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Nitric Oxide Inhibits Indoleamine 2,3-Dioxygenase Activity in Interferon-γ Primed Mononuclear Phagocytes*

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Indoleamine 2,3-dioxygenase (IDO) and nitric oxide synthase are part of the anti-tumor and antimicrobial activities of mononuclear phagocytes induced by interferon-γ (IFNγ). As IDO is a heme-containing enzyme and NO, the product of nitric oxide synthase-initiated arginine degradation, is a regulator of heme enzymes, we investigated whether NO is capable of modulating IDO activity in IFNγ-primed mononuclear phagocytes. Authentic NO gas or the NO-generating compound, diethylamine dinitric oxide adduct, dose-dependently inhibited IDO activity in cell lysates prepared from IFNγ-primed human peripheral blood mononuclear cells, as assessed by the ascorbate/methylene blue assay for IDO. In contrast, neither nitrite nor nitrate affected IDO activity. Exposure of intact IFNγ-primed human peripheral blood mononuclear cells or monocyte-derived macrophages to any of the NO-generating compounds, sodium nitroprusside, glyceryl trinitrate, S-nitroso-N-acetylpenicillamine, or diethylamine dinitric oxide adduct, resulted in inhibition of both the consumption of tryptophan from and formation of its metabolite, kynurenine, in the culture medium. The observed inhibition of IDO activity was not due to toxicity of the NO generators and was abrogated by the co-addition of oxyhemoglobin, an antagonist of NO function. Comparable concentrations of nitrite or nitrate did not inhibit IDO activity in intact cells. In contrast to human cells, addition of IFNγ to murine macrophages, cultured in complete RPMI 1640 medium, readily induced nitric oxide synthase. Others have reported that such treatment does not induce IDO activity in these cells. However, induction of IDO activity was observed in murine macrophages when the synthesis of reactive nitrogen species was inhibited, by using arginine-free medium and/or the nitric oxide synthesis inhibitor, N^G-monomethyl-L-arginine. Together, these results demonstrate that both exogenous and endogenous NO inhibit IDO activity and that oxidative arginine and tryptophan metabolism in IFNγ-primed mononuclear phagocytes are functionally related. Our study thereby provides an insight into how these cells may regulate some of their antimicrobial and anti-tumor activities.

Interferon-γ (IFNγ),[1] a key mediator of inflammation produced by activated T-cells, modulates the antimicrobial and anti-tumor activities of monocytes and macrophages (1). IFNγ is known to prime mononuclear phagocytic cells for enhanced production of reactive oxygen and nitrogen species (1) as well as to induce a number of enzymes involved in the tumoricidal and bacteriocidal activities of these cells (1–3). Among the latter are indole 2,3-dioxygenase (IDO; EC 1.13.11.17) (4–6) and nitric oxide synthase (EC 1.14.23) (3), the initial and rate-limiting enzymes of tryptophan and arginine degradation pathways respectively.

The ubiquitous IDO catalyzes the oxidative cleavage of the pyrrole ring of tryptophan and several other indoleamines to form *N*-formylkynurenine, which itself decomposes to kynurenine and formic acid. Purified IDO has been shown to require superoxide anion radical ($O_2^{\cdot-}$) as a substrate and co-factor for maximal activity (7, 8); however, whether this applies to IDO activity within intact cells remains unknown. Exposure of *human* mononuclear phagocytes and a wide variety of other normal or neoplastic mammalian cells to IFNγ *in vitro* causes induction of IDO (Fig. 1) (5, 9, 10). While in most cells such induction results in formation of kynurenine, only *human* mononuclear phagocytes further metabolize the kynurenine to 3-hydroxyanthranilic acid (9) and quinolinic acid (11) (Fig. 1). For reasons presently not understood, the cells release these tryptophan metabolites into the culture medium (9). Elevated levels of tryptophan metabolites have been demonstrated in experimental animals and human patients suffering from a variety of inflammatory diseases or treated with interferon or interferon inducers (see *e.g.* Refs. 12–14), indicating that oxidative tryptophan metabolism along the kynurenine pathway is operative *in vivo* under these conditions. Increasing evidence suggests that the antimicrobial and anti-proliferative activity of IFNγ in *human* cells *in vitro* is at least partly due to induction of IDO within the target cell and the resulting depletion of tryptophan, the least available essential amino acid (15). For example, the growth inhibitory activity of IFNγ on several obligate intracellular parasites within human macrophages or fibroblasts is overcome by supplementing the medium with tryptophan (4, 16). Also, introduction of sense but not antisense IDO cDNA into a mouse cell line resulted in the cells' ability to deplete the medium of tryptophan, and this was associated with their ability to inhibit the growth of *Toxoplasma gondii* (17). Furthermore, cell lines with reduced IDO activity show reduced antimicrobial response to intracellular parasites (18). Because aminophenolic tryptophan metabolites produced along the kynurenine pathway are powerful antioxidants, induction of IDO in mononuclear phagocytes has also been suggested to represent a local extracellular antioxidant defense (13, 19), perhaps to protect host tissue from inadvertant oxida-

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[1] The abbreviations used are: IFNγ, interferon-γ; DEANO, diethylamine dinitric oxide adduct; GTN, glyceryl trinitrate; IDO, indoleamine 2, 3-dioxygenase; LPS, lipopolysaccharide; MDM, monocyte-derived macrophages; NMA, N^G-monomethyl-L-arginine; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; SNP, sodium nitroprusside; SNAP, *S*-nitroso-*N*-acetylpenicillamine; OxyHb, oxyhemoglobin.

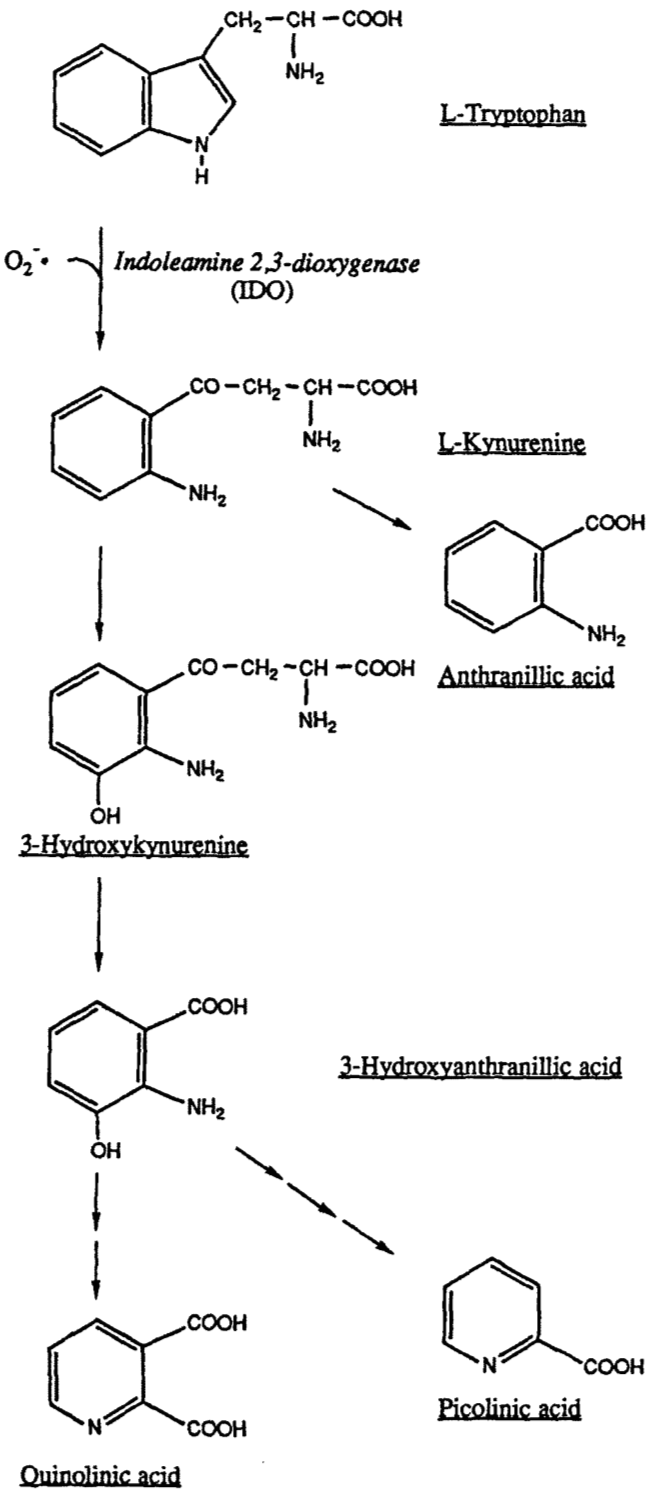

FIG. 1. **IFNγ-mediated induction of the L-tryptophan degradation pathway in mononuclear phagocytes.**

tive damage that could occur during inflammatory conditions.

Nitric oxide synthase catalyzes the formation of the nitric oxide radical (NO) from the terminal guanidino nitrogen of L-arginine (20). Murine macrophages synthesize reactive nitrogen species (including NO, nitrite, and nitrate) subsequent to exposure to IFNγ alone or in combination with microbial products (*i.e.* LPS) or tumor necrosis factor *in vitro* (3, 21). Degradation of arginine and concomitant reactive nitrogen species production are associated with the IFNγ-mediated anti-microbial and anti-tumor activities of *murine* macrophages, rather similar to the aforementioned tryptophan degradation in *human* macrophages *in vitro*. Indeed, inhibition of NO synthesis by either depletion of the culture medium of L-arginine or use of nitric oxide synthase inhibitors decreases the IFNγ-mediated microbicidal and tumoricidal activities of murine macrophages (22, 23). NO is thought to exhibit cytostatic and cytotoxic effects via its ability to inhibit iron- and iron/sulfur-containing enzymes involved in respiration and DNA synthesis (reviewed in Ref. 3). Furthermore, NO may react with O_2^{-}, a product of the macrophages respiratory burst, to form peroxynitrite which itself is cytotoxic (24). In addition to its cytotoxic activity, NO has a role as a diverse biological messenger (25).

A puzzling finding has been that in *human* PBMC (26) and macrophages (9) IFNγ or IFNγ/LPS potently induces IDO activity but not nitric oxide synthase activity (27) while in *murine* macrophages IFNγ or IFNγ/LPS potently induces nitric oxide synthase but not IDO activity (16). IFNγ-induced arginine and tryptophan metabolism have been suggested to be functionally related in activated mononuclear phagocytes. Thus, high concentrations of added picolinic acid, a product that can be formed from tryptophan (Fig. 1), has been demonstrated to modulate the catabolism of arginine to reactive nitrogen species in murine macrophages *in vitro* (28). However, additional factors may regulate nitric oxide synthase- and IDO-initiated degradative pathways in these cells.

As IDO is a cytosolic enzyme containing heme iron for which NO has a high affinity (29), we investigated if NO was capable of modulating IDO activity. We show here that exogenous and endogenous NO inhibit IDO activity in IFNγ-primed human and murine mononuclear phagocytes, in support of a possible regulatory role for NO on IDO-initiated tryptophan metabolism.

EXPERIMENTAL PROCEDURES

Materials—Kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, EDTA, and lipopolysaccharide (LPS) were obtained from Sigma. L-Tryptophan and chloroacetic acid were from Merck; L-arginine, ascorbic acid, and methylene blue were from Aldrich; sodium nitrite and nitrate from BDH; and N^G-monomethyl-L-arginine acetate (NMA) from Calbiochem. Oxyhemoglobin (OxyHb) was prepared by reduction of mouse hemoglobin (Sigma) with sodium dithionite (Merck) as previously described (30). Human recombinant IFNγ (rh-IFNγ) was from Boehringer Mannheim, while recombinant murine IFNγ (rm-IFNγ) was generously donated by Boehringer Ingelheim Pty. Ltd. and given to us by Dr. W. Jessup (Heart Research Institute). RPMI 1640 (powder) and phosphate-buffered saline were obtained from Sigma, while Ham's F-10 medium was from Life Technologoies, Inc. LiquiPure water (MODULAB) was used for all buffers and aqueous solutions which were subsequently treated with Chelex 100 (Bio-Rad) to remove contaminating transition metals. Acetonitrile (HPLC quality) was from Mallinckrodt, while Microspin ultrafilters (10,000 molecular weight cut-off) were from Activon.

NO and NO Generators—Nitric oxide gas (99% purity) (CIG, Australia) was obtained from Matheson Gas products (Dorsey, MD). Saturated NO solutions (≈2 mM) were prepared on ice, in vials sealed with a rubber septum, by bubbling authentic NO gas through three in-line gas washing bottles containing saturated solutions of NaOH (to remove traces of NO_2^{-} and N_2O_3), and then through argon-deoxygenated water for 30 s at room temperature. Aliquots were removed anaerobically using a hypodermic syringe and added directly to the IDO assay reaction mixture to give the indicated final NO concentrations. Sodium nitroferricyanide dihydrate (sodium nitroprusside, SNP) was from Aldrich, while glyceryl trinitrate (GTN) was purchased from David Bull Laboratories, Victoria, Australia. Diethylaminodinitric oxide adduct sodium salt (DEANO) was synthesized as described previously (31, 32) with minor modifications. Briefly, an anhydrous diethylamine diethyl ether solution was flushed with N_2 and cooled to −78 °C (ethanol dry ice). Nitric oxide gas was slowly added to the reaction mixture, and the colorless product that precipitated from the solution was filtered off after stirring the reaction mixture for 10 h. The precipitate was washed with dry ether and finally resuspended in dry ether. A slightly less than stoichiometrical amount of NaOEt in EtOH was added and the solution concentrated until it became clear. Following filtration, the solution was

placed into ice-cold ether, the resulting colorless crystals filtered, washed with ether and chloroform, and found to be 99.9% pure DEANO by UV spectroscopy (33). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was synthesized using methods previously described (34, 35).

Isolation and Culture of Human PBMC and Monocyte-derived Macrophages—PBMC from white blood cell concentrates ("buffy coats"; kindly provided by the N.S.W. Red Cross Blood Transfusion Service, Sydney) or freshly collected blood were isolated by density-centrifugation on Lymphoprep (NYCOMED, Norway) and cultured as previously described (19). PBMC preparations consisted of ≈20% monocytes and 80% lymphocytes. For preparation of monocyte-derived macrophages (MDM), monocytes were first purified from PBMC (isolated from buffy coat) using counter-flow centrifugal elutriation and cultured as described previously (19, 36).

For cellular experiments involving addition of NO generators to IFNγ-primed PBMC and MDM, cells were cultured in tryptophan-supplemented (≈70–100 μM) Ham's F-10 medium. Human plasma concentration of tryptophan is ≈50 μM. F-10 medium was used for these experiments as it does not contain proteins that could possibly scavenge the NO generated. PBMC and MDM were primed for 18–24 h with 250 units/ml of rh-IFNγ before exposure to various concentrations of the indicated NO generator for a further 12 or 24 h, after which time the levels of nitrite and tryptophan and its kynurenine pathway metabolites were determined in the culture medium. IDO activity is defined in these experiments as the level of tryptophan consumed from the culture medium, and/or the level of kynurenine formed. In some experiments, NO generators were co-incubated with OxyHb (10 μM). Cell viability was assessed by trypan blue exclusion.

Isolation and Culture of Resident Peritoneal Macrophages—Resident murine peritoneal macrophages were isolated from Quackenbush-Swiss (QS) strain mice as previously described (37). Cells were cultured in 6-well tissue culture plates (35-mm diameter wells; Costar) at a concentration of 5×10^6 cells/well in 2 ml of tryptophan- and arginine-free RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum and where indicated, with L-tryptophan (≈100 μM) and L-arginine (≈1.2 mM; *i.e.* the standard concentration of L-arginine in RPMI 1640 medium). The concentration of arginine in human plasma is ≈80 μM. Cells were activated by the addition of rm-IFNγ (500 units/ml) and LPS (2.5 μg/ml), either alone or in combination. Where indicated, cells were cultured in the presence of the nitric oxide synthase inhibitor NMA (100 μM). Controls consisted of cells not treated with IFNγ or LPS. The levels of tryptophan and its kynurenine pathway metabolites and nitrite present in the culture medium were determined after 48 h of incubation.

IDO Assay—IDO activity was assessed using the ascorbate/methylene blue assay (38) and cell lysates prepared from IFNγ-primed (250 units/ml for 48 h) human PBMC, cultured in RPMI 1640 medium supplemented with 10% heat-inactivated human serum and L-tryptophan (≈100 μM), as the source of the enzyme. Cells (5×10^6 cells/ml) were washed twice with phosphate-buffered saline and then lysed in H_2O (15×10^6 cells/ml H_2O) either with three rapid cycles of freezing (liquid nitrogen) and thawing (37 °C) or by placing the cells on ice for 30–60 min. The cell lysate was centrifuged for 5 min at 10,000 × *g* and the resulting supernatant used immediately for the IDO assay.

For experiments with authentic NO gas, the IDO assay was carried out under 2% O_2 to minimize the reaction of NO with O_2 but still provide sufficient O_2 for the dioxygenase-catalyzed reaction, while experiments with DEANO were carried out under normal O_2 tension. The IDO enzyme assay mixture contained ascorbic acid (10 mM), methylene blue (25 μM), L-tryptophan (200 μM), catalase (0.2 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4), 3.5 mM EDTA. The reaction was initiated by the addition of the enzyme assay mixture to the cell lysate in a 1:1 (v/v) ratio and was carried out at 37 °C in a shaking water bath. The test compound of interest was added at the beginning of the incubation. Following incubation for 30 or 60 min, 200-μl aliquots were removed and the reaction terminated by filtering the aliquot through microspin ultrafilters (10,000 M_r cut-off; IDO has a M_r of ≈40,000 (5)). The concentration of tryptophan and kynurenine in the ultrafiltrates were determined by HPLC.

HPLC Analysis of Tryptophan and Kynurenine Pathway Metabolites—Ultrafiltrates were analyzed for tryptophan, kynurenine, anthranilic acid, and 3-hydroxyanthranilic acid as described previously (19).

Nitrite Assay—The NO produced by cultured cells or the synthetic NO generators was estimated by measurements of nitrite using the Griess reagent (40). We are aware that this method is not a direct measurement of NO nor nitrate, and underestimates total NO synthesis.

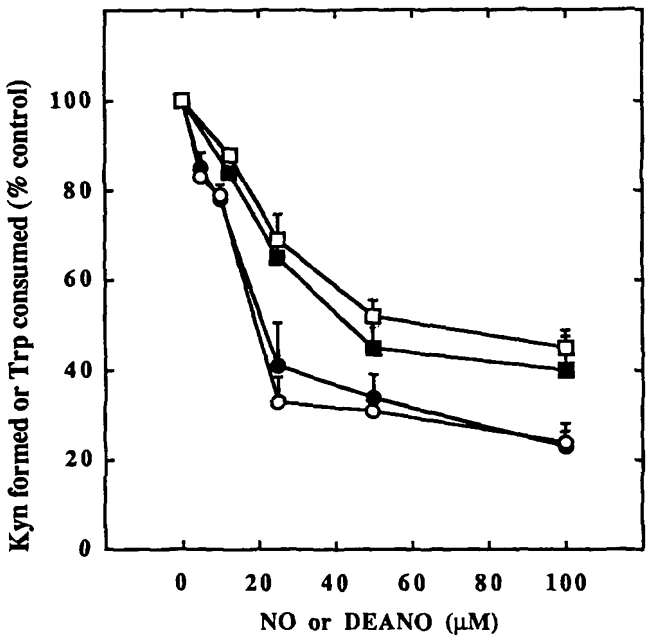

FIG. 2. **Inhibition by NO and DEANO of IDO activity in cell lysates from IFNγ-primed human PBMC.** The indicated amounts of NO (*circles*) or DEANO (*squares*) were added to 1 ml of reaction buffer containing the cell lysate of ≈10×10^6 cells. The reaction mixture was incubated for 30 min at 37 °C and the levels of kynurenine formed (*closed symbols*) and tryptophan consumed (*open symbols*) determined. The kynurenine production in the IDO assay was linear for the first 60 min of incubation in control and treated samples. Experiments with NO were carried out under 2% O_2 while those for DEANO were under normal O_2 tension. The data shown represent mean ± S.E. of four independent experiments. The 100% values for tryptophan consumed and kynurenine formed were both ≈3–5 μM.

RESULTS

As documented previously by others (9, 26), incubation of human PBMC or MDM in RPMI 1640 + 10% fetal calf serum or 10% human serum and in the presence of IFNγ resulted in the induction of IDO activity as indicated by the time-dependent decrease of tryptophan from and concomitant accumulation of kynurenine, anthranilic acid, and 3-hydroxyanthranilic acid in the culture medium (not shown). IFNγ also induced similar tryptophan degradation and metabolite formation in PBMC or MDM cultured in Ham's F-10 medium. Thus, in this medium, IFNγ (250 units/ml)-primed PBMC or MDM degraded ≈40–50 μM tryptophan after 24 h of incubation. 3-Hydroxyanthranilic acid and kynurenine plus anthranilic acid accounted for ≈3 and 65% of the tryptophan degraded by PBMC, respectively, while with MDM 3-hydroxyanthranilic acid and kynurenine plus anthranilic acid accounted for ≈20–25% and ≈30–40% of the tryptophan lost, respectively. Although detected, 3-hydroxykynurenine, a precursor of 3-hydroxyanthranilic acid, did not accumulate to concentrations >0.2 μM. Recently, it has been reported that quinolinic acid (which we did not measure) accounts for a significant proportion of the tryptophan degraded by MDM (11). In the case of PBMC, only monocytes and not lymphocytes are capable of degrading tryptophan along the kynurenine pathway (26).

Inhibition of IDO Activity in Cell Lysates by NO—Authentic NO gas and the NO generator DEANO inhibited IDO activity in cell lysates of IFNγ-primed human PBMC in a concentration-dependent fashion (Fig. 2). The degree of inhibition of tryptophan consumption and kynurenine formation corresponded for both NO and DEANO. NO gas (IC_{50} ≈ 20 μM) was more potent than DEANO (IC_{50} ≈ 50 μM) even though 2 mol of NO are potentially released per mol of DEANO. This can be ratio-

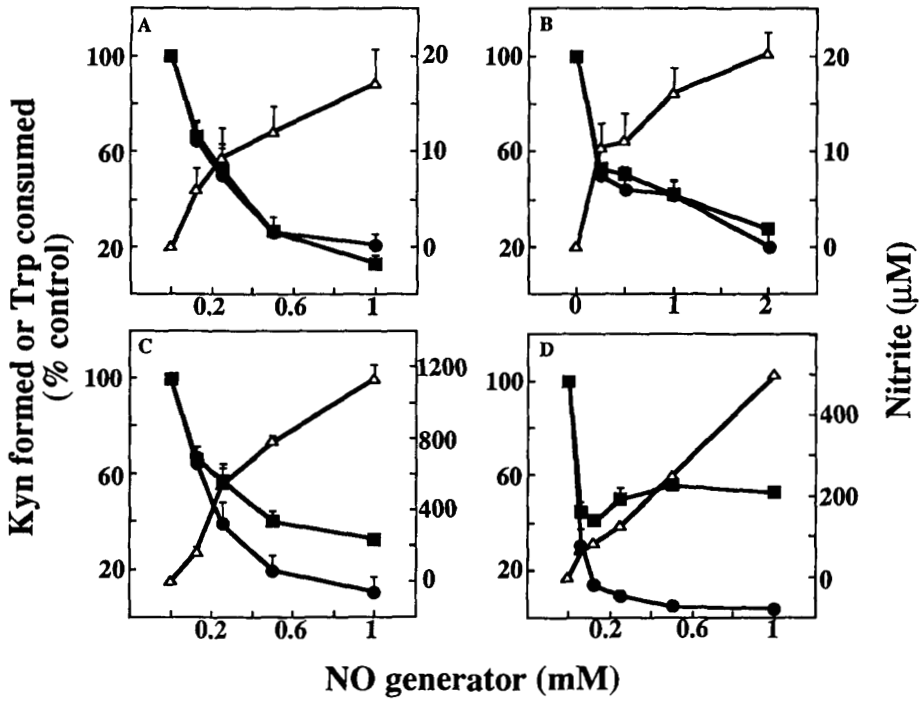

FIG. 3. **NO-generating compounds inhibit conversion of tryptophan into kynurenine by IFNγ-primed, intact human PBMC.** Isolated human PBMC (5×10^6 cells/ml), cultured under nonadherent conditions in 1 ml of tryptophan-supplemented Ham's F-10 medium, were primed by preincubation for 24 h with rh-IFNγ (250 units/ml). Cells were then exposed to the indicated concentrations of GTN (*A*), SNP (*B*), DEANO (*C*), and SNAP (*D*) in the presence of rh-IFNγ (250 units/ml) for a further 24 h. Following incubation, the levels of extracellular nitrite (*open triangles*) and kynurenine (*closed circles*) and tryptophan (*closed squares*) were determined. Values represent the mean ± S.E. of three to four independent experiments. The 100% values for tryptophan consumed and kynurenine formed were 24.8 ± 4.2 and 15.0 ± 1.0 μM, respectively.

nalized by the fact that in the case of NO gas, NO was added as a bolus at the start of the reaction (zero time point in Fig. 2), while DEANO released NO over the time scale of minutes ($t_{1/2} \approx$ 2.0 min at pH 7.4). Full inhibition of IDO by NO gas was noted within the initial 5 min of the incubation and this inhibition was significantly maintained over the entire 90 min of the reaction (data not shown). Nitrite and nitrate, the stable end products of the reaction of NO with O_2 and H_2O, had no significant effect on IDO activity even when added to the reaction mixture at concentrations of up to 1 mM (data not shown).

Inhibition of IDO Activity in IFNγ-primed Intact Human PBMC and MDM by NO-generating Compounds—Exposure of IFNγ-primed human PBMC (Fig. 3) and MDM (Table I) to either of the four chemically dissimilar NO-generating compounds, SNP, GTN, SNAP, or DEANO, resulted in the inhibition of tryptophan consumption from and accumulation of kynurenine in the culture medium. Cells remained largely viable (viability >90%) during exposure to the NO-generators, indicating that the observed inhibition of IDO activity was not due to toxicity of the test compounds. Furthermore, comparable concentrations of added nitrite or nitrate did not inhibit IDO activity (data not shown). For PBMC, GTN and SNP dose-dependently inhibited kynurenine formation and tryptophan degradation to a similar extent. In contrast, DEANO and SNAP inhibited kynurenine formation to a greater extent than tryptophan degradation (Fig. 3). The same trends applied for MDM with the exception that SNP inhibited kynurenine formation more than tryptophan consumption (Table I). Separate cell-free control experiments demonstrated that at the concentrations tested of the four compounds only SNAP degraded tryptophan and DEANO and SNAP, only at the highest concentration tested, directly degraded kynurenine (Table II). DEANO and SNAP, at the highest concentration, produced high levels of nitrite which directly degraded kynurenine (Table II). SNAP, in addition to NO, also produces thiyl radicals that may mediate the degradation of tryptophan. These direct effects of DEANO and SNAP largely accounted for the discrepancies in the extent of inhibition of tryptophan degradation and kynurenine formation observed for these compounds in Fig. 3 and Table II. When cultured in the presence of MDM, SNP appeared to have a

TABLE I
Inhibition of IDO activity in IFNγ-primed human MDM by NO generators

1×10^6 MDM cultured in RPMI 1640 medium, supplemented with 10% human serum, and 100 μM L-tryptophan were primed with IFNγ (250 units/ml) for 18 h. The cells were subsequently washed and then cultured in F-10 medium supplemented with L-tryptophan (100 μM). Cells were then exposed to the NO generators at the indicated concentrations for 24 h in the presence of IFNγ (250 units/ml) after which the levels of tryptophan, kynurenine, and nitrite in the culture medium were determined.

NO generator	Kynurenine formation[a]	Tryptophan consumption[a]	Nitrite
	% control		μM
GTN (0.5 mM)	14.4 ± 2.0	13.2 ± 3.1	31.5 ± 2.1
(0.2 mM)	27.4 ± 3.7	25.7 ± 4.7	24.4 ± 1.1
SNP (1.0 mM)	14.8 ± 0.4	27.4 ± 2.4	34.2 ± 5.2
SNAP (0.5 mM)	11.5 ± 2.3	39.7 ± 15.6	56.0 ± 8.6
DEANO (0.5 mM)	23.8 ± 1.8	37.5 ± 2.1	502 ± 9.9
DEANO (0.5 mM) (conditioned)[b]	65.6	80.1	507

[a] 100% values for tryptophan consumed and kynurenine formed were 52.6 ± 7.0 and 21.0 ± 5.1 μM, respectively. Results are the mean ± S.D. for two separate experiments carried out in duplicate except for DEANO (conditioned) which was the result from one experiment carried out in duplicate.

[b] DEANO was preincubated in F-10 medium for 30 min before addition of such treated medium to IFNγ-primed MDM.

degrading effect on kynurenine, the reason(s) for which is not known. It was evident that GTN was metabolized to a greater extent by MDM than by PBMC. This was reflected in a greater potency of inhibition of IDO by GTN in MDM compared to PBMC.

The extent of inhibition of IDO caused by the various compounds was not dependent on the level of nitrite detected extracellularly. For example, DEANO which produced large amounts of extracellular NO over a time scale of minutes was not as potent as GTN which is thought to be metabolized continuously by the cells over the entire 24 h. Also the half-life of the NO generators determined if the inhibition was time-dependent or not. For example, inhibition of IDO caused by GTN but not DEANO was time-dependent (not shown). Furthermore, DEANO-conditioned media (*i.e.* medium that had been

TABLE II
Certain NO generators directly degrade tryptophan and kynurenine in Ham's F-10 medium

Cell-free experiments were carried out in F-10 medium supplemented with L-tryptophan (80 μM) and L-kynurenine (20 μM). NO generators were added at the indicated concentrations to the medium and incubated for 24 h at 37 °C and 5% CO_2. Following incubation, the levels of tryptophan, kynurenine, and nitrite in the medium were determined. Results are the means of one to two separate experiments carried out in duplicate.

Compound	mM	Kynurenine	Loss kynurenine	Tryptophan	Loss tryptophan	Nitrite
		μM	%	μM	%	μM
Control		20	0	80	0	0
SNAP	1.0	15.2	23.7	47.8	41.1	675
	0.5	19.8	0	52.3	34.6	175
	0.25	20.1	0	58.3	27.1	100
	0.125	19.9	0	64.8	19.0	79
DEANO	1.0	15.5	22.3	80.1	0	1250
	0.5	16.8	16.0	79.7	0	591
	0.25	17.9	10.3	80.3	0	345
	0.125	19.1	4.5	80.6	0	182
Nitrite	2.0	14.0	30.0	74.2	7.2	1950
	1.0	16.1	19.4	79.7	0	950
	0.5	18.7	6.4	80.0	0	575
	0.25	20.1	0	79.9	0	306
GTN	1.0	20.0	0	80.1	0	6.7
	0.5	20.3	0	80.3	0	4.8
	0.25	19.8	0	80.2	0	2.6
SNP	2.0	20.1	0	80.4	0	16.6
	1.0	19.7	0	79.8	0	10.2
	0.5	20.3	0	80.5	0	6.8

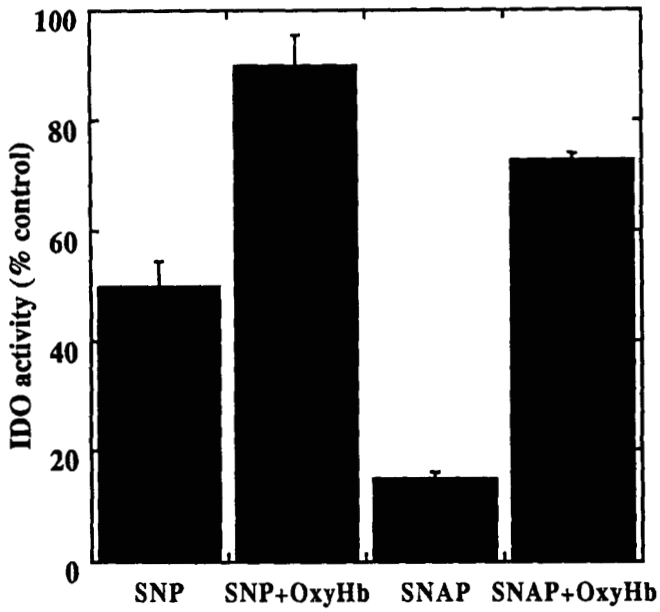

FIG. 4. **OxyHb abrogates the ability of SNP and SNAP to inhibit IDO activity in IFNγ-primed PBMC.** Experiments were carried out as described in legend to Fig. 3 except that OxyHb (10 μM) was added where indicated in addition to SNAP (0.25 mM) or SNP (0.5 mM). Values represent the mean ± S.E. of two independent experiments carried out in duplicate. IDO activity was measured as the loss of tryptophan with concomitant formation of kynurenine (for SNP) or formation of kynurenine (SNAP). Addition of OxyHb alone stimulated IDO activity in the PBMC by ≈30–40%. This increase was corrected for by expressing the results for cells treated with OxyHb and the NO-generator as a percentage of the values for cells treated with OxyHb alone. Nitrite levels detected in SNAP- and SNP-treated samples in the absence of OxyHb were 67 and 12.5 μM.

preincubated with DEANO for 30 min, such that virtually all the NO was released, before incubation with MDM) was markedly less potent in inhibiting IDO activity than DEANO added freshly to the MDM (Table I). Some of the inhibitory activity of DEANO-conditioned media may be due to the formation of somewhat more stable products capable of mediating NO effects such as *S*-nitrosothiols (35, 41). Co-incubation of SNAP or SNP with OxyHb (10 μM), a well established antagonist of the actions of NO, significantly reversed the inhibitory effect of these NO generators on IDO activity in IFNγ-primed PBMC (Fig. 4). This extent of reversal corresponded to an inhibition of nitrite and hence NO formed of both SNAP- (≈70% inhibition) and SNP-treated (≈60% inhibition) samples.

Induction of IDO Activity in IFNγ- or IFNγ/LPS-primed Murine Macrophages—Previous work by others (3, 16) has shown that in murine macrophages, nitric oxide synthase but not IDO is induced by IFNγ. To test whether a lack of measurable IDO activity was the result of NO-mediated inhibition, we activated murine macrophages with IFNγ or IFNγ/LPS under culture conditions that did or did not allow formation of reactive nitrogen species (Fig. 5). Murine peritoneal macrophages cultured in arginine-supplemented medium in the presence of IFNγ or IFNγ/LPS showed the characteristic induction of nitric oxide synthase as indicated by significantly greater levels of nitrite detected in the medium than in non-stimulated control cells (Fig. 5*A*, *+Arg*). LPS strongly enhanced IFNγ-mediated nitric oxide synthase induction while IDO activity was not induced under these conditions, consistent with previous reports. In contrast to a previous report (16), addition of IFNγ to mouse macrophages, cultured in arginine-containing medium, resulted in a measurable induction of IDO activity as indicated by both tryptophan consumption and kynurenine formation (Fig. 5, *B* and *C*, *+Arg*). In the presence of arginine (+Arg), the extent of nitrite formation elicited by IFNγ treatment varied between experiments using different batches of isolated cells and this variance was reflected inversely in varying degrees of IDO induction. Thus, when the nitrite levels measured were high (*i.e.* 25 nmol/10^6 cells), only 0.4 nmol of kynurenine were formed per 10^6 cells. However, when nitrite levels were <5 nmol/10^6 cells, ≈1.0 nmol of kynurenine was formed per 10^6 cells. Even more kynurenine was formed from tryptophan when IFNγ in the absence or presence of LPS was added to mouse macrophages cultured in arginine-free (–Arg) or NMA-containing (+NMA) medium or both (–Arg + NMA), *i.e.* when NO formation was inhibited (Fig. 5). The maximum observed IDO activity was ≈5–10 times less than that measured in human MDM when expressed on a per cell basis. In contrast to human MDM, murine macrophages did not produce detectable amounts of 3-hydroxyanthranilic acid under conditions of measurable IDO activity (not shown). The presence of LPS did not appear to affect the IFNγ-induced IDO induction observed in mouse peritoneal macrophages in the presence of NMA and/or in arginine-free medium (Fig. 5).

DISCUSSION

The present study demonstrates that both exogenous and endogenous NO inhibit IDO activity in IFNγ-primed mononuclear phagocytes *in vitro*. This is supported by several lines of evidence. Thus, authentic NO or the NO-generating compound, DEANO, inhibited IDO activity in cell lysates prepared from IFNγ-primed human PBMC. Compounds that release exogenous NO (*i.e.* SNP, SNAP, and DEANO) or are thought to release NO after enzymatic metabolism within cells such as macrophages, endothelial cells, and smooth muscle cells (*i.e.* GTN) (42, 43), all inhibited IDO activity in intact IFNγ-primed human PBMC and MDM at non-toxic concentrations. Once produced, NO readily reacts with O_2 and H_2O to form nitrite

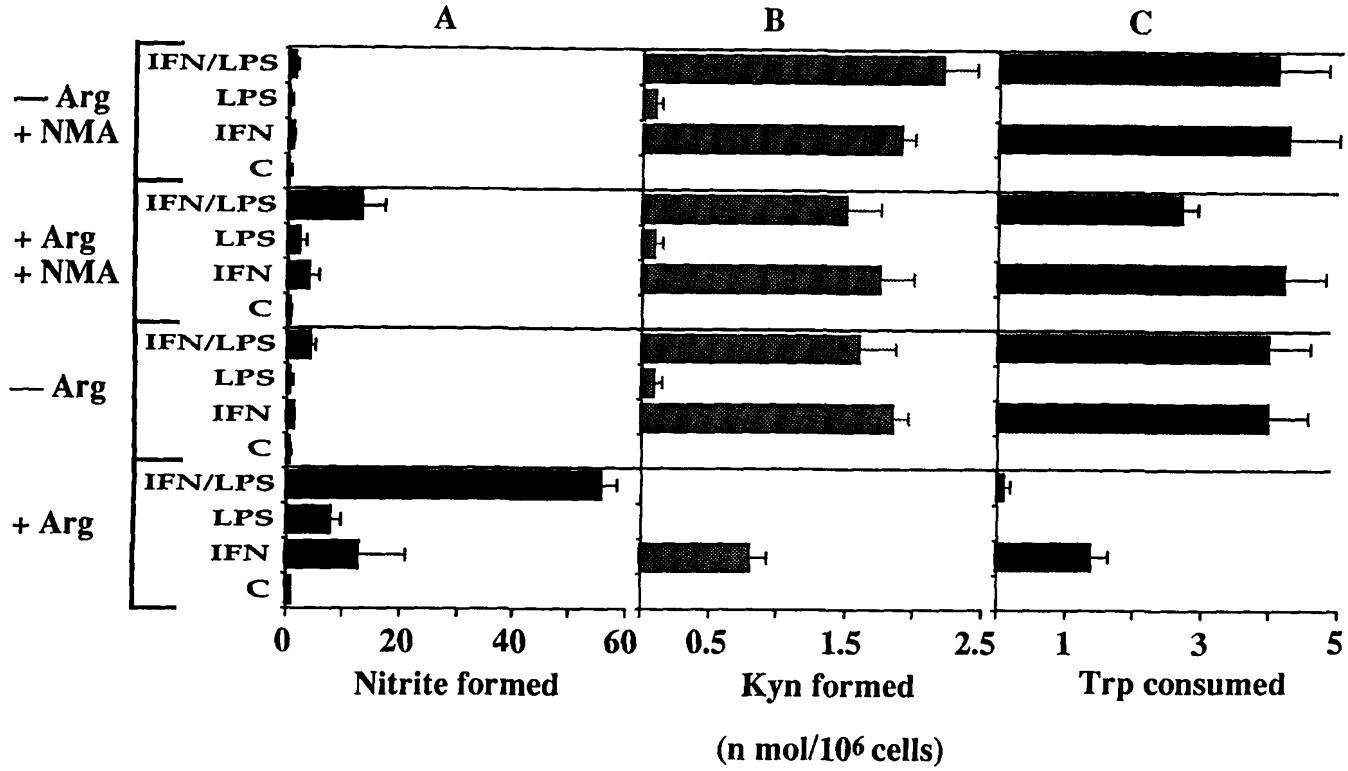

FIG. 5. **Inhibition of nitric oxide synthase activity in IFNγ-activated murine macrophages results in induction of IDO activity.** Resident murine peritoneal macrophages (5×10^6 cells/well) were isolated and cultured for 48 h in 2 ml of tryptophan and arginine-free RPMI 1640 medium supplemented with 10% human serum, L-tryptophan (100 μM), and L-arginine (1.2 mM). rm-IFNγ (500 units/ml) and LPS (2.5 μg/ml), either alone or in combination, were added where indicated and NO synthesis was inhibited by culturing the cells under arginine-free conditions and/or adding the nitric oxide synthase inhibitor NMA (100 μM). Control wells were not treated with IFNγ or LPS. In control wells, small amounts of kynurenine (0.14 ± 0.02 nmol/10^6 cells) were detected, which were subtracted from all other values. Following incubation, the levels of tryptophan, kynurenine, and nitrite present in the culture medium were determined as described under "Experimental Procedures." Results are the mean ± S.E. for five independent experiments.

and nitrate. However, sodium nitrite or nitrate, even at concentrations exceeding those detected in the experiments with authentic NO gas and the various NO generators, did not affect IDO activity in cell lysates or intact IFNγ-primed human PBMC. Also, DEANO-conditioned media was much less potent in inhibiting IDO than freshly added DEANO. Furthermore, the inhibitory activity of the NO-generating compounds, SNP and SNAP, on IFNγ-induced IDO activity in intact PBMC was reversed by OxyHb, which effectively binds NO (29). Finally, inhibition of the formation of endogenous NO in IFNγ-activated *murine* macrophages resulted in a significant induction of IDO activity that was not observed when these cells produced large amounts of reactive nitrogen species and hence NO.

A plausible mechanism by which NO inhibits IDO is via interaction with the heme iron present at the active site of the enzyme, because NO has a high affinity for heme iron and such binding may interfere with the conversion of ferric to ferrous iron required for IDO catalysis (38). It is becoming increasingly apparent that NO is capable of inhibiting or activating a variety of heme iron containing enzymes directly (44–47), including nitric oxide synthase (48). Also, the cytotoxic action of macrophage-derived NO on tumor cells and microorganisms is a result of inhibition of iron and iron-containing enzyme activity through NO binding to iron centers in proteins such as aconitase and ribonucleotide reductase (3, 49).

Another mechanism by which NO could inhibit IDO activity is via the ability of NO to rapidly react with and thereby remove O_2^- proposed to be required for IDO enzyme activity (7, 8). However, Sono (38) showed that when 25 μM methylene blue and 10 mM ascorbic acid are used as co-factors in the IDO assay, as in this study, reduced methylene blue rather that O_2^- acts as the electron donor for the ferric dioxygenase. This makes it unlikely that direct reaction of NO with O_2^- was responsible for inhibition of IDO activity observed in our experiments with cell lysates. Although there is some evidence for a role of O_2^- in the activation of IDO in enterocytes (50), the situation in mononuclear phagocytes is not clear at present. In enterocytes, treatment with the xanthine oxidase inhibitor allopurinol inhibited IDO activity while treatment with the superoxide dismutase inhibitor, diethyldithiocarbamate, stimulated IDO activity. However, we have observed that in IFNγ-primed PBMC, allopurinol does not effect IDO activity while diethyldithiocarbamate in fact inhibited IDO activity.[2] Together, our findings suggest that there are distinct differences in the regulation of IDO in enterocytes and mononuclear phagocytes and that inhibition of IDO by NO in mononuclear phagocytes is more likely to occur independent of O_2^- scavenging.

From the experiments with NO-generators and intact, IFNγ-primed PBMC and MDM it was apparent that nitrite levels detected in the culture medium did not correlate with the extent of IDO inhibition. This may be due to differences in the location and rate with which the compounds release the NO. DEANO's ($t_{1/2} \approx 2.0$ min) half-life is in the order of minutes while that for SNP ($t_{1/2} \approx 18$ h), GTN and SNAP ($t_{1/2} \approx 2$ h) is hours. Also, GTN is thought to release NO intracellularly while SNAP, DEANO, and SNP release NO extracellularly. We did not attempt to measure intracellular nitrite levels.

In contrast to a previous report by others (16), we show here that IFNγ does induce IDO activity in murine macrophages, even under conditions where relatively small amounts of NO

[2] S. Thomas and R. Stocker, unpublished data.

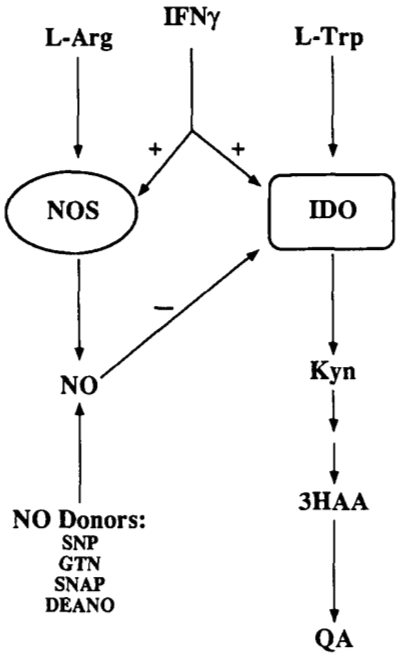

FIG. 6. **Proposed model for NO-mediated regulation of IDO in IFNγ-primed mononuclear phagocytes.**

are synthesized. However, in the presence of IFNγ and LPS, where large amounts of NO were produced, induction of IDO in mouse macrophages was not observed. LPS contamination may therefore explain why in the previously published work IDO activation by IFNγ alone was not observed. The inhibition by LPS was due to increased NO formation, as in arginine-containing medium, +NMA, or arginine-free medium ± NMA, LPS no longer inhibited tryptophan degradation.

It has been suggested that the antimicrobial and anti-tumor activities of mononuclear phagocytes are interrelated (3), although to date little is known about such interrelationships and their regulation. Picolinic acid, an end product of tryptophan metabolism, has been shown to act synergistically with IFNγ in inducing NO synthesis when added to murine macrophages and this has been proposed to represent a link between the tryptophan and arginine degradative pathways in mononuclear phagocytes (28). However, there is no evidence that we are aware of that human or murine macrophages synthesize picolinic acid. Furthermore, even if produced it is not known whether the high levels of picolinic acid required to obtain such an effect would be reached. In contrast, we demonstrate here for the first time that inhibition of IDO by NO represents a connection between the arginine- and tryptophan-dependent anti-growth pathways (Fig. 6) and that this connection is physiologically feasible, as demonstrated by the murine macrophage experiments. Also, inhibition of IDO by NO may be applicable to IFNγ-exposed cells other than mononuclear phagocytes.

The *in vivo* relevance of a regulatory action of NO on IDO, if any, is unknown. However, one may anticipate that the various lines of antimicrobial defenses of mononuclear phagocytes represent a coordinate, partly overlapping temporal sequence of events. IDO induction may represent an early line of defense against invading pathogens or tumor cells while nitric oxide synthase induction may represent a later line of defense. This is consistent with a report demonstrating that addition of exogenous tryptophan to MDM infected with *Chlamydia psittaci* at days 1 to 3, but not after more extended culture periods, reversed the inhibitory activity of IFNγ (51). Through regulation of IDO, NO would prevent total depletion of tryptophan and/or other indoleamines important in cellular function of the host cell and may also prevent accumulation of high concentrations of potentially toxic metabolites of the kynurenine pathway, such as 3-hydroxyanthranilic acid and quinolinic acid (11).

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REFERENCES

1. Nathan, C. F., and Yoshida, R. (1988) in *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J. I., Goldstein, I. M., and Snyderman, R., eds) pp. 229–251, Raven Press, New York
2. Murray, H. W. (1988) *Semin. Hematol.* **25,** 101–111
3. Nathan, C. F., and Hibbs, J. B., Jr. (1991) *Curr. Opin. Immunol.* **3,** 65–70
4. Pfefferkorn, E. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 908–912
5. Taylor, M. W., and Feng, G. S. (1991) *FASEB J.* **5,** 2516–2522
6. Carlin, J. M., Ozaki, Y., Byrne, G. I., Brown, R. R., and Borden, E. C. (1989) *Experientia (Basel)* **45,** 535–541
7. Hayaishi, O., Hirata, F., Ohnishi, T., Henry, J. P., Rosenthal, I., and Katoh, A. (1977) *J. Biol. Chem.* **252,** 3548–3550
8. Ohnishi, T., Hirata, F., and Hayaishi, O. (1977) *J. Biol. Chem.* **252,** 4643–4647
9. Werner-Felmayer, G., Werner, E. R., Fuchs, D., Hausen, A., Reibnegger, G., and Wachter, H. (1989) *Biochim. Biophys. Acta* **1012,** 140–147
10. Takikawa, O., Kuroiwa, T., Yamazaki, F., and Kido, R. (1988) *J. Biol. Chem.* **263,** 2041–2048
11. Heyes, M. P., Saito, K., and Markey, S. P. (1992) *Biochem. J.* **283,** 633–635
12. Yoshida, R., Urade, Y., Tokuda, M., and Hayaishi, O. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4084–4086
13. Christen, S., Peterhans, E., and Stocker, R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2506–2510
14. Heyes, M. P., Saito, K., Crowley, J. S., Davis, L. E., Demitrack, M. A., Der, M., Dilling, L. A., Elia, J., Kruesi, M. J. P., Lackner, A., Larsen, S. A., Lee, K., Leonard, H. L., Markey, S. P., Martin, A., Milstein, S., Mouradian, M. M., Pranzatelli, M. R., Quearry, B. J., Salazar, A., Smith, M., Strauss, S. E., Sunderland, T., Swedo, S. W., and Tourtellotte, W. W. (1992) *Brain* **115,** 1249–1273
15. Aune, T. M., and Pogue, S. L. (1989) *J. Clin. Invest.* **84,** 863–875
16. Murray, H. W., Szuro-Sudol, A., Wellner, D., Oca, M. J., Granger, A. M., Libby, D. M., Rothermel, C. D., and Rubin, B. Y. (1989) *Infect. Immun.* **57,** 845–849
17. Habara-Ohkubo, A., Shirahata, T., Takikawa, O., and Yoshida, R. (1993) *Infect. Immun.* **61,** 1810–1813
18. Thomas, S. M., Garrity, L. F., Brandt, C. R., Schobert, C. S., Feng, G. S., Taylor, M. W., Carlin, J. M., and Byrne, G. I. (1993) *J. Immunol.* **150,** 5529–5534
19. Christen, S., Thomas, S. R., Garner, B., and Stocker, R. (1994) *J. Clin. Invest.,* in press
20. Iyengar, R., Stuehr, D. J., and Marletta, M. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6369–6373
21. Drapier, J. C., Wietzerbin, J., and Hibbs, J. B., Jr. (1988) *Eur. J. Immunol.* **18,** 1587–1592
22. Lepoivre, M., Boudbid, H., and Petit, J.-F. (1989) *Cancer Res.* **49,** 1970–1976
23. Adams, L. B., Hibbs, J. B., Jr., Taintor, R. R., and Krahenbuhl, J. L. (1990) *J. Immunol.* **144,** 2725–2729
24. Zhu, L., Gunn, C., and Beckman, J. S. (1992) *Arch. Biochem. Biophys.* **298,** 452–457
25. Lowenstein, C. J., and Snyder, S. H. (1992) *Cell* **70,** 705–707
26. Carlin, J. M., Borden, E. C., Sondel, P. M., and Byrne, G. I. (1989) *J. Leukocyte Biol.* **45,** 29–34
27. Padgett, E. L., and Pruett, S. B. (1992) *Biochem. Biophys. Res. Commun.* **186,** 775–781
28. Melillo, G., Cox, G. W., Radzioch, D., and Varesio, L. (1993) *J. Immunol.* **150,** 4031–4040
29. Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992) *Science* **258,** 1898–1902
30. Salvemini, D., De Nucci, G., Gryglewski, R. J., and Vane, J. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6328–6332
31. Drago, R. S., and Paulik, F. E. (1960) *J. Am. Chem. Soc.* **82,** 96–98
32. Drago, R. S., and Karstetter, B. R. (1961) *J. Am. Chem. Soc.* **83,** 1819–1822
33. Maragos, C. M., Morley, D., Wink, D. A., Dunams, T. M., Saavedra, J. E., Hoffman, A., Bove, A. A., Isaac, L., Hrabie, J. A., and Keefer, L. K. (1991) *J. Med. Chem.* **34,** 3242–3247
34. Field, L., Dilts, R. V., Ravichandran, R., Lenhert, P. G., and Carnahan, G. E. (1978) *J. Chem. Soc. Chem. Comm.* 249–250
35. Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J., and Gruetter, C. A. (1981) *J. Pharmacol. Exp. Ther.* **218,** 739–749
36. Fogelman, A. M., Seager, J., Hokom, M., and Edwards, P. A. (1979) *J. Lip. Res.* **20,** 379–388
37. Jessup, W., Darley-Usmar, V., O'Leary, V., and Bedwell, S. (1991) *Biochem. J.* **278,** 163–169
38. Sono, M. (1989) *J. Biol. Chem.* **264,** 1616–1622
39. Deleted in proof
40. Ding, A. H., Nathan, C. F., and Stuehr, D. J. (1988) *J. Immunol.* **141,** 2407–2412
41. Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 444–448

42. Salvemini, D., Mollace, V., Pistelli, A., Anggard, E., and Vane, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 982–986
43. Salvemini, D., Pistelli, A., Mollace, V., Änggård, E., and Vane, J. (1992) *Biochem. Pharmacol.* **44,** 17–24
44. Craven, P. A., and DeRubertis, F. R. (1983) *Biochim. Biophys. Acta* **745,** 310–321
45. Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., and Needleman, P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7240–7244
46. Molina y Vedia, L., McDonald, B., Reep, B., Brüne, B., Di Silvio, M., Billiar, T. R., and Lapetina, E. G. (1992) *J. Biol. Chem.* **267,** 24929–24932
47. Clancy, R. M., Leszczynska-Piziak, J., and Abramson, S. B. (1992) *J. Clin. Invest.* **90,** 1116–1121
48. Rengasamy, A., and Johns, R. A. (1993) *Mol. Pharmacol.* **44,** 124–128
49. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, Z., and Rachlin, E. M. (1988) *Biochem. Biophys. Res. Commun.* **157,** 87–94
50. Hayaishi, O. (1985) *Biken J.* **28,** 39–49
51. Byrne, G. I., Carlin, J. M., Merkert, T. P., and Arter, D. L. (1989) *Infect. Immun.* **57,** 1318–1320