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.

Methods

Alveolar Macrophage Donors

CD rats (Crl:CD(SN)BR; 250–350 g) and Syrian golden hamsters (LaJ: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 described 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

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Abbreviations used: AM, alveolar macrophages; 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.
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 CACAAGGCACATCGGATTC (sense) and TGCATACACCTCAACCCGAG (antisense), which correspond to the murine macrophage iNOS (21), and AGTTTCTGGCAGCCAAAGG (sense) and TTAAGTCTGTTGGCCGAG (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 potentiating 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              | -         | -             | -            | -           |
| 1 ng/ml LPS       | 101.9±23.8b | -             | -            | ND          |
| 10 ng/ml LPS      | 225.9±54.5 | -             | -            | ND          |
| 1000 ng/ml LPS    | 257.6±50.1 | -             | -            | ND          |
| 1 U/ml IFN-γ      | 265.4±55.1 | -             | -            | ND          |
| 10 U/ml IFN-γ     | 150.0±15.0 | -             | -            | ND          |
| 100 U/ml IFN-γ    | 124.7±6.8  | -             | -            | ND          |
| 278.2±35.8        | -         | -             | -            | -           |

ND, not determined. *Below detection limit of 0.5 nmol. 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±23.8b | -             | -            | ND          |
| 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±22.4 | -             | -            | ND          |
| 1 ng/ml LPS + 100 U/ml IFN-γ | 278.2±21.9 | -             | -            | -           |

ND, not determined. *Values are means±SEM. **Below detection limit of 0.5 nmol.
staining with a polyclonal rabbit antiamouse 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 toxins.

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