Defective Nitric Oxide Production by Alveolar Macrophages during *Pneumocystis* Pneumonia

Mark E. Lasbury¹, Chung-Ping Liao¹, Chadi A. Hage², Pamela J. Durant¹, Dennis Tschang¹, Shao-Hung Wang³, Chen Zhang¹, and Chao-Hung Lee^{1,4}

¹Department of Pathology and Laboratory Medicine, and ²Division of Pulmonary, Allergy, Critical Care, and Occupational Medicine, Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana; ³Department of Microbiology and Immunology, National Chiayi University, Chiayi City, Taiwan; and ⁴Graduate Institute of Clinical Medical Science and Department of Laboratory Medicine, China Medical University, Taichung, Taiwan

The effect of nitric oxide (NO) on *Pneumocystis* (Pc) organisms, the role of NO in the defense against infection with Pc, and the production of NO by alveolar macrophages (AMs) during *Pneumocystis* pneumonia (PCP) were investigated. The results indicate that NO was toxic to Pc organisms and inhibited their proliferation in culture. When the production of NO was inhibited by intraperitoneal injection of rats with the nitric oxide synthase inhibitor L-N⁵-(1-iminoethyl) ornithine, progression of Pc infection in immunocompetent rats was enhanced. Concentrations of NO in bronchoalveolar lavage fluids from immunosuppressed, Pc-infected rats and mice were greatly reduced, compared with those from uninfected animals, and AMs from these animals were defective in NO production. However, inducible nitric oxide synthase (iNOS) mRNA and protein concentrations were high in AMs from Pc-infected rats and mice. Immunoblot analysis showed that iNOS in AMs from Pc-infected rats existed primarily as a monomer, but the homo-dimerization of iNOS monomers was required for the production of NO. When iNOS dimerization cofactors, including calmodulin, were added to macrophage lysates, iNOS dimerization increased, whereas incubation of the same lysates with all cofactors except calmodulin did not rescue iNOS dimer formation. These data suggest that NO is important in the defense against Pc infection, but that the production of NO in AMs during PCP is defective because of the reduced dimerization of iNOS.

Keywords: *Pneumocystis*; nitric oxide; iNOS; alveolar macrophage; calmodulin

Complex interactions between host and pathogen occur during *Pneumocystis* pneumonia (PCP), triggering both innate and adaptive immune responses. Studies showed that the alveolar macrophage (AM) is an important cell for the clearance of *Pneumocystis* organisms from the lungs during PCP (1, 2). The clearance of organisms by AMs is mediated by direct phagocytosis (2–4), the production of toxic metabolites (5, 6), and the modulation of inflammatory cytokines (2–4). Some of these functional abilities are defective (7, 8), and the number of AMs (7, 9) is decreased during PCP. The reasons for the reduced function of AMs are largely unknown.

AMs are an important source of nitric oxide (NO) in the lung during infection (10). NO is a short-lived, freely diffusible molecule with many functions in normal physiology (11) and in response to cytokines (12), infectious agents, or bacterial products (13). It has an antimicrobial effect on organisms such

CLINICAL RELEVANCE

This research reveals the mechanism by which infection with *Pneumocystis* causes a defect in the production of nitric oxide in alveolar macrophages. This information will enable the development of methods to prevent this defect and enhance host defense ability against infection with *Pneumocystis*.

as *Toxoplasma gondii* (14), *Leishmania donovani* (15), and *Cryptococcus neoformans* (16). NO also causes a redistribution of the actin pool in some cells (17). This reorganization is mediated by calmodulin, because the inhibition of calmodulin activity suppresses redistribution, despite high concentrations of NO (17). Calmodulin was shown to be an important molecule for the survival of AMs, but its concentration in AMs is decreased during PCP (18).

Pneumocystis (Pc) organisms were shown to stimulate AMs from humans and rats to produce NO (19), and both chemically produced NO (20) and IFN- γ -stimulated NO (21) are toxic to Pc organisms. However, elevated concentrations of inducible nitric oxide synthase (iNOS) mRNA and protein in AMs in Pc-infected, CD4⁺ cell-depleted mice did not reduce the organism burden or slow disease progression (22, 23). In this study, we investigated this problem further, and found that the inability of the iNOS protein to form dimers was a cause of this defect.

MATERIALS AND METHODS

Reagents

(Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino] diazen-1-ium-1,2-diolate (Spm NONOate), diethylammonium (Z)-1-(N,N-diethylamino) diazen-1-ium-1,2-diolate (DEA NONOate), and L-N⁵-(1-iminoethyl)ornithine (L-NIO) were purchased from Cayman Chemical (Ann Arbor, MI). Tetrahydrobiopterin (BH4) was purchased from Alexis Biochemicals (Lausen, Switzerland). Heme was obtained from MP Biochemicals (Irvine, CA), and L-arginine hydrochloride was purchased from Fisher Bioreagents (Pittsburgh, PA). Anti-rat and anti-mouse iNOS antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was obtained from US Biological (Swampscott, MA). Calmodulin protein was purchased from ANIARA (Manson, OH). All other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless stated otherwise.

Short-Term Culture of Pc Organisms

Pc organisms were grown on monolayers of human embryonic lung (HEL 299) cells (CCL-137; ATCC, Manassas, VA) on 24-well culture dishes in 0.5 ml minimum essential medium containing 2 mM L-glutamine, 17.9 mM sodium bicarbonate, 0.1 mM nonessential amino acids, 10% FCS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C and 5% O₂, 10% CO₂, and 85% nitrogen. *Pneumocystis carinii* inoculum was isolated and added to cultures as previously described (24).

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Correspondence and requests for reprints should be addressed to Chao-Hung Lee, Ph.D., Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Fesler Hall, 1120 South Drive, Room 419, Indianapolis, IN 46202-5113. E-mail: chlee@iupui.edu

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Rodent Models of PCP

The immunosuppression of rats with dexamethasone (7) and of mice with anti-CD4 antibody (1, 25), the transtracheal inoculation of animals with *Pc* organisms (7), bronchoalveolar lavage (BAL) (26), and the isolation of AMs from BAL fluids (7) were performed as previously described.

Infection of Mice with *Histoplasma capsulatum*

Histoplasma capsulatum (IU-CT) yeasts were grown in Histoplasma Macrophage Medium (HMM) broth, as described previously (27). Six-week-old C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) were anesthetized with 5% isoflurane and intranasally instilled with 50 μ l inoculum containing 1×10^6 yeast of *H. capsulatum*. At 7 and 14 days after infection, BAL fluids and AMs were obtained from these mice by BAL.

Assessment of Nitrate/Nitrite Concentrations

The nitrite assay was performed using a NO Quantitation Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions.

RNA Isolation and Real-Time RT-PCR

Total RNA was isolated from AMs using the TRIzol reagent, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Real-time RT-PCR for rat or murine iNOS was performed using Assays-on-Demand Gene Expression Kits (Applied Biosystems, Foster City, CA) on a Smartcycler (Cepheid, Sunnyvale, CA). Ribosomal protein S8 (RPS8) mRNA was assayed in an identical manner as a control, as described elsewhere (28). Organism viability was estimated based on the ribosomal RNA levels of *Pc* mitochondrial large-subunit ribosomal RNA gene, as described elsewhere (29).

Immunoblot Blot Analysis of iNOS Protein

Determinations of protein concentration and Western blotting were performed as described elsewhere (30). For the determination of iNOS dimer formation, electrophoresis was performed under partially denatured conditions to allow the iNOS dimer to remain intact, as previously described (31). Immunoblot autoradiographs were analyzed using the ImageJ software package, which is a Java-based image processing program developed at the National Institutes of Health (32).

Statistical Analysis

Comparisons between the mean values of treatment and control groups and data regarding the effects of NO on *Pc* proliferation over time were analyzed using the Mann-Whitney U test. $P < 0.05$ was considered significant.

RESULTS

Production of NO by Chemical Generators in Culture Media, and Effects of NO on *Pc* Viability

Spm NONOate and DEA NONOate were incubated in complete medium, and the release of NO from these chemicals was induced by decreasing the pH of the medium from 8.0 to 7.4 with 1 N HCl. At pH 7.4 and 37°C, the half-life of DEA NONOate is 2.1 minutes, and that of Spm NONOate is 39 minutes (product information; Cayman Chemical). The pH of medium in a well of each condition was checked daily and maintained at 7.0–7.4. Both chemicals produced NO in a dose-dependent manner. The production of NO (measured as nitrites) by DEA NONOate (0.1 mM) peaked ($20.2 \pm 2.0 \mu\text{M}$) at 15 minutes after lowering the pH; little NO was produced after that time. The production of NO by Spm NONOate (0.1 mM) was still increasing 60 minutes after the change in pH ($23.7 \pm 1.8 \mu\text{M}$ at 60 minutes). Lower concentrations (0.01 mM) of Spm NONOate and DEA NONOate generated roughly 30% as much NO as the higher concentrations ($6.9 \pm 1.2 \mu\text{M}$ for Spm NONOate, and $6.5 \pm 2.0 \mu\text{M}$ for DEA NONOate). The times

for peak production were the same as those for the higher concentrations of each compound. All values shown are means $\pm$ SDs.

To test the toxicity of these compounds, a half million *P. carinii* organisms were incubated with carrier (0.01 mM NaOH) or NO generators, and then assessed for viability by measuring *Pc* mitochondrial large-subunit ribosomal RNA concentrations at 2 or 4 hours (Figure 1). Compared with carrier-treated control concentrations at 4 hours, 0.01 mM Spm NONOate decreased *Pc* viability by 17%, whereas 0.1 mM Spm NONOate reduced it by 33% at 2 hours and 43% at 4 hours (Figure 1). *Pc* organisms incubated with 0.01 mM or 0.1 mM DEA NONOate did not show significant decreases in viability at either time point, probably because of the shorter duration of NO production by DEA NONOate.

Suppression of *Pc* Proliferation in Short-Term Culture by NO

Pc was cultured on HEL cell monolayers. High (0.1 mM) and low (0.01 mM) concentrations of NO generators were included in the culture media and replenished every 24 hours. Separate cultures treated with carrier (0.1% DMSO in saline) or trimethoprim/sulfamethoxazole (Tpm/Smx, 50/250 $\mu\text{g}/\text{ml}$) were used as negative and positive controls, respectively. The cultures were examined under an inverted microscope every other day to assess the health of the monolayer. Any culture showing signs of contamination by other microorganisms (bacteria or fungi) was discarded. Organisms were sampled and counted every other day, as described previously (24). Untreated cultures showed a significant increase in numbers of organisms after 7 days of culture (21.3 ± 4.1 organisms per $\times 1,000$ microscopic field; Figure 2). Tpm/Smx inhibited the propagation of organisms in culture by 89% (2.4 ± 0.4 organisms per $\times 1,000$ microscopic field; $P < 0.0001$ versus untreated controls) at the same time point (7 days). DEA NONOate at either concentration and Spm NONOate at 0.01 mM exerted minimal effects (Figure 2), whereas Spm NONOate at 0.1 mM suppressed 97% of *Pc* growth at 7 days (0.58 ± 0.1 organisms per $\times 1,000$ microscopic field). These results indicate that the NO generated was inhibitory to *Pc* replication in short-term culture.

Figure 1. Effect of NO on *Pneumocystis* organisms. *P. carinii* organisms were incubated with 0.01 or 0.1 mM (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazene-1-ium-1,2-diolate (Spm NONOate) or diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-ium-1,2-diolate (DEA NONOate) for 2 or 4 hours, and then assayed for viability by real-time RT-PCR for *P. carinii* mitochondrial large-subunit ribosomal RNA. * $P < 0.05$ versus carrier (0.01 mM NaOH) controls at the same time point. § $P < 0.05$ versus same condition at 2 hours.

Figure 2. Inhibitory effect of NO on proliferation of *Pneumocystis* (Pc) in short-term culture. *P. carinii* organisms were inoculated onto human embryonic lung cell monolayers in 24-well plates, and numbers of organisms in cultures were counted every other day. Some wells contained trimethoprim/sulfamethoxazole (Tmp/Smx; 50/250 μ g/ml) or the nitric oxide generators Spm NONOate or DEA NONOate at 0.1 mM or 0.01 mM, and the media were adjusted to pH 7.4 daily. Results are averages $\pm$ SDs of quadruplicate wells of each condition in five independent experiments.

To control for the toxicity of NO to HEL cells, the health of the monolayer was monitored in each condition. Without Pc, HEL cell culture alone exhibited only $5.4\% \pm 1.0\%$ cell death, but $54.6\% \pm 6.6\%$ of Pc-infected cells died ($P < 0.05$ versus controls) after 7 days of culture. Spm NONOate at 0.1 mM inhibited 97% of Pc growth, as already mentioned, but caused only $10.7\% \pm 2.3\%$ cell death by Day 7, indicating that the suppression of Pc growth in cultures by NO generators was not attributable to damage of the cell monolayer by NO.

Increased Pc Proliferation *In Vivo* by Suppressing the Production of NO

To determine if NO participates in the control of Pc proliferation *in vivo*, uninfected (Normal) and Pc-inoculated Normal (Normal-Pc) rats were injected (intraperitoneally) daily with L-NIO at 2 mg/kg to inhibit the production of NO, and then assessed for organism burden and NO concentrations in AMs and BAL fluid. NO is quickly converted to nitrites and nitrates *in vivo* (33–35). Nitrates can be converted to nitrites through the action of nitrate reductase. Therefore, NO concentrations in AMs and BAL fluid were determined by measuring nitrites after the conversion of nitrates to nitrites, using nitrate reductase in a NO quantitation kit. AMs were isolated, incubated for 1 hour in saline, lysed by sonication, and assayed for nitrite concentrations.

AMs from rats treated with L-NIO for 1 week produced 1.5 ± 0.2 μ M (normalized to 1 mg of AM protein) of nitrites. This amount was 62% less than that (3.9 ± 0.5 μ M) produced by AMs from untreated rats ($P < 0.05$; Figure 3). The ability to produce NO by AMs was further impaired with longer treatment of L-NIO. After 2 weeks of L-NIO injections, the production of nitrites in AMs decreased by 73% (1.0 ± 0.1 μ M versus 3.7 ± 0.2 μ M for untreated controls; $P < 0.05$; Figure 3).

The response of AMs to stimulation by IFN- γ and *Escherichia coli* organisms to produce NO was then assessed. One hour of incubation with 10 ng/ml IFN- γ and 5×10^6 *E. coli* organisms increased the production of nitrites in AMs from Normal rats by approximately threefold (from 3.9 ± 0.5 μ M to 11.6 ± 2.2 μ M for those treated with carrier for 1 week, and

Figure 3. Effect of L-N⁵-(1-iminoethyl)ornithine (L-NIO) on alveolar macrophage (AM) nitrite concentrations and *Pneumocystis* burden. (A) Uninfected (Normal) and Pc-challenged (Normal-Pc) immunocompetent rats received intraperitoneal injections daily with 2 mg/kg of L-NIO, starting 2 days after inoculation with Pc. AMs were isolated from these rats 1 or 2 weeks after initiation of L-NIO treatment, and assessed for nitrite concentrations with or without stimulation of the AMs with 10 ng/ml IFN- γ and 5×10^6 *Escherichia coli*. Nitrite concentrations are expressed in μ M relative to 1 mg AM protein in cell lysate. (B) Numbers of *Pneumocystis* organisms (both trophic and cyst forms) in Normal-Pc rats after L-NIO treatment were determined by examining Giemsa-stained lung impression smears. Numbers of organisms consist of average counts from 10 microscopic fields at $\times 1,000$ magnification. * $P < 0.05$, compared with untreated group.

from 3.7 ± 0.2 to 10.2 ± 1.1 μ M for those treated with carrier for 2 weeks). In contrast, AMs from L-NIO-treated rats increased the production of nitrites by only 1.4-fold and 1.2-fold, respectively, from 1.5 ± 0.2 μ M to 2.1 ± 0.4 μ M for those treated for 1 week, and from 1.0 ± 0.1 μ M to 1.2 ± 0.2 μ M for those treated for 2 weeks (Figure 3). AMs from Normal-Pc rats that had been inoculated with Pc for 1 week or 2 weeks produced significantly more nitrites (14.7 ± 2.0 μ M and 15.3 ± 1.8 μ M, respectively; $P < 0.05$, versus controls at the same time point). Stimulation with IFN- γ and *E. coli* did not increase the concentrations of nitrites produced by AMs at either time point.

In contrast to the increase in nitrite production in Normal rats inoculated with Pc, AMs from L-NIO-treated Normal-Pc rats did not show an increase in the production of nitrites in response to Pc inoculation (3.2 ± 0.4 μ M after 1 week, and 2.3 ± 0.2 μ M after 2 weeks of L-NIO treatment; $P < 0.05$ for each, versus Normal-Pc rats). IFN- γ and *E. coli* also failed to stimulate further production of nitrites in these cells (3.0 ± 0.3 μ M for 1 week, and 2.1 ± 0.3 μ M for 2 weeks; Figure 3).

Organism burdens in Normal-Pc rats with or without L-NIO treatment were also determined. After 1 week of infection, impression smears of lungs from Normal-Pc rats showed an average of

TABLE 1. NITRITE CONCENTRATIONS IN BALF AND AMs FROM RATS AND MICE WITH PCP*

Sample	Animal	Normal	Suppressed†	4 Weeks of PCP	7 Weeks of PCP	IFN- γ /Escherichia coli ‡	Normal-Pc§
BALF	Rat	3.2 $\pm$ 0.2	3.4 $\pm$ 0.6	NA	2.1 $\pm$ 0.3*	NA	9.6 $\pm$ 1.3#
	Mouse	2.3 $\pm$ 0.4	2.2 $\pm$ 0.2	1.3 $\pm$ 0.1¶	1.0 $\pm$ 0.1*	NA	NA
AMs	Rat	4.2 $\pm$ 0.3	4.5 $\pm$ 0.4	NA	0.8 $\pm$ 0.1*	1.0 $\pm$ 0.1¶	15.6 $\pm$ 1.2#
	Mouse	4.8 $\pm$ 0.4	4.3 $\pm$ 0.3	2.0 $\pm$ 0.2¶	1.7 $\pm$ 0.2*	2.1 $\pm$ 0.3¶	NA

Definition of abbreviations: AMs, alveolar macrophages; BALF, bronchoalveolar lavage fluid; NA, not assessed; Pc, *Pneumocystis*; PCP, *Pneumocystis* pneumonia. All values are mean $\pm$ SD.

* BALF and AM lysates were treated with nitrate reductase to convert nitrates to nitrites before the determination of nitrite concentrations. Nitrite concentrations in the first 5 ml rat BALF or the first 1 ml mouse BALF are expressed in μ M, and those in AMs (1×10^6 from rats, and 5×10^5 from mice) are expressed in μ M relative to 1 mg of protein in cell lysates.

† Rats were immunosuppressed with dexamethasone, and mice were immunosuppressed with anti-CD4 antibody.

‡ Preincubated with 10 ng/ml IFN- γ and 5×10^6 *Escherichia coli* for 1 hour before assay.

§ Normal rats challenged with *P. carinii*.

¶ $P < 0.05$ versus immunosuppressed control samples.

$P < 0.05$ versus normal control samples.

0.6 $\pm$ 0.1 trophic forms and 0.1 $\pm$ 0.1 cysts per $\times 1,000$ microscopic field (Figure 3). Rats treated with L-NIO showed higher burdens, with an average of 1.9 $\pm$ 0.2 trophic forms and 0.2 $\pm$ 0.1 cysts at the same time point. These represent 3.1-fold and twofold increases in trophic forms and cysts, respectively, and both increases were statistically significant ($P < 0.05$ versus Normal-Pc). At 2 weeks, the difference in organism burden between untreated and L-NIO-treated rats was even larger. Normal-Pc rats had begun to clear the organisms, and both trophic form (0.5 $\pm$ 0.1) and cyst (0.1 $\pm$ 0.1) numbers were reduced or unchanged. In contrast, organism burdens in L-NIO-treated rats had increased from 1.9 $\pm$ 0.2 trophic forms/field to 3.6 $\pm$ 0.2 trophic forms/field, and from 0.2 $\pm$ 0.1 cysts/field to 0.6 $\pm$ 0.1 cysts/field at 1 week ($P < 0.0001$ versus Normal-Pc and Normal-Pc + L-NIO; Figure 3). These results demonstrate that AMs do produce NO in response to infection with Pc, and that the inhibition of NO production hampers the clearance of Pc from the lungs of immunocompetent rats. Because L-NIO-treated rats were not immunosuppressed, they all cleared the infection and survived.

Low Nitrite Concentrations in BAL Fluids of Pc-Infected Rats and Mice

To investigate the production of NO in the lung during PCP, the first 5 ml of BAL fluids from uninfected and Pc-infected immunosuppressed rats were assayed for nitrite concentrations. The results showed that BAL fluids from dexamethasone (Dex)-Pc rats contained 35% less nitrites than those from either Normal or Dex rats ($P < 0.05$; Table 1). BAL fluids from Normal-Pc rats had threefold more nitrites than those from Normal rats that were not inoculated with Pc, and 4.5-fold more than those from Dex-Pc rats ($P < 0.05$; Table 1).

Previous results indicate that CD4-depleted-Pc mice respond differently from rats in the timing of decreases in numbers of AMs (9). Therefore, BAL fluid (first 1 ml) nitrites were assessed at both 4 and 7 weeks of infection, and were found to contain 41% (1.3 $\pm$ 0.1 μ M) and 55% (1.0 $\pm$ 0.2 μ M), respectively, less nitrites than those from CD4-depleted mice ($P < 0.05$; Table 1). These data suggest that the decrease in nitrites in BAL fluid during PCP is not species-specific, and is not related to the method of immunosuppression.

Decreased Nitrite Concentrations in AMs during PCP in Rats and Mice

To assess the production of NO by AMs from rats and mice with PCP, AM-produced nitrites were measured after 1 hour of culture. Nitrite concentrations were first assayed in AM samples treated with saline alone, to determine background values of

nitrites for the assay. AMs from Dex-Pc rats ($n = 15$) were found to produce only 20% as much NO ($P < 0.05$ versus Dex controls; Table 1) as those from Normal or Dex rats ($n = 12$ each). Stimulation of these cells with IFN- γ and *E. coli* did not significantly increase nitrite concentrations. Likewise, CD4-depleted-Pc mice from early (4-week) and late (7-week) infections contained significantly lower nitrite concentrations ($P < 0.05$; Table 1; $n = 15$ –20). These results indicate that AMs from PCP rats and mice are defective in the production of NO.

Production of NO in AMs from *H. capsulatum*-Infected Mice

To determine whether the defect in NO production by AMs is specific to infection with Pc, mice were infected with the yeast form of *H. capsulatum* by intranasal instillation. At 7 days (mid-stage) and 14 days (late stage) after infection, nitrite concentrations in AMs and BAL fluid were measured. The nitrite concentrations in both AMs and BAL fluid from mice infected for 7 days exhibited no significant difference, compared with uninfected controls. However, 5.56-fold and 4.62-fold increases in nitrite concentrations in AMs and BAL fluid, respectively, were evident 14 days after infection (Table 2). These results suggest that in contrast to infection with Pc, the production of NO in AMs increases during *H. capsulatum* infection.

iNOS Transcription in PCP Rats and Mice

Real-time RT-PCR for iNOS mRNA concentrations was performed to determine if the defect in NO production was attributable to the reduced transcription of the iNOS gene. RNAs from two million AMs from Normal, Dex, and Dex-Pc rats, or one million AMs from Normal, CD4-depleted, or CD4-depleted-Pc mice were converted to cDNAs and used for the real-time RT-PCR. As shown in Figure 4, iNOS transcripts, normalized to RPS8, were 14.6-fold higher in AMs from Dex-Pc rats than in those from Dex rats ($P < 0.05$). This finding is in

TABLE 2. NITRITE CONCENTRATIONS IN BALF AND AMs FROM *H. CAPSULATUM*-INFECTED MICE

	Uninfected	<i>H. capsulatum</i> -Infected	
		Day 7	Day 14
BALF	0.29 $\pm$ 0.35	0.26 $\pm$ 0.41	1.34 $\pm$ 0.34*
AMs	0.18 $\pm$ 0.20	0.10 $\pm$ 0.32	1.00 $\pm$ 0.12*

BALF and AM lysates were treated with nitrate reductase to convert nitrates to nitrites before the determination of nitrite concentrations. Nitrite concentrations in the first 1 ml BALF are expressed in μ M, and those in AMs (5×10^5) are expressed in μ M relative to 1 mg of protein in the cell lysates. All values are mean $\pm$ SD.

* $P < 0.05$ versus uninfected control.

Figure 4. Inducible nitric oxide synthase (iNOS) mRNA concentrations in AMs during *Pneumocystis pneumonia* (PCP). Concentrations of iNOS mRNA relative to those of control gene ribosomal protein S8 (RPS8) in AMs from *Pneumocystis*-infected rats (A) and mice (B) were determined by real-time RT-PCR. Dexamethasone (Dex)-Pc AMs were from rats that had been infected with *P. carinii* for 6 weeks. CD4-depleted-Pc (CD4-depl-Pc) AMs were from mice infected with *P. murinii* for 4 weeks. Normal-Pc AMs were isolated from normal rats 17 days after inoculation with *P. carinii*. Results represent mean fold change $\pm$ SD relative to normal controls from 4–7 animals in each group. * $P < 0.05$ versus immunosuppressed group (Dex or CD4-depl). # $P < 0.05$ versus Normal group.

direct contrast to the low nitrite concentrations in AMs from Dex-Pc rats (Table 1). iNOS mRNA concentrations in AMs from Normal and Dex rats were very similar, indicating that immunosuppression by dexamethasone did not significantly affect iNOS transcription in AMs. AMs from Normal-Pc rats had increased iNOS transcripts, compared with those from Normal rats (14.7-fold; $P < 0.05$). As with rats, real-time RT-PCR results indicated that AMs from CD4-depleted-Pc mice had higher concentrations (13.6-fold) of iNOS mRNA than those from CD4-depleted mice (Figure 4). These results indicate that the upregulation of iNOS transcription in AMs is a normal immune response to Pc.

iNOS Protein Concentrations in Rats and Mice with PCP

To determine whether the iNOS mRNA detected in AMs from rats was translated, Western immunoblotting was performed. Equal amounts of AM-soluble protein from Normal, Dex, and Dex-Pc rats were electrophoresed on a polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was reacted with antibody against the iNOS protein, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. The iNOS protein was detected in all samples (Figure 5A), and AMs from Dex-Pc rats were found to produce significantly more iNOS protein than AMs from Normal or Dex rats.

The CD4-depleted murine model of PCP was also assessed for the expression of iNOS protein. As shown in Figure 5B, the amount of iNOS protein was low in AMs from Normal and CD4-depleted mice, but AMs from those infected with *P.*

Figure 5. iNOS protein concentrations in AMs from PCP rats and mice. (A) Total soluble protein from AMs of Normal, Dex, and Dex-Pc rats was separated by SDS-PAGE and reacted with an anti-iNOS antibody. (B) AM proteins from Normal, CD4⁺ lymphocyte-depleted (CD4-depl), and CD4-depleted Pc-infected (CD4-depl-Pc) mice were treated as described for rat AMs in A. CD4-depl-Pc mice were infected with *P. murina* for 4 or 7 weeks before being killed. The iNOS protein shown here is the 130-kD monomer. Results are representative of four independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

murina for either 4 or 7 weeks contained significantly increased concentrations of iNOS protein.

iNOS Dimerization in AMs during PCP

To determine if the decreased production of NO by AMs was attributable to reduced iNOS dimerization, partially denaturing immunoblotting was performed as described previously (31) to identify both the iNOS dimer (260 kD) and monomer (130 kD) in AM lysates. As shown in Figure 6A, pooled AMs from three Normal or Dex rats had approximately equal levels of iNOS monomer and dimer. This ratio was shifted in AMs from Dex-Pc rats with high concentrations of the iNOS monomer and low concentrations of the iNOS dimer (Figure 6A). Image analysis of the immunoblots showed that Normal and Dex animals dimerized 46–58% of their AM iNOS (Figure 6B). In contrast, AMs from Dex-Pc rats dimerized only 15% of their iNOS protein (Figure 6B).

Effects of Cofactors on iNOS Dimerization

To determine whether the low iNOS dimer concentrations were attributable to a reduced availability of cofactors necessary for iNOS dimerization, AM lysates from Normal, Dex, or Dex-Pc rats were incubated for 30 minutes with iNOS dimerization cofactors in sterile saline containing 1 mM PMSF before being analyzed for iNOS dimers. These cofactors included heme, tetrahydrobiopterin (BH₄), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), arginine, and in some conditions, calmodulin. The concentrations of cofactors used were similar to those previously reported (36, 37).

Normal or Dex AM lysates, supplemented with all cofactors, showed similar concentrations of iNOS dimers (Figure 7A). For Dex-Pc AM lysates, concentrations of iNOS monomers were high and those of iNOS dimers were low without the presence of cofactors. The addition of all cofactors significantly increased the concentrations of iNOS dimers, whereas the concentrations of monomers decreased (Figure 7A). However, when calmod-

Figure 6. iNOS dimer formation in rat AMs during PCP. (A) Total soluble protein from rat AMs was separated by SDS-PAGE under partially denaturing conditions, as previously described (31), and subjected to Western blotting with an anti-iNOS antibody. Pooled AMs from three Normal and Dex rats and five Dex-Pc rats were used. The molecular weights of the iNOS monomer and dimer are 130 and 260 kD, respectively. Image analysis of four independent Western blots was performed by normalizing iNOS monomer and dimer signals to that of the control protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) Average iNOS dimer concentrations $\pm$ SDs are plotted as a percentage of iNOS monomer concentrations. * $P < 0.05$, versus Dex control samples.

ulin was not included among the cofactors, Dex-Pc AM lysates contained very low concentrations of iNOS dimers (Figure 7A). Image analysis of three independent immunoblots (Figure 7B) showed that $17.9\% \pm 1.9\%$ of iNOS proteins were dimerized without the addition of any cofactors, and that $18.4\% \pm 2.1\%$ of iNOS proteins were dimerized when the cell lysate was incubated with all cofactors except calmodulin. In contrast, $62.0\% \pm 9.8\%$ of iNOS proteins were dimerized when the cofactors included $5 \mu\text{M}$ calmodulin (Figure 7B).

DISCUSSION

To survive in the lung, Pc must evade the actions of AMs. We previously showed that infection with Pc renders AMs defective in phagocytosis (8) and induces AM apoptosis (1, 7, 9). In this study, we found that AM defense is further weakened during infection with Pc because of decreased NO production. We first demonstrated that Pc is susceptible to NO produced *in vitro* by the NO generators, Spm NONOate or DEA NONOate. Spm NONOate at a concentration of 0.1 mM also inhibited the proliferation of Pc in culture on HEL monolayers (Figure 2). This inhibition was attributable to effects of NO on the organism rather than on the feeder HEL cells. These data agree with previous results on the toxicity of NO to Pc (20, 21), and confirm that its action is directed against the organism.

The production of NO by AMs is mediated by iNOS, and the regulation of iNOS expression occurs primarily at the transcriptional level. Therefore, Shellito and colleagues determined iNOS mRNA and protein concentrations in AMs from uninfected and Pc-infected CD4 cell-depleted mice and severe combined immunodeficiency (SCID) mice, to investigate the role of NO in host defense against infection with Pc (23).

Figure 7. iNOS dimer formation in AM lysates after incubation with cofactors and calmodulin. (A) AMs from Normal ($n = 3$), Dex ($n = 3$), and Dex-Pc ($n = 5$) rats were pooled and sonicated on ice. AM lysates were incubated with iNOS dimerization cofactors, including heme ($10 \mu\text{M}$), tetrahydrobiopterin ($10 \mu\text{M}$), flavin adenine dinucleotide (FAD) ($4 \mu\text{M}$), flavin mononucleotide (FMN) ($4 \mu\text{M}$), nicotinamide adenine dinucleotide phosphate (NADPH) (2 mM), and arginine (2 mM), with or without calmodulin ($5 \mu\text{M}$), in sterile saline solution containing 1 mM PMSF. After 30 minutes of incubation, cell lysates were subjected to partially denaturing Western blot analysis, as described in the legend for Figure 6. (B) Histogram represents average iNOS dimerization levels $\pm$ SDs from three independent trials. * $P < 0.05$ versus Dex controls.

Because the organism burden remained high despite elevated concentrations of iNOS mRNA and protein in the AMs of these mice, they concluded that iNOS is an unlikely participant in host defense against Pc, and speculated that NO is not important in this regard, but they did not measure concentrations of NO or nitrites (23). In the present study, we also found that concentrations of both iNOS mRNA and protein had increased in AMs from Pc-infected animals (Figures 4 and 5), but the production of NO by these cells was defective (Table 1). This finding explains why the organism burden was high even though iNOS concentrations were high. The iNOS detected by Shellito *et al.* (23) may not have been functional. Therefore, insufficient NO was produced to kill the organisms. The defect in NO production was not evident in AMs from *H. capsulatum*-infected mice (Table 2). Because *H. capsulatum* can infect immunocompetent animals, these *H. capsulatum*-infected mice were not immunosuppressed. Therefore, whether host immunosuppression or Pc infection itself is responsible for the decreased production of NO in AMs remains to be confirmed.

We found that AMs from immunocompetent rats challenged with Pc responded to the organism by producing large amounts of NO, and the concentrations remained high for 2 weeks while the animals were clearing the organisms. These data suggest that the production of NO is a normal host response to infection with Pc in immunocompetent animals. This possibility is supported by the observation that lower concentrations of NO in L-NIO-treated Normal-Pc rats were accompanied by in-

creased organism burdens (Figure 3). The increase in organism burden was more pronounced after 2 weeks of treatment with L-NIO, suggesting that NO plays a role in the control of Pc proliferation during the normal immune response.

When AMs from normal rats were stimulated with IFN- γ and *E. coli*, the production of NO increased approximately threefold (Table 1). Surprisingly, no additional production of NO was evident when AMs from normal rats challenged with Pc were treated with IFN- γ and *E. coli*. This lack of response to stimulation with IFN- γ and *E. coli* in the production of NO was also evident in AMs from Pc-infected rats (Table 1). These results suggest that infection with Pc can render the production of NO in AMs defective. This is confirmed by the findings that AMs from Pc-infected rats produced very low concentrations of NO, and that these AMs were also unresponsive to stimulation with IFN- γ and *E. coli* (Table 1). The expression of iNOS in AMs may be maximally induced during infection with Pc. Therefore, additional response does not occur when the cells are stimulated with IFN- γ or *E. coli*.

The decrease in production of NO in Dex-Pc rats was not attributable to dexamethasone, because the antibody-mediated immunosuppression of mice by the depletion of CD4⁺ cells revealed the same phenomenon. Previous studies indicate that these mice undergo a transient increase in AM number before a profound decrease by 7 weeks of infection (9). The low nitrite concentrations in murine lungs at 4 weeks of infection, when the number of AMs is maximal, suggest that the production of NO in AMs is suppressed even at early stages of infection.

In addition to the regulation of transcription, the production of iNOS can also be regulated at the translational or post-translational level. Transforming growth factor- β can promote the degradation of iNOS protein and decrease translation of the transcript (36). Imidazole-based antifungal agents (37) and carbon monoxide (38) can inhibit the dimerization of iNOS or the activity of the dimer. Cofactor availability and binding also modulate the stability of iNOS, because the suppression of BH4 by cytokines results in reduced iNOS dimerization (39). In this study, iNOS protein synthesis increased during PCP (Figure 5), indicating that the reduction in NO production during PCP was not attributable to defects in the production of the iNOS protein.

iNOS must homo-dimerize to form a functional enzyme and requires the cofactors BH4, heme, arginine, NADPH, FAD⁺, FMN, and calmodulin for this action (40, 41). iNOS converts arginine to citrulline and NO (40, 42). If arginine concentrations are low, the iNOS protein will not dimerize (41). The dimerization of iNOS is initiated by the binding of calmodulin, FAD⁺, and FMN to the monomer, followed by the addition of a heme molecule near the N-terminus of the monomer. This stimulates the addition of another iNOS monomer, which is stabilized by arginine and BH4 (41). The results of our study showed that the dimerization of iNOS monomers occurs normally in AMs from uninfected and Pc-challenged immunocompetent animals, but is reduced in AMs from Dex-Pc rats (Figures 6 and 7).

The observation that the addition of calmodulin and other cofactors, but not the other cofactors alone, rescued iNOS dimer formation (Figure 7) suggested that the available calmodulin is insufficient in AMs during PCP. This conclusion is supported by our recent results indicating that calmodulin mRNA and protein concentrations are significantly decreased in AMs during PCP (18). Calmodulin is a ubiquitous calcium-sensing protein. The binding of calcium to calmodulin results in an altered conformation and permits it to bind other proteins. iNOS dimerization requires apocalmodulin (calcium-free calmodulin) as a cofactor (40, 43).

The results of this study suggest that the production of NO is an important host response to Pc, and that the defect in iNOS

dimerization confers a survival advantage to the organism. AMs attempt to increase the production of NO by upregulating the expression of iNOS, but fail to achieve this goal because of the decreased availability of calmodulin for dimerization. Because calmodulin downregulation also contributes to AM defects in survival and granulocyte macrophage colony-stimulating factor (GM-CSF) signaling (18), additional research is required to define the mechanisms that prevent calmodulin downregulation and restore iNOS dimerization. Although the inhalation of NO in conjunction with Tmp/Smx and prednisolone was used to treat PCP (44, 45), the roles of iNOS and NO in the defense against Pc infections in humans are not known. This animal study will serve as a model for further studies on the involvement of NO in human PCP.

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