Nitric Oxide Synthase Is Not a Constituent of the Antimicrobial Armature of Human Mononuclear Phagocytes

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Nitric oxide synthase (NOS) has received immense interest as an antimicrobial and antitumoral effector system of mononuclear phagocytes from rodents. Because there is increasing doubt that an analogous system exists in human macrophages, NOS was reexamined in these cells. Under tightly controlled conditions, with murine macrophages as positive controls, human macrophages failed to secrete nitric oxide (<0.1 μmol/10^6 cells/24 h), even after activation with endotoxin, interferon-γ, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, bacteria, or proliferating lymphocytes. The discrepancy between murine and human macrophages depended on neither the anatomic source (blood, peritoneum), the agent used for activation, nor the duration of activation. NOS activity was paralleled by metabolization of L-arginine to L-citrulline. Exogenous tetrahydrobiopterin, an essential cofactor of NOS not synthesized by human macrophages, did not support NOS activity in human macrophages. Also, no NOS activity was found in cellular subfractions of human macrophages. It appears that in humans, the inducible high-output NOS is not conserved as an antimicrobial system of macrophages.

Nitric oxide (NO) has recently been brought into focus as an antimicrobial and antitumoral effector system of mononuclear phagocytes with activity against fungi [1, 2], bacteria [3, 4], parasites [5–8], and tumor cells [9–15]. While there is general agreement that phagocytes from mice and rats synthesize abundant NO from L-arginine [1, 7, 16–19], demonstration of high-output NO synthase (NOS) activity in human mononuclear phagocytes remains controversial. On one hand, it has been proposed that the antimicrobial activity of human blood–derived macrophages, seen after prolonged activation against *Mycobacterium avium–Mycobacterium intracellulare* [3] or *Trypanosoma cruzii* [20], depends on NO. On the other hand, human peritoneal, alveolar, and blood-derived macrophages have not been found to secrete substantial amounts of NO [21–24], even after treatment with endotoxin (lipopolysaccharide, LPS) and interferon-γ (IFN-γ). Also nitrite, a descendant of NO that is unstable under physiologic conditions, was not detectable in supernatants from human macrophages stimulated with LPS and IFN-γ [23–25]. Furthermore, alveolar and peritoneal macrophages have not been found to metabolize appreciable amounts of L-arginine, the substrate of NOS [21].

Because of the significance attributed to the effector function of NO produced by mouse and rat macrophages [26–28] and the continuous efforts put into studies of NOS in mononuclear phagocytes [8, 20, 29–32], we decided to reexamine NOS activity in human macrophages.

Materials and Methods

Phagocytes. Human mononuclear cells were isolated from heparinized blood of normal volunteers by a Ficoll gradient (Pharmacia, Uppsala, Sweden), and lymphocytes were washed away after adhesion of the mononuclear phagocytes to the bottoms of culture wells, giving a monocyte preparation of >95% purity as determined by Giemsa stain [33]. Human peritoneal macrophages were obtained by centrifugation, washing, and adhesion purification of dialysate from patients undergoing chronic peritoneal dialysis or with malignant ascites. Mouse peritoneal macrophages were obtained from female 8- to 10-week-old ICR, BALB/c (Institut für Tierzucht, University of Zurich), or C3H/HeN mice (WIGA, Sulzfeld, Germany) as resident cells or collected 5–6 days after stimulation with 2 mL of 10% thioglycolate broth (Difco, Detroit) or 2% starch (Merck, Zurich) by lavage with 10 mL of cold Gey's balanced salt solution (GIBCO Europe, Basel, Switzerland) supplemented with 10 units/mL heparin (Hoffmann La Roche, Basel). Blood-derived (heparinized blood, Ficoll) and thioglycolate-induced (20 mL/rabbit) peritoneal macrophages were obtained from 2-kg New Zealand White rabbits (Madöring, Füllinsdorf, Switzerland), and mononuclear phagocytes were prepared as described above.

Cell cultures. Mononuclear phagocytes were cultured in 24- or 6-well cluster plates (Falcon Plastics, Oxnard, CA) for measurement of NO secretion, NOS, amino acid metabolism, or biopterin synthesis. Phagocytes were seeded at a density of 0.4–0.5 × 10^5 mononuclear phagocytes/cm^2 of culture surface and cultured with 1 mL of medium/10^6 cells. Medium 199 (GIBCO) was supplemented with 15% fetal calf serum (GIBCO) for mice and 20% human or 20% rabbit serum for human or rabbit phagocytes, respectively, in addition to 500 μmol L-arginine (Fluka,

Buchs, Switzerland) and 50 μg/mL gentamycin (Schering, Kenilworth, NJ). Medium was changed every 48 h.

Cytokines, hormones, and stimulants. Human recombinant IFN-γ was from Biogen (Geneva), recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) from Schering, recombinant tumor necrosis factor-α (rTNFα) from Knoll (Ludwigshaven, Germany), and recombinant human interleukin-2 (rIL-2) from Hoffmann La Roche. Mouse recombinant IFN-γ was a gift of K. Frei (Institute for Clinical Immunology, University of Zurich). LPS (*Escherichia coli* O26:B6, Boivin extraction) was from Difco; purified protein derivative (PPD) for in vitro use was from the Statens Serumsinstitut (Copenhagen). All supplements were directly dissolved and diluted in medium 199 with the exception of tetrahydrobiopterin (BH₄; B. Schircks Laboratories, Jona, Switzerland), which was dissolved in 0.05 M HCl as a 100 mM stock solution. In some experiments, heat-killed bacteria (10⁸/mL) of *Listeria monocytogenes* (strain EGD) or *Moraxella catarrhalis* (gift of R. Keller, Arbeitsgruppe für Immunbiologie, University of Zurich Medical School) were used to induce NOS [34].

Quantitation of nitrite. To 150 μL of Griess reagent (0.05% N-1-naphthylethylenediamine dihydrochloride, 0.5% sulfanilamide 2.5% phosphoric acid), 50 μL of supernatant from macrophage cultures was added and incubated at room temperature for 10 min in a 96-well microplate (Dynatech, Kloten Switzerland). Absorbance was read at 570/630 nm in a MicroELISA Autoreader MR 580 (Dynatech). Sodium nitrite was used as NO_2^- standard diluted in complete medium, and the measured amount of NO_2^- was related to the number of cells in the corresponding well. Cell counts were obtained electronically by a counter (Coulter, Hialeah, FL) as described [33, 35]. Under these conditions, the detection limit was 0.1 μM nitrite/10⁶ cells or 10 pmol/microwell. Activation of human macrophages by cytokines was assessed by monitoring antilisterial activity and H_2O_2 secretion by methods described previously [35].

Assay for NOS. Macrophage monolayers from 6-well cluster plates (35 mm) were washed twice with 15 mM HEPES, pH 7.5, and were harvested by scraping in 500 μL of the same buffer containing 1 mg/mL 2-macroglobulin. Then 50 mL of this suspension was collected for determination of cell numbers (see above). Cells were disrupted by sonication and centrifuged for 30 min at 50,000 g at 4°C. NOS activity in the supernatants was measured as described [34] with the following modifications: The reaction mixture contained 15 mM HEPES, pH 7.5, 1 mM MgCl₂, 1 mM dithioerythritol (DTE), 60 μM BH₄, 0.1 mM NADPH, 1 mM L-arginine, and macrophage lysate in a total volume of 300 μL. The mixture was incubated in the dark at 37°C for 6 h. The reaction was stopped by freezing at −70°C. Samples were assayed for nitrite formation using the Griess reaction as described above. Then 100 μL of the assay mixture was incubated with 100 μL of Griess reagent and read against a sodium nitrite standard dissolved in 15 mM HEPES, pH 7.5, containing MgCl₂, DTE, NADPH, and L-arginine at concentrations used in the assay mixture.

Amino acid analysis. Amino acid contents in macrophage culture supernatants were quantitated by a routine procedure using an LC 5001 Autoanalyzer equipped with BTC 2710 cation exchange resin (Biotronik, Maintal, Germany). Samples

Table 1. Comparison of nitrite (NO_2^-) secretion by macrophages from different species and anatomic sources.

Cell source	Pretreatment for 48 h	NO_2^- (μmol/10⁶ cells/day)
Human blood	None	<0.1
	LPS	<0.1
	IFN-γ	<0.1
	IFN-γ + LPS	<0.1
	IFN-γ + LPS + BH₄	<0.1
Human peritoneal	None	<0.1
	LPS	<0.1
	IFN-γ + LPS	<0.1
	IFN-γ + LPS + BH₄	<0.1
Mouse resident	None	1.9 ± 0.4
	LPS	5.5 ± 0.1
	IFN-γ	59.9 ± 5.0
Mouse thioglycolate	None	1.8 ± 0.4
	LPS	10.5 ± 1.3
	IFN-γ	44.3 ± 4.4
	IFN-γ + LPS	51.3 ± 4.4
	IFN-γ + LPS + BH₄	54.7 ± 1.5
Mouse starch	None	6.64 ± 1.4
	LPS	15.6 ± 2.2
	IFN-γ	25.5 ± 2.2
	IFN-γ + LPS	38.3 ± 2.9
Rabbit blood	None	<0.1
	LPS	<0.1
Rabbit thioglycolate	None	<0.1
	LPS	<0.1

NOTE. Human peritoneal macrophages were from peritoneal dialysate fluid (1 experiment) or from inflammatory ascites (2 experiments). Peritoneal macrophages from rodents were either resident or obtained after intraperitoneal stimulation with 10% thioglycolate or 2% starch. Data are mean ± SE from triplicate experiments. LPS, *Escherichia coli* endotoxin (lipopolysaccharide), 200 ng/mL; IFN-γ, recombinant interferon-γ from corresponding species, 100 units/mL; BH₄, tetrahydrobiopterin, 0.5 mmol.

were deproteinated by centrifugation through Ultrafree-MC NMWL filter units with a cutoff of 10,000 Da (Millipore, Bedford, MA). Analysis was developed by a stepwise gradient of lithium citrate ranging from 0.15 to 1.4 N Li. Amino acids were quantitated by the ninhydrin reaction at 570 and 440 nm.

Results

When we compared the amount of NO secreted by human, murine, or lapine mononuclear phagocytes, we found in five independent experiments that macrophages from mice but not from humans or rabbits secreted NO in amounts detectable by quantification of nitrite in culture supernatants (table 1). Human and lapine mononuclear phagocytes consistently failed to build up detectable nitrite concentrations in culture supernatants, even after stimulation with LPS or IFN-γ, while the same stimuli resulted in a 2.5- to 30-fold increase of nitrite concentrations in supernatants from murine peritoneal macrophages. To control for activation of human macrophages, listericidal and H_2O_2 activity
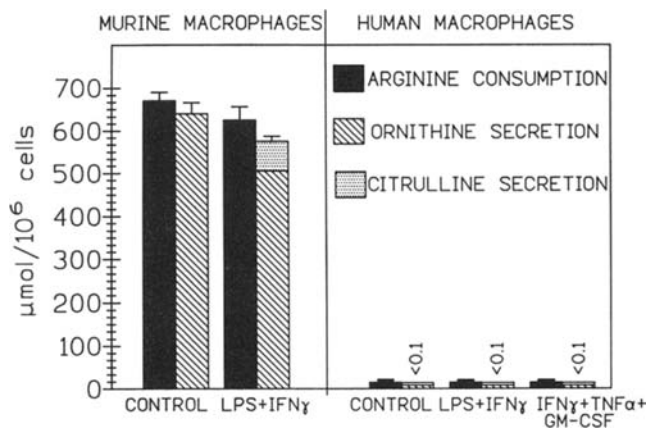
Figure 1. Comparison of consumption of L-arginine and release of ornithine and citrulline into culture medium by mouse peritoneal and human blood-derived macrophages. Endotoxin (lipopolysaccharide, LPS), 200 ng/mL; interferon-γ (IFN-γ), 100 units/mL; granulocyte-macrophage colony-stimulating factor (GM-CSF), 100 ng/mL; tumor necrosis factor-α (TNFα), 100 units/mL. Concentrations measured in 48-h culture supernatants (mean ± SD from triplicate wells).

were monitored after treatment with IFN-γ and LPS. Activated cells showed an increase of H_2O_2 secretion by a factor of 4–9 and of listericidal activity by a factor of 2–5 (not shown).

The difference between the NOS activity of human, lapine, and murine macrophages did not appear to depend on the anatomic source of the phagocytes, because not only blood-derived but also peritoneal macrophages from humans and rabbits gave equivalent results (table 1). Also, when peritoneal cells were tested, there was no interspecies difference in whether mouse or rabbit macrophages were elicited or resident or in human cells whether macrophages were obtained from peritoneal dialysate fluid or from inflammatory ascitic effusions (table 1).

Because human mononuclear phagocytes did not synthesize tetrahydrobiopterin (BH_4), an essential cofactor of NOS, we investigated also whether supplementation of exogenous BH_4 would give rise to the secretion of NO by human macrophages. Even under these conditions, no NO was produced by human cells (table 1).

Next we studied whether stimuli other than LPS or IFN-γ, known to activate macrophages, would induce NOS activity in human mononuclear phagocytes or whether a prolonged stimulation for up to 12 days would be effective. Stimulation with IFN-γ (at 100 units/mL), IFN-γ plus LPS (200 ng/mL), IFN-γ plus LPS plus BH_4 (0.5 mmol), GM-CSF (100 ng/mL), GM-CSF plus TNFα (100 units/mL), GM-CSF plus IFN-γ, or GM-CSF plus TNFα plus IFN-γ for up to 12 days did not induce any detectable nitrite secretion (<0.1 μmol/10^6 cell/day). Similarly stimulation with 10^8 heat-killed *Listeria monocytogenes* or *Moraxella catarrhalis*, both potent

stimuli of high-output NOS in murine macrophages [34, 36], or a complete effector system of cell-mediated immunity consisting of autologous sensitized lymphocytes, PPD, and Il-2 [33] completely failed to induce NOS activity. In contrast, the same stimuli studied in parallel provoked nitrite production of 16–54 μmol/10^6 cells/24 h in thioglycolate-elicited murine macrophages.

It has been speculated that the failure to demonstrate NO production by human mononuclear phagocytes in vitro might represent an in vitro artifact because cytokines were found to augment urinary nitrate output in humans [22]. On the basis of such speculations, one could consider the possibility that nitrite measurement in culture supernatants would not be appropriate to detect NO production by human macrophages, for example because NO would be quenched and would not transform to nitrite. To exclude this possibility, we followed the fate of L-arginine, the substrate of NOS, in human and murine macrophages. Neither blood-derived nor peritoneal human macrophages consumed substantial amounts of L-arginine compared with murine macrophages, even after full stimulation with LPS, IFN-γ, GM-CSF, or TNFα, and accordingly produced neither NO nor citrulline, the deamination product of NOS (figures 1, 2). Furthermore, human mononuclear phagocytes, as distinct from murine macrophages, also did not metabolize detectable amounts of L-arginine in the urea cycle to ornithine and urea (figures 1, 2). When we compared quantitatively the consumption of L-arginine by human macrophages to that of 18 other amino acids, we found that L-arginine (which was purposely enriched in culture medium) was not consumed by human macrophages in excess of other amino acids. This indicates that L-arginine was principally used by human phagocytes for protein synthesis. Also in this respect, human cells contrasted with murine macrophages, which consumed 50–80
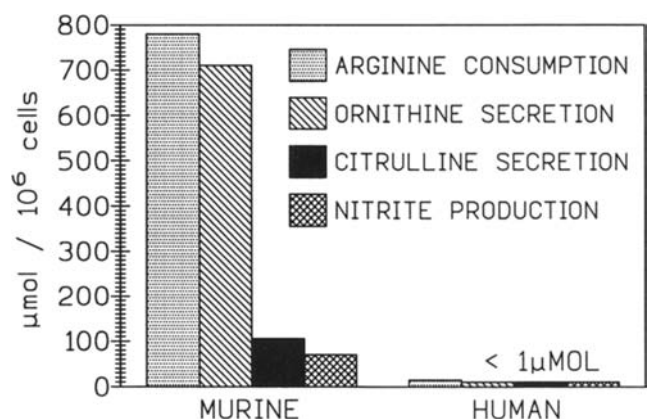

Figure 2. Comparison of L-arginine consumption, ornithine, citrulline, and nitrite secretion by human and murine peritoneal macrophages stimulated by combination of 200 ng/mL endotoxin and 100 units/mL interferon-γ for 48 h. Concentrations measured in 48-h culture supernatants (mean from duplicate wells).

Table 2. Summary of differences between NOS activity, tetrahydrobiopterin synthesis, and L-arginine metabolism in human and murine macrophages.

Parameter (per 10^6 cells/24 h)	Murine macrophages	Human macrophages
Nitrite secretion (μmol)	2–60	<0.1
L-arginine consumption (μmol)	600–800	10–20
Citrulline production (μmol)	Up to 60	<0.1
Ornithine production (μmol)	500–700	<0.1
Urea production (μmol)	800–1100	<0.1
Cytosolic NOS activity (μmol/10^6 cells/h)	1.4–2.2	<0.3
Cytosolic tetrahydrobiopterin (pmol/mg)	10–90	<0.03

NOTE. Cytosolic NOS activity was measured in duplicate experiments with cell lysates from activated (100 units of interferon-γ/mL and 200 ng of endotoxin/mL for 48 h) and nonactivated cells and (5×10^7 human and murine macrophages/assay). Lower values were from nonactivated cells, higher values from activated cells. No activity was found in vesicular fraction of murine and human cells or total lysates from human cells. Sensitivity level of assay was 0.3 μmol/10^6 cells/h. Comparison of cytosolic tetrahydrobiopterin, essential cofactor of NOS, is from [38, 39].

times more L-arginine than any of the other L-amino acids (not shown).

Finally, we turned to a direct measurement of NOS activity in cell lysates and subcellular fractions of human and murine macrophages. In a system in which NADPH and BH_4, two essential cofactors of NOS, as well as the substrate L-arginine are supplied in optimal concentrations [37], we were unable to detect NOS activity in subcellular fractions of human macrophages, while murine macrophages consistently showed NOS activity that increased after induction by LPS and IFN-γ (table 2).

Discussion

NO has been proposed as an important antimicrobial and antitumoral effector system of mononuclear phagocytes from laboratory animals. More and more observations on antimicrobial and cytotoxic effects of NO and the regulation of its production by macrophages appear in the literature, reflecting the enormous interest in this effector system (reviewed in [26–28, 40]). Our increasing knowledge of the NOS of rodent macrophages contrasts with the scarcity of data on a counterpart of this enzyme system in human mononuclear phagocytes.

Previous studies have cast doubt on the existence of a high-output NOS in human mononuclear phagocytes that could be induced to produce NO in consequential amounts [21, 23–25]. Only two studies have proposed that human macrophages produce NO by demonstrating nitrite in a micromolar range in supernatants from human blood–derived macrophages stimulated for extended periods with GM-CSF

and TNFα [3] or IFN-γ combined with TNFα [20]. In those two studies, however, it was not excluded that the terminal oxidases of the microorganisms, cocultured with macrophages, rather than the phagocytes had produced the nitrite recovered from supernatants [3, 20].

To end this controversy, we extended previous studies on NOS activity, NO production, and amino acid metabolism in human mononuclear phagocytes. Our attempts to induce macrophages to secrete NO in detectable amounts (≤0.1 μmol/10^6 cells/24 h) by activation with LPS, IFN-γ, TNFα, GM-CSF, intact bacteria, or proliferating lymphocytes, all potent stimuli for the induction of NOS in murine or rat macrophages, completely failed, while parallel studies with murine macrophages confirmed previous observations that resident and elicited peritoneal macrophages secret NO in a micromolar range, an activity that increases after activation up to 40–60 μmol/10^6 cells/24 h. Even a prolonged activation for up to 12 days was without effect on the capability of human macrophages to produce NO. The disparity between human and murine cells did not depend on the anatomic source of the phagocyte, because human peritoneal cells and blood-derived macrophages, in accordance with previous reports [21, 23], behaved comparably. Furthermore, rabbit mononuclear phagocytes derived from the blood or from the peritoneal cavity concurrently did not produce NO. Accordingly, it has previously been reported that thioglycolate-elicited macrophages from rabbits do not metabolize L-arginine to NO and citrulline [41].

A failure to detect NOS activity in human mononuclear phagocytes could theoretically be explained by a deficiency of BH_4, an essential cofactor of NOS [38, 39, 42]. Human mononuclear phagocytes lack the enzyme systems required for the complex biosynthesis of BH_4 and would, therefore, in clear distinction to murine macrophages, depend on an exogenous BH_4 source for NO synthesis, a hitherto neglected idea. However, even when BH_4 was substituted or when macrophages were cultured in the presence of proliferating lymphocytes that could provide BH_4 [39], no NO was produced.

Another explanation for the failure to demonstrate NO production by human macrophages could be that in contrast to murine systems, for some reasons NO could not be measured as nitrite in culture supernatants. Also, this possibility was excluded by the observation that human blood–derived and peritoneal macrophages, in contrast to murine cells, do not metabolize L-arginine to citrulline.

Finally, the absence of detectable NOS activity in total cell lysates, the cytosol, or the vesicular fraction of activated human macrophages speaks against an artificial deficit in substrate or cofactors of NOS in vitro. This is because all cofactors for maximal enzyme activity are provided in the assay that worked in parallel wells with murine cell lysates (this study) and in lysates of human hepatocytes [43], human cells that indeed have NOS activity.

In summary, these rigidly controlled studies, each accom-

panied by positive control experiments, showed that human macrophages, even when activated by numerous stimuli, have no NOS compared with the high activity of the inducible high-output NOS in murine macrophages. The failure to document NOS activity in human macrophages has been regarded in the past by some authorities as an in vitro artifact or has prompted speculations on unprecedented cofactors operative in human but not murine NOS in macrophages. These speculations must be strictly limited to macrophages because the in vitro demonstration of inducible high-output NOS activity is not associated with obstacles in human hepatocytes or their lysates [43].

Therefore, it is time to change our assumptions and to accept that human macrophages do not possess the high-output NOS of murine macrophages as they do not possess systems for the synthesis of its important cofactor, BH_4. Interspecies variations in the spectrum of nonoxidative killing systems of phagocytes are not without precedent, as shown with the defensin family of antibiotic peptides [44, 45]. We propose that in humans and most probably also in the rabbit, the inducible high-output NOS of macrophages that is operative in several other mammal species is not conserved as an antimicrobial system, possibly because phagocytic killing systems are redundant in mammals.

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