L-Arginine is not the limiting factor for nitric oxide synthesis by human alveolar macrophages *in vitro*

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ABSTRACT: Unlike murine mononuclear phagocytes, human macrophages do not release high amounts of nitric oxide (NO) *in vitro* despite the presence of nitric oxide synthase (NOS). To determine whether this limited NO synthesis *in vitro* is due to limited availability of the NOS substrate L-arginine, and putative NOS inhibiting factors present in foetal serum preparations, both alveolar macrophages (AM) and monocyte derived macrophages (MDM) were incubated in various circumstances.

Nitrite production measured using stimulated AM was typically $<5 \text{ pmol} \cdot \text{min}^{-1} \cdot 10^{-6}$ cells. A range of stimuli were tested, but without result. Furthermore, incubation of MDMs with normal human serum or purified bovine serum albumin instead of foetal calf serum failed to enhance NO production. Moreover, neither the use of arginase inhibitors nor the addition of surplus L-arginine resulted in an increased NO synthesis. Interestingly, addition of the NOS intermediate N ω -hydroxy-L-arginine (100 μM) to AM led to nitrite release, which was unaffected by the NOS inhibitor amino guanidine showing that this effect is NOS independent.

It is concluded that the limited nitric oxide production of human macrophages *in vitro* can neither be explained by limited availability of L-arginine, nor by nitric oxide synthase inhibiting substances in foetal serum. Furthermore, it is shown that nitrite release from N ω -hydroxy-L-arginine by alveolar macrophages is nitric oxide synthase independent.

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Nitric oxide (NO) is a well-documented effector molecule in rodents. Inflammatory cells, such as neutrophils and macrophages, release high amounts of NO upon immunostimulation [1, 2]. Moreover, studies performed in inducible nitric oxide synthase (NOS2 or iNOS) knock-out mice reveal that NO plays a crucial role in the defence against the protozoan *Leishmania major* [3]. Unlike rodent mononuclear phagocytes and granulocytes, both human macrophages and monocytes do not release significant amounts of NO *in vitro* when stimulated with the classical iNOS inducers interferon gamma (IFN- γ) and lipopolysaccharide (LPS). Despite this limited NO release, the induction of nitric oxide synthase (NOS) has been demonstrated by WEINBERG *et al.* [4] using immunohistochemistry and Western blots. Furthermore, the formation of 3-nitrotyrosine, a footprint of NO derived oxidants, has been demonstrated in human inflammatory disorders, *e.g.* asthma [5] and inflammatory bowel disease [6]. For an extensive review about iNOS induction and NO production by human mononuclear phagocytes, the reader is referred to WEINBERG *et al.* [7].

At present, it is unclear why human mononuclear phagocytes do not release high amounts of NO *in vitro* despite the fact that iNOS is inducible and low

amounts of NO formation are detectable [4]. Theoretically, this lack of high output NO synthesis could be due to an *in vitro* artefact, since *in vivo* NO production during inflammatory processes seems evident [8].

There are several possible explanations for the limited NO output by human inflammatory cells *in vitro*. Theoretically, essential immunomodulating factors could be missing in the incubation medium. In contrast, the medium could very well contain factors that prevent NO synthesis. An example is docosahexaenoic acid, which has been demonstrated to inhibit the induction of iNOS by murine macrophages [9]. Concentrations $\leq 150 \mu\text{M}$ have been found in foetal serum [9], which is a common constituent of culture media. The concentration in adult serum, however, is typically 50 times lower. Furthermore, the micro-environment (*i.e.* extracellular matrix) could be of importance for macrophage function [10].

According to MURPHY and NEWSHOLME [11], the lack of NO release by human mononuclear phagocytes is due to an increased breakdown of the NOS substrate L-arginine by the enzyme arginase. MURPHY and NEWSHOLME [11] claim that pre-incubating monocyte derived macrophages (MDM) with low levels of L-arginine prevents the induction of arginase. Resupplying the cells with L-arginine at the time of

Fig. 1. Schematic overview of L-Arginine metabolism by nitric oxide synthase and Arginase. Please note that *N*ω-hydroxy-L-arginine is an endogenous inhibitor of arginase. NOS: nitric oxide synthase; NO[•]: nitric oxide radical.

immunostimulation would thus result in enhanced NO synthesis, since breakdown of L-arginine by arginase is prevented (fig. 1 illustrates a schematic overview of L-arginine metabolism).

In this study, an attempt was made to induce NO output by human mononuclear phagocytes. For this purpose, both freshly isolated alveolar macrophages (AM) and MDM were used. Firstly, a variety of stimuli were screened on their ability to induce NO release. Secondly, the incubation circumstances (*i.e.* serum source and concentration, coating of the incubation plates) were varied. Moreover, whether the lack of high output NO synthesis *in vitro* can be explained by a limited availability of the NOS substrate L-arginine was tested.

Materials and methods

Alveolar macrophages

Nonsmoking, healthy volunteers underwent bronchoalveolar lavage (BAL), as described previously [12]. In short, four aliquots of 50 mL sterile phosphate-buffered saline (PBS) of 37°C were instilled into the lateral segment of the right middle lobe and recovered by gentle suction (-40 cmH₂O) after each aliquot. The BAL fluid was collected and immediately placed in ice. After collection, the BAL fluid was centrifuged for 5 min at 400×*g* (4°C). After washing (three times with PBS) the cells were resuspended in K-medium (which comprised of Roswell Park Memorial Institute 1640 medium supplemented with 10% foetal bovine serum, 10 mM Hepes, 4 mM glutamate, 2 mM pyruvate, 50 µg·mL⁻¹ gentamicin, penicillin/streptomycin and 100 µM β-mercapto-ethanol (all from Gibco-BRL, Merelbeke, Belgium) and incubated at 37°C with 5% carbon dioxide (CO₂) for 2 h in plastic petri dishes. Thereafter, nonadherent cells were washed away (three times with PBS); the adherent cells were scraped off and incubated in sterile 96-well plates (costar) at a concentration of 1×10⁶ cells·mL⁻¹. The cells were incubated overnight with and without stimuli, as described in the results section, at 37°C at 5% CO₂. Finally, the supernatant was collected and kept at -20°C until nitrite measurement.

Monocyte derived macrophages

MDMs were derived from mononuclear cells obtained by density gradient centrifugation of heparinized buffy coats (Red Cross Bloodbank, Utrecht,

the Netherlands) on Ficoll-paque (Amersham Pharmacia, Pharmacia, Uppsala, Sweden). The PBMC layer was collected, washed three times in Hank's balanced salt solution (HBSS) and incubated in 96-well flat-bottom microtitre plates (Corning Costar, Bodenheim, Germany), or in collagen I precoated plates (Becton & Dickinson, San Diego, CA, USA). Cells were incubated in Iscove's Modified Dulbecco' Medium (IMDM) containing different concentrations of foetal calf serum (FCS) or normal human serum (HS) (CLB, the Netherlands) with or without recombinant human granulocyte/monocyte-colony stimulating factor (rhGM-CSF) (Genzyme, Cambridge, MA, USA) for 5 days at 37°C and 5% CO₂ prior to stimulation. Twenty-four hours after the addition of stimuli the medium was collected and nitrite concentrations were measured.

Murine macrophages

Murine J774A.1 (ATCC, Manassas VA, USA) macrophages were maintained in K-medium and cultured at 37°C with 5% CO₂. The cells were passaged every 3 days. Cells were incubated overnight in flatbed 96-well plates (Costar) at 10⁶ cells·mL⁻¹ with and without mouse IFN-γ (Genzyme) (50 U·mL⁻¹) and LPS (10 µg·mL⁻¹) (Sigma, St Louis, MO, USA). After incubation, the medium was immediately analysed for nitrite content.

L-Arginine depletion

L-Arginine depletion is discussed according to MURPHY and NEWSHOLME [11]. Monocytes were isolated as described earlier. After washing, the cells were incubated for 48 h in L-arginine free Dulbecco's Minimal Essential Medium (DMEM) (Gibco-BRL) containing 0.1% fatty acid free bovine serum albumin (Merck, Darmstadt, Germany) and 100 International Units (IU) of penicillin and streptavidin (Gibco-BRL), at 37°C with 5% CO₂. After 48 h the cells were incubated for another 20 h with and without stimuli and L-arginine, as described in the Results section.

Denitrification of Nω-hydroxy-L-arginine by hydrogen peroxide

Hydrogen peroxide concentrations ranging from 0.02–1% were added to 100 µM of *N*ω-hydroxy-L-arginine (Alexis, Switzerland) dissolved in K-medium, and incubated for 2 h at 37°C with 5% CO₂. Thereafter, the nitrite concentration was measured.

Nitrite measurements

Nitrite concentrations were measured using the Griess reaction [13]. Griess reagent (100 µL: 1% sulphanilamide and 0.1% naphthyl-ethylenediamide in 5% phosphoric acid) was added to 100 µL of sample medium. After a 10-min incubation at room

temperature, the optical density was measured at 550 nm using a microplate reader (Benchmark, Biorad, Veenendaal, the Netherlands). Calibration curves were made with NaNO_2 dissolved in the incubation medium. The Griess assay used could discriminate between nitrite concentrations of $\geq 1 \mu\text{M}$, as assessed by the calibration curves made using authentic nitrite solutions.

Results

Effect of various stimuli on nitrite accumulation by alveolar macrophages and monocyte derived macrophages

Various immune and nonimmune stimuli were tested for their ability to induce NO synthesis in both human AM and MDMs. None of the tested stimuli were able to increase nitrite production in human cells (table 1). Nonetheless, stimulation of murine macrophages with IFN- γ /LPS resulted in high concentrations of nitrite in the culture medium.

Effect of different serum concentrations and source on nitrite production by monocyte derived macrophages

Addition of different concentrations of either normal HS or FCS to cells stimulated with a combination of IFN- γ ($200 \text{ U} \cdot \text{mL}^{-1}$), tumour necrosis factor (TNF)- α ($200 \text{ U} \cdot \text{mL}^{-1}$) and interleukin (IL)-1 β ($50 \text{ U} \cdot \text{mL}^{-1}$) with and without LPS did not induce nitrite accumulation (table 2). Coating of the microtitre plates with collagen I in the same circumstances as described earlier gave similar results.

Table 1. – Effect of different stimuli on nitrite production by human alveolar macrophages, human monocyte derived macrophages (MDMs) and murine J774.A macrophages *in vitro*

Stimulus	Alveolar macrophages	MDMs	Murine macrophages
LPS IFN- γ $10 \mu\text{g} \cdot \text{mL}^{-1}$, $200 \text{ U} \cdot \text{mL}^{-1}$	-	-	++
TNF- α $200 \text{ U} \cdot \text{mL}^{-1}$	-	-	ND
IFN- γ $200 \text{ U} \cdot \text{mL}^{-1}$	-	-	+
IL-1 β TNF- α IFN- γ (Cytomix)	ND	-	ND
LPS $10 \mu\text{g} \cdot \text{mL}^{-1}$	-	-	+
IL-1 β $50 \text{ U} \cdot \text{mL}^{-1}$	-	-	ND
GM-CSF $200 \text{ U} \cdot \text{mL}^{-1}$	-	ND	ND
IL-4 $50 \text{ U} \cdot \text{mL}^{-1}$	-	ND	ND
FMLP $1 \times 10^{-7} \text{ M}$	ND	-	ND
PMA 10 nM	ND	-	ND

LPS: lipopolysaccharide; IFN- γ : interferon gamma; TNF- α : tumour necrosis factor- α ; IL: interleukin; GM-CSF: granulocyte-macrophage colony-stimulating factor; FMLP: Formyl-Met-Leu-Phe $1 \times 10^{-7} \text{ M}$; PMA: Phorbol myristate acetate 10 nM . -: <5 ; +: >10 ; ++: $>50 \text{ pmoles nitrite min}^{-1} 10^6 \text{ cells}^{-1}$; ND: not determined.

Table 2. – Effect of serum source on nitrite accumulation by stimulated monocyte derived macrophages *in vitro*

Serum added	Cytomix	Cytomix/ LPS	Cytomix/ Coat*
No Serum	-	-	-
Normal human serum 1%	-	-	-
Normal human serum 5%	-	-	-
Normal human serum 10%	-	-	-
Foetal calf serum 1%	-	-	-
Foetal calf serum 5%	-	-	-
Foetal calf serum 10%	-	-	-

–: no detectable nitrite accumulation; Cytomix: interferon- γ , tumour necrosis factor- α , interleukin-1 β ; Coat*: Collagen I.

Effect of arginase inhibition on nitrite accumulation by alveolar macrophages and monocyte derived macrophages

Figure 2 shows that the addition of the NOS intermediate and arginase inhibitor *N* ω -hydroxy-L-arginine ($100 \mu\text{M}$) to stimulated AMs resulted in a significant ($p < 0.05$, $n=5$) production of nitrite. This release, however, could not be inhibited by the NOS inhibitor aminoguanidine (1 mM). Similar results were obtained using MDMs ($n=2$). Furthermore, the arginase inhibiting amino acid L-valine (10 mM) failed to enhance nitrite accumulation (data not shown).

*Hydrogen peroxide releases nitrite from *N* ω -hydroxy-L-arginine*

Hydrogen peroxide dose-dependently released nitrite from $100 \mu\text{M}$ of *N* ω -hydroxy-L-arginine after 2 h of incubation in a cell-free system (fig. 3).

Fig. 2. – Effect of *N* ω -hydroxy-L-arginine ($100 \mu\text{M}$) on nitrite release by human alveolar macrophages ($n=5$) and monocyte derived macrophages ($n=2$) *in vitro* in combination with stimulation (interferon gamma/tumour necrosis factor- α $200 \text{ U} \cdot \text{mL}^{-1}$). The addition of 1 mM of the nitric oxide synthase inhibitor aminoguanidine did not change the effect. □: monocyte derived macrophages; ▨: alveolar macrophages. IFN- γ : interferon gamma; TNF- α : tumour necrosis factor- α ; AG: aminoguanidine. *: $p < 0.05$.

Fig. 3.—Denitrification of *N*ω-hydroxy-L-arginine by hydrogen peroxide (H₂O₂). *N*ω-hydroxy-L-arginine (100 μM) was incubated for 2 h in the presence of increasing concentrations of hydrogen peroxide in medium.

Effect of L-arginine depletion/repletion on nitrite synthesis

Pre-incubation of monocytes in L-arginine free medium for 48 h did not result in an enhanced NO synthesis upon stimulation. Furthermore, replenishment of L-arginine after this period had no effect (table 3).

Discussion and conclusions

At present, it is still unclear why human macrophages and monocytes fail to produce NO in levels comparable to murine cells, especially since NO production during human inflammatory processes *in vivo* seems evident [5, 8]. Despite the lack of NO production *in vitro*, previous work by WEINBERG *et al.* [4] clearly demonstrated that the NO producing enzyme iNOS can be brought to expression in both human macrophages and monocytes, as shown by Western blots and immunohistochemistry. Interestingly, the same group showed that the essential iNOS cofactor tetrahydrobiopterin (BH₄) is absent in human mononuclear phagocytes. Replenishment of intracellular BH₄ levels, however, also failed to activate the enzyme. However, it cannot be excluded that, as such, NO is a less important defence mechanism in humans than in rodents. Thus, NO release by

Table 3.—Effect of L-arginine depletion/repletion on nitrite accumulation by monocytes *in vitro*

Stimulus	No addition	+1 mM L-arginine
Control	-	-
LPS IFN-γ 10 μg·mL ⁻¹ , 200 U·mL ⁻¹	-	-
IFN-γ 200 U·mL ⁻¹	-	-
LPS 10 μg·mL ⁻¹	-	-

LPS: lipopolysaccharide; IFN-γ: interferon gamma. -: <5 pmol nitrite min⁻¹ 10⁶ cells⁻¹.

human inflammatory cells could also be limited *in vivo*, despite the presence of iNOS.

According to MURPHY and NEWSHOLME [11], the limited NO production by human mononuclear phagocytes is due to an increased breakdown of the NOS substrate L-arginine by arginase. They suggest that incubation of human mononuclear phagocytes with L-arginine concentrations as high as 1 mM (which is normal in culture media) induces the enzyme arginase. Arginase in turn breaks down L-arginine, putatively depleting the substrate for NOS synthesis. In the present study, however, culturing the cells without L-arginine, using the same procedure as described by MURPHY and NEWSHOLME [11], did not result in enhanced NO synthesis. Moreover, addition of the arginase inhibitors L-valine or *N*ω-hydroxy-L-arginine also failed to induce NOS-dependent nitrite accumulation. In agreement with the present data, WEINBERG *et al.* [4] reported that addition of L-arginine to mononuclear phagocytes in concentrations high enough to avoid breakdown by arginase did not result in an increased NO production. It is, therefore, concluded that low NO output by human mononuclear phagocytes is not due to a limited L-arginine availability.

Foetal or neonatal calf serum is a common constituent of cell culture media. Foetal serum contains docosahexaenoic acid, which has been demonstrated to inhibit the induction of NOS by murine macrophages [9]. In theory, compounds like these could account for the limited NO production by human mononuclear phagocytes. The present data show that neither this compound nor other constituents of FCS account for the lack of high output NO release, since incubation of MDMs without any serum, with fatty acid free bovine serum albumin or with adult human serum did not boost NO synthesis.

Despite the fact that neither human mononuclear phagocytes nor granulocytes release high amounts of NO, there is a report stating that these cells release peroxynitrite, the coupling product of NO and superoxide. The evidence in this paper, however, is based solely on oxidation of the aspecific peroxynitrite probe 123-dihydrorhodamine without evidence for NO production [14]. Considering the fact that 123-dihydrorhodamine oxidation by human neutrophils is completely dependent on myeloperoxidase activity [15], it is doubtful that human neutrophils release peroxynitrite.

Interestingly, incubating both AM and MDMs with the NOS intermediate *N*ω-hydroxy-L-arginine, which is also a potent inhibitor of arginase [16], resulted in an increased release of nitrite in the culture medium. This nitrite accumulation, however, could not be inhibited by the NOS inhibitor aminoguanidine, showing that it is an NOS independent effect. Moreover, addition of hydrogen peroxide to *N*ω-hydroxy-L-arginine in a cell free system also resulted in nitrite release. Finally, recent data demonstrated that denitrification *N*ω-hydroxy-L-arginine by human neutrophils does not release NO but directly leads to nitrite/nitrate formation instead [17]. Interestingly, NAGASE *et al.* [18] showed that hydrogen peroxide also releases NO from L-arginine, as confirmed by

chemiluminescence. It is unclear whether nitrite/NO release from *N*ω-hydroxy-L-arginine and L-arginine reflect physiological mechanisms.

In summary, the present data show that the limited nitric oxide release by human mononuclear phagocytes can neither be explained by a limited availability of the nitric oxide synthase substrate L-arginine, nor by putative inhibiting factors present in foetal calf serum. It is concluded that great care should be taken whilst interpreting results concerning nitric oxide and its metabolites (*i.e.* peroxynitrite) obtained by the use of human granulocytes and mononuclear phagocytes *in vitro*.

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