absorbance for IDO-transfectants (black bars) to Vo-transfectants (white bars).

Lower levels of superoxide anions in IDO-transfected cells

We used a two-tailed, unpaired test with the Welch correction to compare mean superoxide anion levels in IDO- and Vo-transfected cells. Steady-state levels of

superoxide anions were significantly lower in cultures of IDO-transfected cells (Fig. 5, black bars) compared to Vo-transfected cells (Fig. 5, white bars) for each culture condition: no purine ($p = 0.029$), with hypoxanthine ($p = 0.021$), with xanthine ($p = 0.031$).

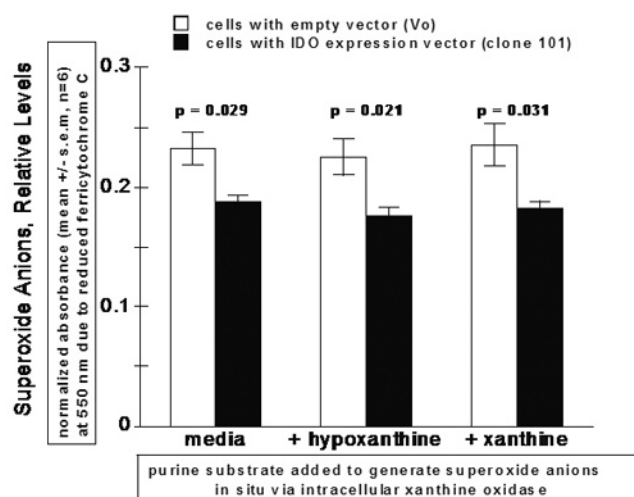


Fig. 5. Levels of superoxide anions are significantly lower (p -values above each pair) in cultures of RAW 264.7 macrophages that overexpress IDO (black bars) compared to cells that do not express IDO (white bars). IDO-transfected cells (clone 101) and vector-transfected (Vo) cells incubated (24 hours) in culture media or media containing 100 μ M purine (+ hypoxanthine or + xanthine). We added ferricytochrome C (to 50 μ M), incubated 37°C for 30 minutes, and measured absorbance (550 nm). Superoxide anions reduce ferricytochrome C; reduced ferricytochrome C absorbs at 550 nm. For each condition (media, hypoxanthine, or xanthine), a 2-tailed t-test with Welch correction was used to compare mean absorbance for IDO-transfectants (black bars) to Vo-transfectants (white bars).

DISCUSSION

To the best of our knowledge, this is the first report that: (1) describes the generation of RAW 264.7 macrophages that constitutively express, enzymatically active IDO; (2) indicates that IDO-expressing cultures have lower steady-state levels of superoxide anions compared to cultures that do not express IDO; (3) shows evidence for diminished protein oxidation in IDO-expressing macrophages compared to macrophages that do not express IDO. It remains to be determined whether IDO expression would lower superoxide anion levels and minimize protein oxidation in other cell types. In addition, it was beyond the scope of the present study to determine whether IDO over-expression directly affects other pro-oxidant or antioxidant systems enzyme systems either directly

or indirectly. In future studies, it would be informative to determine whether IDO activity alters other ROS-generating or ROS-scavenging pathways.

We expected to see much higher levels of superoxide anions and subsequent oxidative damage in vector only (Vo)-transfectants after exposure to purines, since under our experimental conditions superoxide anions (rather than hydrogen peroxide) are supposedly a product of xanthine oxidase catalyzed oxidation of xanthine or hypoxanthine [61, 62]. In our protocol, superoxide anion formation depended exclusively upon the presence of intracellular (endogenous) xanthine oxidase activity, which is present in all cells. It is conceivable that the addition of exogenous xanthine to the cell cultures may have produced higher levels of superoxide anions and consequent oxidative damage.

Nonetheless, it appears that protein nitration is diminished in cells that constitutively express IDO. While our data do not show a direct cause and effect relationship between IDO activity and oxidative damage, our results are consistent with the postulate that IDO activity may have an antioxidant function [11, 25-27]. IDO activity could mitigate oxidative damage by multiple mechanisms: (1) consuming superoxide anions [68, 69], which should limit the formation of other ROS; (2) generating downstream products, including 3-hydroxykynurenine and 3-hydroxyanthranilic acid, which are free radical scavengers [11, 26, 70, 71]; (3) increasing cellular NAD levels, which indirectly helps to maintain cell viability [72, 73]. RAW 264.7 macrophages probably possess the enzymes required make 3-hydroxykynurenine, 3-hydroxyanthranilic acid and NAD [73, 74]; therefore, we cannot dismiss the potential contribution of those molecules to the apparent antioxidant effect of IDO.

Activated macrophages are a major source of superoxide anions [75-77]; it is possible that macrophage IDO activity curbs superoxide anion production, thereby minimizing oxidative damage to macrophages and nearby cells. We predict that cells or tissues that express IDO are less vulnerable to superoxide anion-dependent oxidative damage, and that the absence, and possibly the inhibition of IDO activity, may predispose cells to superoxide anion-dependent oxidative stress. IDO is a cytosolic enzyme that consumes superoxide anions, an activity that may be functionally analogous to cytosolic Cu/Zn superoxide dismutase (SOD1) [78, 79], which degrades superoxide anions to hydrogen peroxide [79-81]. We speculate IDO and SOD1 are functionally similar systems that mitigate superoxide anion-dependent oxidative damage, and that IDO and SOD1 expression are cell type and circumstance specific. It would be interesting to determine the relative distribution and activity of SOD1 and IDO in various tissues, especially during oxidative stress.

Curiously, IDO activity is present in many non-lymphoid tissues including lung [69, 82], intestine [2], placenta [15, 83], stomach [84]; kidney [85]; brain [86, 87], lens of the eye [88], and cultured cells [17, 89-91]; unfortunately, there are limited studies that describe the immunohistochemical localization of IDO or the physiological function of IDO in those non-lymphoid tissues. We speculate that

the major function of non-macrophage IDO activity may be to mitigate superoxide anion-dependent oxidative damage to cellular macromolecules.

CONCLUSIONS

The work presented here and the work of others [25-27, 72, 92] suggests that IDO may be an antioxidant enzyme. We postulate that IDO dysfunction may contribute to the oxidative stress that occurs in an array of diseases and disorders, and suggest that the antioxidant role of IDO should be investigated more thoroughly.

Acknowledgements. We thank Patricia F. Toole and Anita Wylds for technical assistance, Ted Johnson for the optimization of HPLC methods for tryptophan and kynurenine detection and Linda Clayton for critical reading of this manuscript. This work was supported by funding from multiple agencies. Gearhart: NIH #F32NS10723, Department of Veterans Affairs, and Combined Intramural Grants Program (Medical College of Georgia) Mellor: NIH #R01AI044219 and #R01HD041187.

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