Expression of Inducible Nitric Oxide Synthase and Formation of Nitric Oxide by Alveolar Macrophages: An Interspecies Comparison

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Nitric oxide (NO) is suggested to play a role in mediating pulmonary injury. However, interspecies differences appear to exist in the ability of alveolar macrophages (AM) to express the inducible nitric oxide synthase (iNOS) and to generate NO. The purpose of this study was to compare iNOS expression and NO production by rat, hamster, monkey, and human AM using the identical experimental conditions *in vitro*. As AM donors, CD rats, Syrian golden hamsters, cynomolgus monkeys, and nonsmoking, healthy human volunteers were used. The AM were obtained by bronchoalveolar lavage and stimulated *in vitro* with various concentrations and combinations of lipopolysaccharide (LPS) and interferon-γ (IFN-γ). The oxidation product of NO, nitrite, was measured in the AM supernatant by the Griess reaction. The expression of iNOS in AM was detected using immunocytochemistry and immunoblotting. The expression of iNOS mRNA was assessed by reverse transcriptase–polymerase chain reaction (RT–PCR). Rat AM, stimulated with either LPS or IFN-γ, produced nitrite in a time- and dose-dependent manner. Combination of LPS and IFN-γ resulted in a significantly enhanced nitrite formation. However, none of the treatments was able to induce hamster, monkey, or human AM to release measurable amounts of nitrite. Whereas expression of iNOS protein was only detected in stimulated rat AM, expression of iNOS mRNA was found in unstimulated and stimulated rat AM, slightly in stimulated hamster AM, but not in monkey and human AM. In conclusion, our findings point to distinct regulatory mechanisms of the NO pathway in AM from these four different species. — *Environ Health Perspect* 105(Suppl 4):1297–1300 (1997)

Key words: nitric oxide, inducible nitric oxide synthase, alveolar macrophages, species differences, rat, hamster, monkey, human

Introduction

Species differences in response to various agents are well known in biomedical research. If such differences are identified and characterized at the cellular and molecular level, they might help to improve the knowledge of the pathomechanisms of certain diseases. For two of the rodent species used in inhalation toxicology, rat and hamster, such species differences have already been reported concerning their pulmonary reactions to inhalation of pure oxygen (*1*), diesel soot (*2*), or mineral fibers (*3*). After inhalation of pathogenic material, alveolar macrophages (AM) constitute one of the first lines of cellular defense. Interaction of AM with particles or fibers might result in the formation of reactive oxygen species, such as superoxide anion, and reactive nitrogen species, such as nitric oxide (NO) (*4*). The reaction of superoxide anion with NO forms a potent oxidant, peroxynitrite, which may contribute to inflammatory tissue damage (*5,6*). The production of NO and other reactive nitrogen intermediates is already well established for cytokine-activated rat and mouse AM (*7–10*). However, the presence of such a pathway in monocytes/macrophages from a number of species, including humans, is the subject of great controversy (*11–16*). Recently, we reported that hamster AM, in contrast to rat AM, lack the ability to express the inducible nitric oxide synthase (iNOS) protein and to release detectable amounts of NO after lipopolysaccharide (LPS) and cytokine stimulation *in vitro* (*7*). The objective of the work presented here was to extend these studies by comparing the iNOS expression and NO formation by AM from the two rodent species rat and hamster and the two primate species monkey and human using the identical experimental conditions *in vitro.*

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Abbreviations used: AM, alveolar macrophage(s); BSA, bovine serum albumin; BAL, bronchoalveolar lavage; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; LPS, lipopolysaccharide; NO, nitric oxide; mRNA, messenger ribonucleic acid; PBS, phosphate-buffered saline; RT–PCR, reverse transcriptase–polymerase chain reaction.

Methods

Alveolar Macrophage Donors

CD rats (Crl:CD(SN)BR; 250–350 g) and Syrian golden hamsters (Lak:LVG(SYR)BR; 120–150 g) were obtained from Charles River (Sulzfeld, Germany) and kept in a conventional, nonbarrier rodent housing unit. Water and standard rodent laboratory diets (ssniff, Soest, Germany) were supplied *ad libitum*. Cynomolgus monkeys were born and raised at the institutional animal holding facilities. The human samples were obtained from nonsmoking male and female volunteers, 20 to 30 years of age, with no history of recent pulmonary disease.

Cell Isolation and Culture

AM were obtained by bronchoalveolar lavage (BAL). Rats and hamsters were anesthetized by an ip injection of sodium pentobarbital (rat, 30 mg/kg bw; hamster, 24 mg/kg bw). The lungs were mobilized and lavaged *in situ* as described by Dörger et al. (*7*). Fiberoptic bronchoscopy with BAL was performed in monkeys under general anesthesia with ketamine (15 mg/kg bw) and xylazine (2 mg/kg bw) and in human volunteers under local anesthesia as decribed by Krombach et al. (*17*) and Behr et al. (*18*). For each species studied, the procedure of processing the BAL samples was identical. The pooled samples were

centrifuged at 300×*g* for 10 min; the cell pellet was washed twice, and resuspended in RPMI 1640 (Seromed, Munich, Germany) supplemented with L-glutamine, gentamycin (0.16 mg/ml), and 10% heat-inactivated fetal bovine serum (Gibco BRL, Eggenstein, Germany). Total cell counts were assessed with a standard hemacytometer (Coulter Electronics, Krefeld, Germany). Air-dried cytocentrifuge smears (500 rpm × 5 min) served to identify the cellular populations after staining with May-Grünwald-Giemsa. The preparations contained about 97 to 100% AM, as characterized by morphologic criteria. The cell viability was determined by trypan blue exclusion and was greater than 90% for rat, hamster, and monkey AM, and greater than 75% for human AM. Lavaged cells were then plated to 96-well flat-bottomed cell culture plates (Nunclon Delta, Roskilde, Denmark) at a density of 0.2×10^6 cells/well and cultured for 2 hr at 37°C and 5% CO_2/95% air. The nonadherent cells were removed with 37°C warm RPMI 1640 medium, and the adherent cells were covered with 200 µl of medium. The AM were treated for 24 hr with either *Escherichia coli* LPS (1, 10, 100, and 1000 ng/ml) or interferon-γ (IFN-γ) (1, 10, and 100 U/ml). LPS was purchased from Sigma Chemie (Taufkirchen, Germany), rat specific IFN-γ came from Innogenetics (Ismaning, Germany), and human recombinant IFN-γ (for human, monkey, and hamster cells) was from Boehringer Mannheim (Mannheim, Germany).

Measurement of Nitric Oxide Production

The NO concentration in AM supernatants was determined by measuring the oxidation product nitrite with the Griess reaction using a microplate assay method as described by Ding et al. (*19*). Nitrite concentrations were calculated from a standard sodium nitrite curve.

Immunocytochemistry

The expression of iNOS protein in AM was determined after stimulation with either 100 ng/ml LPS or 10 ng/ml LPS plus 100 U/ml IFN-γ for 20 hr at 37°C. Immunocytochemistry was performed as described earlier (*7*) using a polyclonal rabbit antimouse iNOS antibody (Dianova, Hamburg, Germany) that cross-reacts with rat and human iNOS according to the manufacturer's instructions. Binding of the antibody to hamster iNOS was verified by immunohistochemistry of pancreatic tissue sections taken from animals 18 hr after bolus injection of LPS (5 mg/kg bw, iv) (data not shown). Binding of the antibody to monkey iNOS has not been explored so far, yet the degree of the homology among amino acid sequences of iNOS between rodents and humans is about 80 to 94% (*20*).

Immunoblotting

Immunoblotting was performed as described previously (*7*). Briefly, AM were cultured with 100 ng/ml LPS or 10 ng/ml LPS plus 100 U/ml IFN-γ for 24 hr at 37°C. After incubation and lysis of cells, cell extracts were run on a sodium dodecyl sulfate (SDS)–polyacrylamide gel and transferred to a nitrocellulose membrane. After application to the iNOS antibody, the membrane was exposed to an alkaline phosphatase-conjugated goat antirabbit IgG and developed with 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT).

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA were extracted from AM after incubation with 10 ng/ml LPS plus 100 U/ml IFN-γ for 16 hr using reverse transcriptase (RT). The cDNA was amplified by polymerase chain reaction (PCR) with a DNA thermal cycler (Perkin Elmer, Cetus Corp., Norwalk, CT). The amplification reaction was carried out as described earlier (*7*). Oligonucleotide primers for iNOS were CACAAGGCCACATCGGATTTC (sense) and TGCATACCACTTCAACCCGAG (antisense), which correspond to the murine macrophage iNOS (*21*), and AGTTTCTGGCAGCAACGG (sense) and TTAAGTTCTGTGTGCCGGCAG (antisense) (MWG-Biotech, Ebersberg, Germany), which correspond to human iNOS (*22*).

Results

Formation of Nitric Oxide

To induce NO formation by AM, we incubated the cells with various concentrations of LPS or IFN-γ. Stimulation with either LPS or IFN-γ resulted in a dose-dependent NO release by rat AM. In contrast, none of these stimuli was able to induce a detectable NO release by hamster, monkey, or human AM (Table 1). Next, we investigated whether stimulation with LPS plus IFN-γ would either increase the NO generation by rat AM or actually induce an NO release by hamster, monkey, or human AM. In this series of experiments, AM were cultured with 1 ng/ml LPS and graded concentrations of IFN-γ at the same time. As shown in Table 2, simultaneous incubation with LPS plus IFN-γ resulted in a potentiated NO production by rat AM, but had no effect on either hamster or primate AM.

Expression of iNOS Protein

To analyze the expression of iNOS protein by rodent and primate AM, we used immunocytochemical and immunoblotting methods. After immunocytochemical

Table 1. Nitrite formation (nmol/mg protein) by rat, hamster, monkey, and human alveolar macrophages upon stimulation with LPS or IFN-γ.

Stimulus	Rat $n = 6$	Hamster $n = 3$	Monkey $n = 3$	Human $n = 3$
None	–[a]	–	–	–
1 ng/ml LPS	101.9 ± 23.8[b]	–	–	–
10 ng/ml LPS	225.9 ± 54.5	–	–	ND
100 ng/ml LPS	257.6 ± 50.1	–	–	–
1000 ng/ml LPS	265.5 ± 55.1	–	–	ND
1 U/ml IFN-γ	15.0 ± 15.0	–	–	ND
10 U/ml IFN-γ	124.7 ± 6.8	–	–	ND
100 U/ml IFN-γ	195.2 ± 35.8	–	–	–

ND, not determined. [a]Below detection limit of 0.5 nmol. [b]Values are means ± SEM.

Table 2. Effect of IFN-γ on LPS-induced nitrite formation (nmol/mg protein) by rat, hamster, monkey, and human alveolar macrophages.

Stimulus	Rat $n = 6$	Hamster $n = 3$	Monkey $n = 3$	Human $n = 3$
1 ng/ml LPS	101.9 ± 23.8[a]	–[b]	–	–
1 ng/ml LPS + 1 U/ml IFN-γ	137.2 ± 21.5	–	–	ND
1 ng/ml LPS + 10 U/ml IFN-γ	181.7 ± 23.4	–	–	ND
1 ng/ml LPS + 100 U/ml IFN-γ	278.2 ± 21.9	–	–	–

ND, not determined. [a]Values are means ± SEM. [b]Below detection limit of 0.5 nmol.

Table 3. Expression of iNOS protein and mRNA in rat, hamster, monkey, and human alveolar macrophages.

Method	Stimuli	Rat	Hamster	Monkey	Human
Immunocytochemistry	None	–	–	–	–
	LPS	++	–	–	–
	LPS + IFN-γ	+++	–	–	–
Immunoblotting	None	–	–	–	–
	LPS	++	–	–	–
	LPS + IFN-γ	+++	–	–	–
RT–PCR	None	++	–	–	–
	LPS + IFN-γ	+++	+	–	–

staining with a polyclonal rabbit antimouse iNOS antibody, the native protein was labeled only in stimulated rat AM, but not in hamster, monkey, or human AM (Table 3). Consistent with the immunocytochemical data, the appearance of a band at approximately 125 kD, corresponding to the molecular weight of iNOS protein, was noted only in stimulated rat AM. In contrast, no specific reactivity was found in hamster, monkey, and human AM, whether untreated or treated (Table 3).

Expression of iNOS mRNA

RT–PCR assay was used to detect iNOS mRNA expression in rat, hamster, monkey, and human AM. Using the mouse iNOS primer, a PCR product of predicted size of 741 bp was found in unstimulated and stimulated rat AM, and in stimulated hamster AM. As reported earlier, iNOS mRNA appeared to be expressed at a lower level in hamster AM compared to rat AM (*7*). In contrast, iNOS transcripts were not found in monkey AM, using mouse or human iNOS primers, nor in human AM.

Discussion

Among two of the rodent species often used in inhalation toxicology, rat and hamster, species differences concerning their pulmonary reactions to inhalation of pure oxygen, diesel soot, and mineral fibers have already been reported (*1–3*). However, the cellular and molecular mechanisms causing such differences remain unclear. Recently, we reported that hamster AM, in contrast to rat AM, lack the ability to express iNOS and to produce NO after stimulation with LPS and/or IFN-γ *in vitro* (*7*). NO and its reactive metabolites may play a crucial role in inflammation, tissue damage, mutagenesis, and carcinogenesis (*23*). The production of NO and other reactive nitrogen intermediates has already been well established for cytokine-stimulated rat and mouse macrophages (*8–10*), whereas the presence and regulation of the NO pathway in monocytes/macrophages from various species, including humans, still remains controversial (*7,11–16,24–27*). Here, we focused our interest on comparing iNOS expression and NO production by AM from two rodent species, rat and hamster, and two primate species, monkey and human, under identical experimental conditions *in vitro*.

Our data presented here confirm previous reports on the dose-dependent NO formation by rat AM (*9,10*) and the lack of NO generation by hamster AM upon incubation with LPS and/or IFN-γ (*7*). Now, we have extended these findings by demonstrating also that AM from two primate species, cynomolgus monkey and human, were not activated by LPS and/or IFN-γ to form detectable amounts of NO. In support of this finding, several studies suggested that primate monocytes/macrophages release no NO, or only modest amounts, after incubation with LPS and/or certain cytokines *in vitro* (*11–14,24,25*).

In addition, we have shown that both iNOS mRNA and protein were expressed in stimulated rat AM. These results agree with those previously reported for rat AM (*7,10,26*). iNOS mRNA was barely transcribed in activated hamster, but not in monkey and human AM, and iNOS protein was not expressed by AM from either species. Nevertheless, recent reports suggest that human AM from patients with lung inflammation occasionally express the iNOS protein (*26*) and that AM from patients with tuberculosis transcribe iNOS mRNA (*27*). However, we had the opportunity to examine AM from a heavy smoker with bronchial carcinoma and did not detect any expression of iNOS protein or iNOS mRNA (unpublished data).

The data reported here extend our previous observations that monkey and human AM, in contrast to rat AM, failed to express iNOS and to generate NO upon stimulation with LPS and/or IFN-γ *in vitro*. Thus, in their inability to express the iNOS protein and to generate NO *in vitro*, hamster AM tend to resemble monkey and human AM more than rat AM. These results suggest marked discrepancies among rodent species concerning the presence and regulation of the high-output NO pathway in AM, whereas among primate species such differences apparently do not exist. If these *in vitro* data on interspecies differences in iNOS expression and NO production are confirmed *in vivo*, they might improve our knowledge of the molecular mechanisms causing the disparate pulmonary responses of different species to inhaled irritants or toxicants.

REFERENCES

1. Bryan CL, Jenkinson SG. Species variation in lung antioxidant enzyme activities. J Appl Physiol 63:597–602 (1987).
2. Brightwell J, Fouillet X, Caasano-Zoppi AL, Bernstein D, Crawley F, Duchosal F, Gatz R, Perczel S, Pfeifer H. Tumours of the respiratory tract in rats and hamsters following chronic inhalation of engine exhaust emissions. J Appl Toxicol 9:23–31 (1989).
3. Wahrheit DB, Hartsky MA. Influences of gender, species, and strain differences in pulmonary toxicological assessments of inhaled particle and/or fibers. In: Toxic and Carcinogenic Effects of Solid Particles in the Respiratory Tract (Mohr U, ed). Washington:ILSI Press, 1994;25–265.
4. Thomas G, Ando T, Verma K, Kagan E. Asbestos fibers and interferon-γ up-regulate nitric oxide production in rat alveolar macrophages. Am J Respir Cell Mol Biol 11:707–715 (1994).
5. Ischiropoulos H, Zhu L, Beckmann JS. Peroxynitrite formation from macrophage-derived nitric oxide. Arch Biochem Biophys 298:446–451 (1992).
6. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 268:L699–L722 (1995).
7. Dörger M, Jesch NK, Rieder G, Hirvonen MR, Savolainen K, Krombach F, Messmer K. Species differences in nitric oxide formation by rat and hamster alveolar macrophages *in vitro*. Am J Respir Cell Mol Biol 16:413–420 (1997).
8. Vodovotz Y, Kwon NS, Pospischil M, Manning J, Paik J,

Nathan C. Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophages with IFN-γ and bacterial lipopolysaccharide. J Immunol 152:4110–4118 (1994).
9. Prokhorova S, Lavnikova N, Laskin DL. Functional characterization of interstitial macrophages and subpopulations of alveolar macrophages from rat lung. J Leukoc Biol 55:141–146 (1994).
10. Pendino KJ, Laskin JD, Shuler RL, Punjabi CJ, Laskin DL. Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. J Immunol 151:7196–7205 (1993).
11. Weinberg JB, Misukonis MA, Shami PJ, Mason SN, Sauls DL, Dittman WA, Wood ER, Smith GK, McDonald B, Bachus KE et al. Human mononuclear phagocyte inducible nitric oxide synthase (iNOS): analysis of iNOS mRNA, iNOS protein, biopterin, and nitric oxide production by blood monocytes and peritoneal macrophages. Blood 86:1184–1195 (1995).
12. Schneemann M, Schoedon G, Hofer S, Blau N, Guerrero L, Schaffner A. Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes. J Infect Dis 167:1358–1363 (1993).
13. Denis M. Human monocytes/macrophages: NO or no NO? J Leukoc Biol 55:682–684 (1994).
14. Padgett EL, Pruett SB. Evaluation of nitrite production by human monocyte-derived macrophages. Biochem Biophys Res Commun 186:775–781 (1992).
15 Cameron ML, Granger DL, Weinberg JB, Kozumbo WJ, Koren HS. Human alveolar and peritoneal macrophages mediate fungistasis independently of L-arginine oxidation to nitrite or nitrate. Am Rev Respir Dis 142:1313–1319 (1990).
16. Adler H, Frech B, Thöny M, Pfister H, Peterhans E, Jungi TW. Inducible nitric oxide synthase in cattle. J Immunol 154:4710–4718 (1995).
17. Krombach F, Fiehl E, Burkhardt D, Rienmüller R, König G, Adelmann-Grill BC, Idel H, Rosenbruch M. Short-term and long-term effects of serial bronchoalveolar lavages in a nonhuman primate model. Am J Respir Crit Care Med 150:153–158 (1994).
18. Behr J, Vogelmeier C, Beinert T, Meurer M, Krombach F, König G, Fruhmann G. Significance of bronchoalveolar lavage for evaluation and management of scleroderma lung disease. Am J Respir Crit Care Med 154:400–406 (1996).
19. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J Immunol 141:2407–2412 (1988).
20. Michel T, Xie QW, Nathan C. Molecular biological analysis of nitric oxide synthases. In: Methods on Nitric Oxide Research (Feelisch M, Stamler JS, eds). Chichester, UK:Wiley, 1996; 161–176.
21. Liu S, Adcock IM, Old RW, Barnes PJ, Evans TW. Lipopolysaccharide treatment *in vivo* induces widespread tissue expression of inducible nitric oxide synthase mRNA. Biochem Biophys Res Commun 196: 1208–1213 (1993).
22. Sirsjö A, Karlsson M, Gidlöf A, Rollman O, Törmä H. Increased expression of inducible nitric oxide synthase in psoriatic skin and cytokine-stimulated cultured keratinocytes. Br J Dermatol 134:643–648 (1996).
23. Gross SS, Wolin MS. Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57:737–769 (1995).
24. Albina JE. On the expression of nitric oxide synthase by human macrophages. Why no NO. J Leukoc Biol 58:643–649 (1995).
25. Zembala M, Siedlar M, Marcinkiewicz J, Pryjma J. Human monocytes are stimulated for nitric oxide release *in vitro* by some tumor cells but not by cytokines and lipopolysaccharide. Eur J Immunol 24:435–439 (1994).
26. Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stamler JS. Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. Am J Respir Cell Mol Biol 9:371–377 (1993).
27. Nicholson S, da Glória Bonecini-Almeida M, Lapa e Silva JR, Nathan C, Xie Q-W, Mumford R, Weidner JR, Calaycay J, Geng J, Boechat N et al Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. J Exp Med 183:2293–2302 (1996).