Constitutive nitric oxide production by rat alveolar macrophages

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Miles, P. R., L. Bowman, A. Rengasamy, and L. Huffman. Constitutive nitric oxide production by rat alveolar macrophages. *Am. J. Physiol.* 274 (*Lung Cell. Mol. Physiol.* 18): L360–L368, 1998.—Results from previous studies suggest that alveolar macrophages must be exposed to inflammatory stimuli to produce nitric oxide (·NO). In this study, we report that naive unstimulated rat alveolar macrophages do produce ·NO and attempt to characterize this process. Western blot analysis demonstrates that the enzyme responsible is an endothelial nitric oxide synthase (eNOS). No brain or inducible NOS can be detected. The rate of ·NO production is ~0.07 nmol·10^6 cells^{-1}·h^{-1}, an amount that is less than that produced by the eNOS found in alveolar type II or endothelial cells. Alveolar macrophage ·NO formation is increased in the presence of extracellular L-arginine, incubation medium containing magnesium and no calcium, a calcium ionophore (A-23187), or methacholine. ·NO production is inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) but not by N^G-nitro-L-arginine, L-N^5-(1-iminomethyl)ornithine hydrochloride, or aminoguanidine. Incubation with ATP, ADP, or histamine also inhibits ·NO formation. Some of these properties are similar to and some are different from properties of eNOS in other cell types. Cellular ·NO levels do not appear to be related to ATP or lactate content. Alveolar macrophage production of ·NO can be increased approximately threefold in the presence of lung surfactant or its major component, dipalmitoyl phosphatidylcholine (DPPC). The DPPC-induced increase in ·NO formation is time and concentration dependent, can be completely inhibited by L-NAME, and does not appear to be related to the degradation of DPPC by alveolar macrophages. These results demonstrate that unstimulated alveolar macrophages produce ·NO via an eNOS and that lung surfactant increases ·NO formation. This latter effect may be important in maintaining an anti-inflammatory state in vivo.

lung surfactant; dipalmitoyl phosphatidylcholine; nitric oxide synthase

NITRIC OXIDE (·NO) is a free radical that is produced by a variety of cell types in the lungs. Its synthesis from L-arginine is catalyzed by nitric oxide synthase (NOS). It is generally accepted that isoforms of the NOS enzyme fall into one of two categories. One category is a constitutive form (cNOS), which is regulated by calcium, and the other is an inducible form (iNOS), which is inducible by cytokines and/or endotoxin and is transcriptionally regulated (10). ·NO generated by cNOS in the lungs is found in endothelial cells (7) and airway neurons (4) and seems to be important in the regulation of vascular and bronchial smooth muscle activity. Recently, we have shown that alveolar type II cells also contain cNOS (24). On the other hand, other lung cell types can generate ·NO by means of an iNOS. For example, iNOS activity has been reported in lung fibroblasts (14), pulmonary artery smooth muscle cells (28), and neutrophils (33) after appropriate stimulation.

Alveolar macrophages are mobile phagocytic cells located within the alveoli and small airways of the lungs. These cells represent a primary line of defense against the adverse effects of inhalation of bacteria and foreign particles. It is known that alveolar macrophages do produce ·NO and that its synthesis is probably due to an iNOS. Several different investigators have reported that rat alveolar macrophages produce large quantities of ·NO in response to inflammatory stimuli such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS; see Refs. 14, 19, 29). These inflammatory stimuli act by causing expression of iNOS mRNA (20, 21). On the other hand, it does not appear that naive alveolar macrophages produce ·NO. For example, Persoons et al. (30) reported that ·NO production by these cells could only be detected after exposure of rats to LPS. Also, Kobzik et al. (17) used immunocytochemical techniques and found no NOS in normal rat alveolar macrophages. However, after in vivo or in vitro treatment with LPS, they found iNOS activity in these cells.

The results from all of these previous studies suggest that naive alveolar macrophages do not produce measurable amounts of ·NO and do not contain measurable amounts of NOS. Furthermore, the available evidence suggests that ·NO formation by these cells occurs only after the induction of iNOS, which can be produced by treatment with inflammatory stimuli, e.g., LPS and/or IFN-γ. Almost all of these previous measurements were made over relatively long periods of time with the cells in culture. As part of another investigation, Ischiropoulos et al. (13) did report that freshly isolated, unstimulated alveolar macrophages do produce ·NO. However, to the best of our knowledge, the production of ·NO by naive alveolar macrophages during relatively short incubation periods has not been studied in detail. In this paper, we report that naive rat alveolar macrophages do produce ·NO and that ·NO formation can be measured during incubation periods as brief as 30 min. Therefore, the objectives of this investigation were to *1*) report this finding, *2*) identify the NOS protein involved, *3*) characterize the NOS involved in terms of factors known to influence NOS isoforms in other cell types, and *4*) study possible roles of NOS in alveolar macrophage function.

METHODS

Isolation of alveolar macrophages. Specific pathogen-free male Sprague-Dawley rats (225–350 g; Hilltop Laboratories,

Scottdale, PA) were used for all experiments. The animals were anesthetized with pentobarbital sodium (150 mg/kg body wt) and exsanguinated by cutting the abdominal aorta. The trachea, heart, and lungs were then removed from the animals intact. Alveolar macrophages were obtained via bronchoalveolar lavage according to the method of Myrvik et al. (27). The lungs from each rat were washed eight times with 5 ml of phosphate-buffered medium (in mM: 145 NaCl, 5 KCl, 9.4 Na_2HPO_4, 1.9 NaH_2PO_4, and 5 glucose; pH 7.4) per gram of lung weight. The cells were separated from the lavage fluid by centrifugation at 300 *g* for 10 min and then washed three times by alternate centrifugation and resuspension in phosphate-buffered medium. After the washing procedure, the cells were resuspended in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.5% bovine serum albumin (BSA) for use in all experiments, except for those done in calcium- and/or magnesium-free medium in which the $CaCl_2$ and/or $MgCl_2$ was omitted from the medium. The number of cells in the suspensions was determined by using an electronic cell counter (model Z_B; Coulter Electronics, Hialeah, FL). The cells used in these experiments were >98% alveolar macrophages, with leukocytes as the contaminating cells. The viability of the alveolar macrophages, as measured by oxygen consumption and trypan blue exclusion, did not change during the incubation periods used in these experiments.

Measurement of ·NO production. The amounts of ·NO produced by alveolar macrophages during various time periods were measured as the stable oxidation products of ·NO, nitrate, and nitrite. The cells (4×10^6/ml) were incubated at 37°C in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.5% BSA. The inclusion of BSA in the medium had no effect on the measured ·NO levels. Some experiments were done in the absence of extracellular calcium and/or magnesium. After the incubation, cells were removed from the medium by centrifugation, and the supernatants were saved for analysis. Each sample (0.3 ml) was first incubated at 37°C for 1 h with *Escherichia coli* nitrate reductase (in a 0.3-ml volume) to convert the nitrate to nitrite. The total nitrite in the samples was then measured using the Greiss reaction (11). Briefly, an aliquot (0.5 ml) of each of the samples that had been incubated with the *E. coli* enzyme was incubated at room temperature for 10 min with the Greiss reagent (in a 1.0-ml volume), and the absorbance at 550 nm was read with a spectrophotometer (model 35; Perkin-Elmer, Norwalk, CT). The amount of nitrate and nitrite in the samples was calculated from a standard curve that was constructed from sodium nitrite standards. Conversion of nitrate to nitrite was checked in each assay by using sodium nitrate standards.

The effects of different NOS inhibitors, L-arginine, a calcium ionophore, dipalmitoyl phosphatidylcholine (DPPC) vesicles, lung surfactant, and other substances on ·NO production by alveolar macrophages were determined. Four different NOS inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical, St. Louis, MO), N^G-monomethyl-L-arginine [L-NMMA; Research Biochemicals International (RBI), Natick, MA], L-N^5-(1-iminomethyl)ornithine hydrochloride (L-NIO; RBI), or aminoguanidine (RBI), were included in some incubations with alveolar macrophages. The final concentration of each inhibitor was 1 mM. In some experiments, various amounts of L-arginine were included in the incubation medium. Five different compounds known to affect NOS in other cell types, acetyl-β-methylcholine chloride (methacholine), bradykinin (acetate salt), histamine (diphosphate salt), ATP, or ADP (all from Sigma), were included in some incubations with alveolar macrophages. The final concentration of all compounds was 0.1 mM. In other experiments, the effects of the calcium ionophore 4-bromo-A-23187 (Calbiochem, La Jolla, CA) on cellular ·NO production were determined. A-23187 was dissolved in dimethyl sulfoxide (DMSO) and added to the incubation mixture so that the final concentration was 1 μM. The amount of DMSO in the final incubation mixture was only 1 μl/ml, a level that has no adverse effects.

In some experiments, alveolar macrophages were incubated with DPPC vesicles or purified lung surfactant. The DPPC vesicles were formed as described previously (16). Briefly, DPPC (Sigma) was dissolved in ethanol. Liposomes were formed by injecting the dissolved lipids into phosphate-buffered medium warmed to 48°C. Then the dispersion was sonicated to form vesicles, and the vesicles were added to the alveolar macrophage incubation mixtures. The amount of ethanol used has no effect on alveolar macrophage function. Pulmonary surfactant was obtained according to a modification (24) of the method of King and Clements (15). Briefly, a concentrated form of cell-free alveolar lavage materials, which was obtained as we described previously (24), was spun at 100,000 *g* for 2 h. The resultant pellet was resuspended in phosphate-buffered medium and applied to a linear sodium bromide density gradient. The gradient was then spun at 81,500 *g* for 15 h in a swinging bucket rotor. The band containing the surfactant was removed and spun at 66,000 *g* for 1 h. This pellet was washed and resuspended in phosphate-buffered medium for use as the surfactant preparation in these experiments. The surfactant disaturated phosphatidylcholines (DSPC) were isolated according to the method of Mason et al. (22) and were measured as DSPC phosphorus (3). There was no nitrate and nitrite in the DPPC vesicle or surfactant preparations. Finally, experiments were performed to determine whether or not the substances that were used in an attempt to alter ·NO production interfered with the assay. None of the substances that we used in this study had any effect on any aspect of the assay for nitrate and nitrite.

Detection of NOS protein. To determine if NOS protein could be detected in alveolar macrophages, Western blot analysis was used. The cells were first sonicated to disrupt cell membranes. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on 100-μg aliquots of cell sonicate protein with 7.5% (wt/vol) polyacrylamide gels. Proteins were transferred to nitrocellulose paper by using an electrophoretic transfer unit (Hoefer Scientific Instruments, San Francisco, CA). The blots were then blocked for 1 h at room temperature in a medium (blocking buffer) containing 50 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, 150 mM NaCl, 2% (vol/vol) BSA, and 0.1% (vol/vol) Tween 20 (pH 7.4). These blots were then incubated for an additional hour at room temperature in blocking buffer containing anti-NOS antibody. Two primary antibodies against two forms of cNOS, i.e., mouse [immunoglobulin (Ig) G_1] monoclonal anti-endothelial NOS (eNOS) and mouse (IgG_{2a}) monoclonal anti-brain NOS (bNOS), and one primary antibody against iNOS, i.e., mouse macrophage (IgG_{2a}) monoclonal anti-iNOS, were used. All antibodies were obtained from Transduction Laboratories in Lexington, KY. These antibodies were diluted 1:500 in blocking buffer. After incubation with the primary antibodies, the blots were washed six times (5 min/wash) at room temperature with medium containing 50 mM Tris·HCl, 150 mM NaCl, and 0.1% Tween 20 (TBS-T; pH 7.4). Then the blots were incubated for 1 h at room temperature in blocking buffer containing the secondary antibody, anti-mouse IgG coupled to horseradish peroxidase (Amersham Life Sciences; Cleveland, OH). After incubation with the secondary antibody, the blots were washed six times (5 min/wash) at room

temperature with TBS-T. Protein bands detected by the antibodies were visualized by enhanced chemiluminescence (Amersham). The standards that were carried through the entire procedure were human endothelial cell lysate (for eNOS), rat pituitary cell lysate (for bNOS), and macrophage lysate prepared from RAW 264.7 cells that had been stimulated with IFN-γ and LPS (for iNOS). All standards were obtained from Transduction Laboratories.

Measurement of alveolar macrophage ATP and lactate levels. The effects of L-NAME, L-arginine, and DPPC vesicles on alveolar macrophage ATP levels were determined. ATP content was measured with the firefly luciferase assay adapted from the method of Wulff and Doppen (32). Cells (1×10^6/ml) were incubated (37°C) for either 2 or 4 h in the absence (control) or presence of L-NAME (1 mM), L-arginine (100 μM), or DPPC vesicles (150 μg/ml). After incubation, the cells were spun at 1,000 *g* for 10 min, and the medium was removed by aspiration. The cells were washed one time and then resuspended so that there were 1×10^6 cells in 0.125 ml of 0.5 M Tris-acetate (pH 7.4). Triton X-100 (0.125 ml; 1:200 in Tris-acetate) was added to disrupt cell membranes. The samples were mixed by vortexing for 10 s and then were analyzed immediately for ATP content. The ATP concentration was determined by measuring the emission of light when 0.05 ml of the sample was mixed with 0.05 ml of firefly lantern extract (Sigma) in 0.4 ml of Tris-acetate. Light emission was recorded with a Lumiaggregometer (model 400; Chrono-Log, Havertown, PA). Cellular ATP content was calculated from a curve of ATP standards (Sigma) and is expressed as nanomoles per 10^6 cells.

The metabolism of glucose via glycolysis was estimated by measuring alveolar macrophage levels of lactate, the major end product of glycolysis. Alveolar macrophages (4×10^6/ml) were incubated (37°C) for 4 h in the absence (control) or presence of L-NAME (1 mM) or L-arginine (100 μM). After incubation, an aliquot (0.3 ml) of the cell suspension was added to perchloric acid (final concentration = 3%) to precipitate cellular protein. The samples were thoroughly mixed and centrifuged in an Eppendorf microcentrifuge at 12,800 *g* for 2 min. Lactate concentrations in the perchloric acid extracts were measured spectrophotometrically at 340 nm with enzymatic methods (12). Results are expressed as nanomoles per 10^6 cells.

Measurement of degradation of DPPC vesicles by alveolar macrophages. The degradation of DPPC vesicles by alveolar macrophages was measured in the absence or presence of L-NAME as we described previously (25). Briefly, alveolar macrophages (2×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.5% BSA. DPPC vesicles were formed as described elsewhere in this manuscript and were added to the incubation mixture so that the final concentration was 50 μg/ml. L-3-Phosphatidyl-[*N-methyl*-^{3}H]choline,1,2-dipalmitoyl (specific activity = 80 Ci/mmol; Amersham Life Science, Arlington Heights, IL) was also included with DPPC at a final concentration of 0.2 μCi/ml. Some samples were incubated in the absence (control) and other samples were incubated in the presence of 1 mM L-NAME. Immediately before and after a 2-h incubation period, DPPC was extracted from the samples (22), and labeled DPPC was determined by counting in a liquid scintillation counter. The amount of labeled DPPC that was degraded during the incubation period is expressed as a percentage of that present before the incubation. The level of ·NO in the samples was also measured as described elsewhere in this manuscript.

Statistical analyses. All comparisons of statistical significance were made by comparing each treatment with the control value (100%) using a one-sample Student's *t*-test. $P < 0.05$ was taken as the limit to indicate significance.

RESULTS

Time course for ·NO production. There is some endogenous ·NO in unincubated, freshly isolated alveolar macrophages. The nitrate and nitrite in supernatant from these cells, which had just been washed three times by centrifugation and resuspension, is 0.013 ± 0.006 nmol/10^6 cells (Fig. 1). The time course for production of additional ·NO by the alveolar macrophages is shown in Fig. 1. ·NO formation proceeds along an approximately linear time course during the 4-h incubation period. The amount of ·NO produced in 4 h is 0.29 ± 0.02 nmol/10^6 cells. These results demonstrate that unstimulated, naive alveolar macrophages do produce ·NO.

Immunochemical detection of NOS protein. Experiments were performed in an attempt to identify which type(s) of NOS may be present in naive alveolar macrophages. Western blot analysis was used. Antibodies against two known types of cNOS, i.e., eNOS and bNOS, and an antibody against iNOS were used. The only positive result was obtained with the anti-eNOS antibody and is shown in Fig. 2. This result demonstrates the presence of an alveolar macrophage protein that reacts with a monoclonal anti-eNOS protein (Fig. 2, *lane 3*). The molecular mass of this protein corresponds to that of an eNOS standard (Fig. 2, *lane 2*). However, no bNOS or iNOS could be detected with the monoclonal anti-bNOS or anti-iNOS antibodies, respectively. Furthermore, no bNOS could be detected when another antibody, a rabbit polyclonal anti-bNOS antibody (Transduction Laboratories), was used. The re-

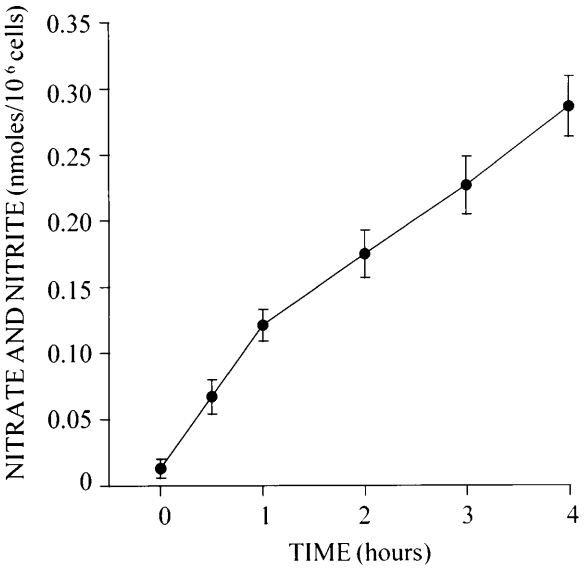

Fig. 1. Time course for production of nitric oxide by alveolar macrophages. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and bovine serum albumin (BSA; 0.5%) for varying times up to 4 h. After the appropriate incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate and nitrite were measured as described in METHODS. Points are mean values for 6 experiments, and bars represent SE.

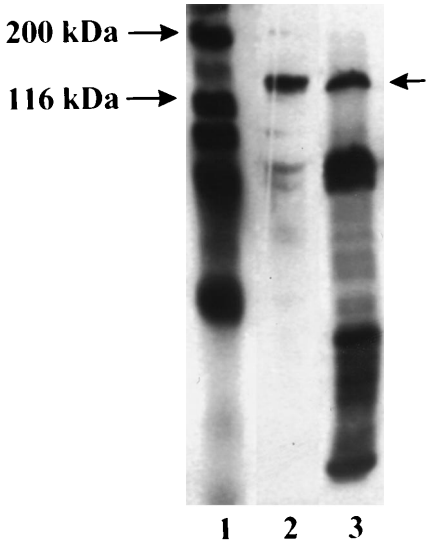

Fig. 2. Western blot analysis of alveolar macrophage proteins with anti-endothelial nitric oxide synthase (eNOS) antibody. Cells were sonicated to disrupt cell membranes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to fractionate 100-μg aliquots of cell sonicate protein. Proteins were then transferred to a nitrocellulose membrane and immunodetected with a monoclonal anti-eNOS antibody (*lane 3*). A standard preparation of eNOS from human endothelial cell lysate was used as a positive control and was carried through the entire procedure (*lane 2*). Molecular-mass markers are shown in *lane 1*. Unlabeled arrow at *right* shows location of the eNOS at a molecular mass of ~140 kDa. Blot is representative of results obtained from 6 different alveolar macrophage preparations.

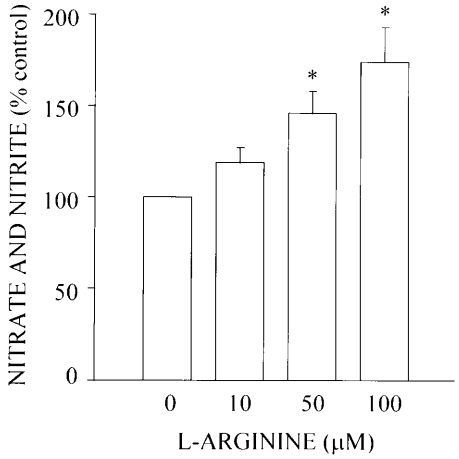

Fig. 3. Effects of extracellular L-arginine on nitric oxide production by alveolar macrophages. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 4 h in the absence (control) and presence of various concentrations of L-arginine. After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate and nitrite were measured as described in METHODS. Nitrate and nitrite production by control cells was 0.25 ± 0.05 nmol/10^6 cells. Values are means ± SE for 6 experiments. *Values are significantly different from no extracellular L-arginine ($P < 0.05$).

sults of these experiments show that there is an endothelial type of cNOS present in unstimulated, naive alveolar macrophages.

Effects of inhibitors, calcium, magnesium, and other compounds that affect eNOS. Because eNOS activity has not been previously reported in alveolar macrophages, experiments were carried out to characterize cellular ·NO production. The relationship between extracellular L-arginine, the substrate for NOS, and ·NO formation is shown in Fig. 3. As the external concentration of L-arginine is increased from 0 to 100 μM, there is a progressive increase in ·NO production. In fact, at 50 and 100 μM L-arginine, the formation of ·NO is increased by ~50 and 75%, respectively. On the other hand, addition of D-arginine has no effect on formation of ·NO; i.e., in the presence of D-arginine (100 μM), ·NO production is 94 ± 7% of control ($n = 5$). The effects of four different NOS inhibitors on alveolar macrophage ·NO production are shown in Table 1. Incubation of the cells with L-NAME leads to a 32% inhibition of ·NO formation. The L-NAME-induced inhibition of ·NO production can be reversed by including L-arginine in the incubation medium; i.e., in the presence of L-NAME and L-arginine (10 mM), ·NO formation is 123 ± 11% of control ($n = 5$). However, none of the other inhibitors, L-NMMA, L-NIO, or aminoguanidine, has any effect on the formation of ·NO. The results of these experiments show that ·NO production by alveolar macrophages is dependent on extracellular L-arginine and can be inhibited, at least partially, by L-NAME.

It is known that cNOS activity is dependent on calcium (10). Therefore, the effects of altering extracellular calcium levels on alveolar macrophage ·NO production were determined. At the same time, effects of extracellular magnesium were also studied, and the results are shown in Table 2. Removal of both calcium and magnesium from the incubation medium or removal of magnesium alone has no effect on cellular ·NO formation. However, if extracellular calcium is removed and magnesium is present in the medium, there is a 58% increase in alveolar macrophage ·NO production. To increase intracellular calcium levels, the cells were exposed to the calcium ionophore A-23187. Incubation of alveolar macrophages with A-23187 leads to a 50% increase in ·NO formation (Table 3). Inclusion of L-NAME in the incubation medium blocks the A-23187-induced increase in ·NO formation; i.e., in the presence of

Table 1. *Effects of L-NAME, L-NMMA, L-NIO, or aminoguanidine on nitric oxide production by alveolar macrophages*

Treatment	Nitrate + Nitrite, %control
Control	100
L-NAME (1 mM)	68 ± 3*
L-NMMA (1 mM)	96 ± 2
L-NIO (1 mM)	101 ± 7
Aminoguanidine (1 mM)	106 ± 3

Values are means ± SE for 6 experiments. Alveolar macrophages (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and bovine serum albumin (BSA; 0.5%) for 4 h in the absence (control) and presence of N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine acetate (L-NMMA), L-N^5-(1-iminoethyl)ornithine hydrochloride (L-NIO), or aminoguanidine. After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate + nitrite production by control cells was 0.25 ± 0.05 nmol/10^6 cells. *Value is significantly different from control ($P < 0.05$).

Table 2. *Effects of extracellular calcium and magnesium on nitric oxide production by alveolar macrophages*

Extracellular Calcium, mM	Extracellular Magnesium, mM	Nitrate + Nitrite, %control
1.8 (Control)	1.0 (Control)	100
0	0	97 ± 2
1.8	0	106 ± 5
0	1.0	158 ± 5*

Values are the means ± SE for 6 experiments. Alveolar macrophages (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing BSA (0.5%) for 4 h in the absence (control) and presence of $CaCl_2$ and $MgCl_2$ in concentrations shown above. After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells (i.e., cells in medium containing both Ca^{2+} and Mg^{2+}) was 0.25 ± 0.05 nmol/10^6 cells. *Value is significantly different from control ($P < 0.05$).

A-23187 and L-NAME (1 mM), ·NO production is 98 ± 9% of control ($n = 5$). These results demonstrate that alveolar macrophage ·NO production is enhanced when the medium contains magnesium and no calcium and when the internal calcium concentration is increased by using a calcium ionophore.

In other cell types, a variety of compounds such as methacholine, bradykinin, histamine, ATP, and ADP can stimulate release of ·NO from eNOS (9, 26). Therefore, we determined the effects of these compounds on ·NO formation by alveolar macrophages, and the results are shown in Table 4. Methacholine is the only compound we tested that stimulates ·NO production; i.e., it produces a twofold increase. The methacholine-induced increase in ·NO is blocked by L-NAME; i.e., in the presence of methacholine and L-NAME (1 mM), ·NO production is 108 ± 9% of control ($n = 5$). Incubation of the cells with three other compounds leads to inhibition of ·NO formation. ATP produces a 45% inhibition, whereas ADP and histamine cause a 20% inhibition. Bradykinin has no effect. The results of these experiments demonstrate that, with the exception of methacholine, these compounds have different effects on ·NO production in alveolar macrophages and endothelial cells.

Alveolar macrophage ATP and lactate contents. It has been shown by other investigators that there is an increase in the rate of glycolysis when ·NO levels are elevated in rat peritoneal macrophages (1, 23). Therefore, we performed experiments to determine if alveolar macrophage ATP and lactate (the major end product of glycolysis) levels are affected when the amounts of cell ·NO are altered by using L-NAME or L-arginine. The results are shown in Table 5. Incubation of alveolar macrophages with L-NAME or L-arginine leads to levels of ·NO production that are 68 and 145% of control, respectively. However, there are no effects of either treatment on cell ATP or lactate content. These results suggest that endogenous alveolar macrophage ·NO levels do not directly affect cellular energy metabolism.

Effects of DPPC vesicles and lung surfactant on ·NO production. We have shown previously that incubation of rat alveolar type II cells with DPPC vesicles leads to an increase in ·NO production from an eNOS (24). As a result of this effect, there is also an increase in type II cell ATP levels. Therefore, experiments were performed to determine the effects of DPPC vesicles on alveolar macrophage ·NO formation. Incubation of the cells

Table 3. *Effects of the calcium ionophore A-23187 on nitric oxide production by alveolar macrophages*

Treatment	Nitrate + Nitrite, %control
Control	100
A-23187 (1 μM)	150 ± 10*

Values are means ± SE for 6 experiments. Alveolar macrophages (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 1 h in the absence (control) and presence of A-23187. After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells was 0.14 ± 0.01 nmol/10^6 cells. *Value is significantly different from control ($P < 0.05$).

Table 4. *Effects of methacholine, ATP, ADP, histamine, or bradykinin on nitric oxide production by alveolar macrophages*

Treatment	Nitrate + Nitrite, %control
Control	100
Methacholine (0.1 M)	205 ± 16*
ATP (0.1 mM)	55 ± 3*
ADP (0.1 mM)	79 ± 5*
Histamine (0.1 mM)	80 ± 8*
Bradykinin (0.1 mM)	110 ± 4

Values are means ± SE for 6 experiments. Alveolar macrophages (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 4 h in the absence (control) and presence of acetyl-β-methylcholine chloride (methacholine), ATP, ADP, histamine (diphosphate salt), or bradykinin (acetate salt). After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells was 0.34 ± 0.06 nmol/10^6 cells. *Values are significantly different from control ($P < 0.05$).

Table 5. *Effects of L-NAME or L-arginine on alveolar macrophage nitric oxide production and cell ATP and lactate content*

Treatment	Nitrate + Nitrite, %control	ATP, %control	Lactate, %control
Control	100	100	100
L-NAME (1 mM)	68 ± 4*	94 ± 5	101 ± 5
L-Arginine (100 μM)	145 ± 10*	95 ± 5	95 ± 4

Values are means ± SE for 5 experiments. Alveolar macrophages [4×10^6/ml for nitric oxide and lactate measurements and 1×10^6/ml for ATP measurements) were incubated (37°C) in phosphate-buffered medium containing calcium, magnesium, and BSA for 4 h in the absence (control) or presence of L-NAME or L-arginine. For determinations of nitrate + nitrite or ATP, the cells were centrifuged. Nitrate + nitrite was measured in the supernatants, and ATP was measured in the cell pellets as described in METHODS. For lactate determinations, a sample of the cell suspension was taken, and the analysis was done as described in METHODS. Nitrate + nitrite, ATP, and lactate levels in control cells were 0.26 ± 0.05, 0.52 ± 0.10, and 56 ± 14 nmol/10^6 cells, respectively. *Values are significantly different from control ($P < 0.05$).

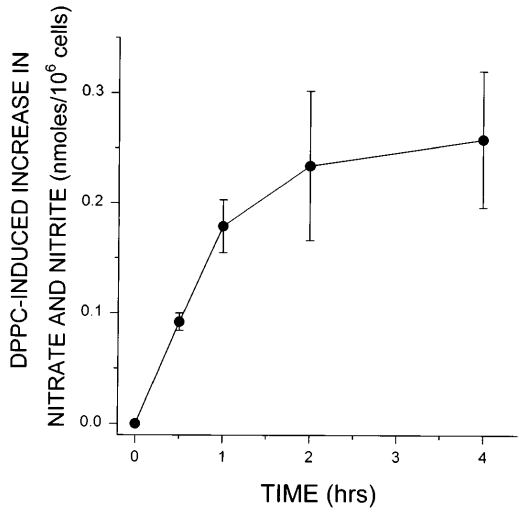

Fig. 4. Time course for dipalmitoyl phosphatidylcholine (DPPC)-induced increase in nitric oxide production by alveolar macrophages. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for varying times up to 4 h in the absence and presence of DPPC vesicles (150 μg/ml). After the appropriate incubation period, cells were centrifuged, and the nitrate and nitrite in the supernatants was measured as described in METHODS. Points were obtained by subtracting nitric oxide production by control cells from that by DPPC-exposed cells at each time point. Values are means ± SE for 5 different experiments.

with DPPC vesicles (150 μg/ml, a concentration that produces a maximal effect) leads to a time-dependent increase in ·NO production that is maximal after 2 h (Fig. 4). The concentration dependence for this effect is shown in Fig. 5. ·NO production increases with the concentration of DPPC vesicles up to a level of 150 μg/ml, where the effect is maximal. The DPPC-induced production of ·NO by alveolar macrophages is completely blocked by L-NAME (Table 6). There is no effect of DPPC on alveolar macrophage ATP levels as there is in type II cells. Incubation of alveolar macrophages with DPPC (150 μg/ml) for 2 h leads to ·NO levels that are 270 ± 40% of control and ATP levels that are 108 ± 8% of control. Thus these results demonstrate that incubation of alveolar macrophages with DPPC vesicles leads to a time- and concentration-dependent increase in cellular ·NO formation.

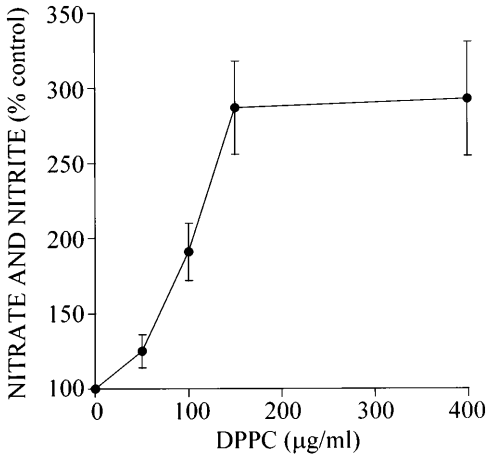

Fig. 5. Effects of different concentrations of DPPC vesicles on nitric oxide production by alveolar macrophages. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 4 h in the absence (control) and presence of various concentrations of DPPC vesicles. After the incubation period, cells were centrifuged, and nitrate + nitrite in the supernatants was measured as described in METHODS. Nitrate + nitrite production by control cells was 0.27 ± 0.03 nmol/10^6 cells. Values are means ± SE for 6 experiments.

Table 6. *Effect of L-NAME on DPPC-induced nitric oxide production by alveolar macrophages*

Treatment	Nitrate + Nitrite, %control
Control	100
DPPC (150 μg/ml)	292 ± 41*
DPPC (150 μg/ml) + L-NAME (1 mM)	69 ± 8*

Values are means ± SE for 6 experiments. Alveolar macrophages (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 4 h in the absence (control) and presence of dipalmitoyl phosphatidylcholine (DPPC) vesicles or DPPC vesicles + L-NAME. After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells was 0.31 ± 0.06 nmol/10^6 cells. *Values are significantly different from control ($P < 0.05$).

In our previous study (24), we determined that the effects of DPPC vesicles were the same as the effects of lung surfactant on alveolar type II cell ·NO levels. Therefore, experiments were performed to compare the effects of lung surfactant and DPPC vesicles on alveolar macrophage ·NO production. The results are shown in Table 7. During a 2-h incubation with lung surfactant (150 μg DSPC/ml) or DPPC vesicles (150 μg/ml), ·NO levels are elevated by 326 and 257%, respectively. Thus lung surfactant and DPPC vesicles have similar effects on alveolar macrophage ·NO formation.

Effect of L-NAME on DPPC degradation by alveolar macrophages. We have shown previously that rat alveolar macrophages degrade DPPC vesicles (and lung surfactant DSPC) in vitro (25). Therefore, experiments were performed to determine if ·NO levels in alveolar macrophages affect the breakdown of DPPC by these cells. The results are shown in Table 8. When alveolar macrophages are incubated with 50 μg DPPC/ml, an

Table 7. *Effects of lung surfactant or DPPC vesicles on nitric oxide production by alveolar macrophages*

Treatment	Nitrate + Nitrite, %control
Control	100
Lung surfactant (150 μg DSPC/ml)	326 ± 32*
DPPC (150 μg/ml)	257 ± 27*

Values are means ± SE for 6 experiments. DSPC, disaturated phosphatidylcholine. Alveolar macrophages (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 2 h in the absence (control) or presence of lung surfactant (prepared as described in METHODS) or DPPC vesicles. After the incubation period, cells were centrifuged, and the supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells was 0.20 ± 0.02 nmol/10^6 cells. *Values are significantly different from control ($P < 0.05$).

Table 8. *Effects of L-NAME on degradation of DPPC vesicles by alveolar macrophages*

Treatment	Degradation of DPPC, %
DPPC (50 μg/ml)	23 ± 2*
DPPC (50 μg/ml) + L-NAME (1 mM)	21 ± 3*

Values are means ± SE for 8 experiments. Alveolar macrophages (4×10^6/ml) were incubated in phosphate-buffered medium containing calcium (1.8 mM), magnesium (1.0 mM), and BSA (0.5%) for 4 h in the presence of DPPC or DPPC and L-NAME. Some of the DPPC was tritium labeled. Amount of labeled DPPC was measured in 0.5-ml samples (cells and medium) before and after the incubation period as described in METHODS. No degradation of DPPC occurs in the absence of cells. Amount of DPPC degraded is expressed as a percentage of that present before the incubation. *Values are significantly different from the lack of any degradation in the absence of cells.

amount that we used previously (25), 23% of the lipid is degraded in 4 h. This amount of degradation is similar to that reported in our previous paper (25). However, the amount of DPPC degraded is not affected by L-NAME. In these experiments, ·NO production over the 4-h incubation period was increased by 200 ± 10% in the presence of DPPC and was not increased in the presence of DPPC and L-NAME; i.e., in the presence of DPPC + L-NAME, ·NO formation was 100 ± 6% of control (n = 8). These results demonstrate that, although DPPC-induced ·NO production is inhibited by L-NAME, there is no effect of the inhibitor on DPPC breakdown. Therefore, alveolar macrophage ·NO levels do not appear to be related to DPPC degradation by the cells.

DISCUSSION

The results of our experiments demonstrate that naive unstimulated rat alveolar macrophages do produce ·NO and that there is a cNOS, which is an eNOS, present in the cells. In addition, we have characterized the eNOS in terms of factors known to influence it in other cell types. For example, alveolar macrophage ·NO production is dependent on extracellular L-arginine. Only one of the four NOS inhibitors tested inhibits ·NO formation. L-NAME inhibits cellular ·NO production by ~30%, but L-NMMA, L-NIO, and aminoguanidine have no effect. NOS activity can also be influenced by calcium and magnesium. When the incubation medium contains magnesium and no calcium, ·NO formation is ~60% greater than with all other extracellular combinations of calcium and/or magnesium. Furthermore, if the intracellular calcium level is increased by using a calcium ionophore, ·NO production is increased by 50%. Five compounds known to stimulate release of ·NO from eNOS in other cell types (9, 26) were studied. Only methacholine stimulates ·NO production by alveolar macrophages. ATP, ADP, and histamine inhibit ·NO formation, and bradykinin has no effect.

It is interesting that the amounts of ·NO produced by the alveolar macrophage eNOS and many of its characteristics discussed above are different from those reported for eNOS activity in rat alveolar type II cells and bovine aortic endothelial cells. For example, the amount of ·NO produced by alveolar macrophages is less than that produced by other cell types. If the maximum rate of ·NO production, i.e., during the first hour of incubation, is calculated on the basis of alveolar macrophage protein for comparison with the other cell types, the value is ~12 pmol·mg protein^{-1}·min^{-1}. This rate is less than that reported for alveolar type II cells (24) or endothelial cells (5), which are 50 and 37 pmol·mg protein^{-1}·min^{-1}, respectively. On a per cell basis, type II cells produce approximately five to six times more ·NO during a 4-h incubation period than do alveolar macrophages. There are also differences in the dependence of ·NO formation on extracellular L-arginine. The eNOS activity in type II cells is independent of extracellular L-arginine (19). The partially purified eNOS from bovine aortic endothelial cells has been reported to have a low requirement for L-arginine (Michaelis constant = 2.9 μM; see Ref. 31), although agonist-stimulated ·NO production by endothelial cells appears to require a small amount (1 μM) of extracellular L-arginine for maximal activity (5). Therefore, it appears that endogenous levels of the substrate are sufficient to support at least basal NOS activity in type II and endothelial cells. However, alveolar macrophage ·NO production can be stimulated by extracellular L-arginine (10–100 μM), possibly indicating some difference in the substrate concentration dependence of the eNOS in these cells.

There are also differences in the response of these three cell types to alterations in extracellular calcium levels. ·NO production by both alveolar type II cells (24) and bovine aortic endothelial cells (5) is sensitive to extracellular calcium. Removal of calcium from the incubation medium leads to reductions in the formation of ·NO by 50 and 35% in endothelial and type II cells, respectively. Changes in extracellular magnesium have no effect in type II cells and have not been studied, as far as we know, in endothelial cells. However, alveolar macrophages respond differently. ·NO production is not altered in these cells if both calcium and magnesium are removed from the incubation medium, but it is increased by 58% if calcium is removed and magnesium remains in the medium. The reason(s) for these responses of alveolar macrophages to changes in extracellular calcium and magnesium is not known. It may be that alveolar macrophages are much less permeable to calcium than are endothelial or type II cells or that magnesium somehow alters internal calcium stores in alveolar macrophages. The important thing, however, is that all three cell types respond to an increase in intracellular calcium in the same manner; i.e., ·NO formation is increased in alveolar macrophages, type II cells, and endothelial cells in the presence of the calcium ionophore A-23187. This demonstrates the calcium dependence of ·NO production.

Alveolar macrophages also respond in a different manner to NOS inhibitors and to compounds known to stimulate eNOS in other cell types. Of the four NOS inhibitors we tested, only L-NAME inhibits ·NO production and only by ~30%. L-NAME has about the same effect in type II cells (24). However, in type II cells, L-NMMA and L-NIO demonstrate the most potent effects, inhibiting by 40–50%. L-NMMA is also a good

inhibitor in endothelial cells (5). Thus L-NMMA inhibits ·NO formation in type II and endothelial cells but has no effect in alveolar macrophages. Of the five compounds known to stimulate ·NO production by eNOS (9, 26), only methacholine enhances NOS activity in alveolar macrophages. Three other compounds, ATP, ADP, and histamine, actually inhibit ·NO formation in alveolar macrophages. Although the reasons for the different responses of alveolar macrophages to these inhibitors and other compounds are not known, it may be that there are different eNOS isoforms in the various cell types.

The role that the eNOS plays in alveolar macrophage function is not known. One possibility is that eNOS activity helps to regulate cellular metabolism. For example, it is known that, when endogenous levels of ·NO are increased in alveolar type II cells, there is an increase in the rate of cellular ATP synthesis and, therefore, an increase in cell ATP levels (24). Also, there are at least two reports of an increase in the rate of glycolysis when ·NO levels are elevated in rat peritoneal macrophages (1, 23). However, we were not able to affect alveolar macrophage ATP or lactate levels by either decreasing cellular ·NO production with L-NAME or increasing ·NO formation with L-arginine. In addition, a DPPC-induced increase in ·NO production of almost threefold does not affect cellular ATP levels. Thus it appears that the eNOS in alveolar macrophages does not affect cellular metabolism as it does in alveolar type II cells.

One of the most interesting findings from our study is the increase in alveolar macrophage ·NO production that occurs during incubation of the cells with DPPC vesicles or lung surfactant. Exposure of the cells to either lung surfactant of DPPC vesicles leads to an approximately threefold increase in ·NO formation. Although we are assuming that the surfactant-induced increase in ·NO levels is due to an increase in its formation, it is also possible that there may be a reduction in ·NO degradation. The DPPC-induced response is both time and concentration dependent and is completely inhibited by L-NAME. We reported a similar finding from our experiments with alveolar type II cells, i.e., exposure of these cells to DPPC or lung surfactant leads to increased production of ·NO (24). Because there is also an increase in type II cell ATP synthesis, as well as the increase in ·NO formation, we postulated that the elevation in ·NO levels in type II cells may be related to surfactant metabolism, e.g., recycling of surfactant. It is known that rat alveolar macrophages are capable of degrading surfactant DSPC or DPPC vesicles in vitro (25). Therefore, it is possible that the DPPC-induced increase in alveolar macrophage ·NO formation may be related to the degradation of DPPC. However, we were able to inhibit the DPPC-induced ·NO formation with L-NAME, but there was no effect on DPPC degradation. Thus it appears that there is no relationship between ·NO production and the degradation of lipids by alveolar macrophages.

Although the physiological role of the DPPC/lung surfactant-induced increase in alveolar macrophage ·NO levels is not known, it does not appear to be related to surfactant metabolism. Our results do suggest that the endogenous ·NO level of alveolar macrophages in the lungs is maintained at a higher level because the cells are continually exposed to surfactant. It is known that ·NO has some beneficial anti-inflammatory effects. For example, ·NO functions as an anti-inflammatory agent by suppressing adhesion and/or activation of neutrophils (18). Other anti-inflammatory effects of ·NO include inhibition of platelet aggregation (2), inhibition of cytokine-induced expression of adhesion molecules (6), and inhibition of neutrophil respiratory burst due to inhibition of NADPH oxidase (8). Therefore, one very important physiological role for the higher alveolar macrophage ·NO levels in the presence of surfactant (i.e., in vivo) may be the maintenance of an anti-inflammatory state. Experiments are currently being done to test this hypothesis.

In summary, naive unstimulated alveolar macrophages do produce ·NO. The NOS responsible appears to be an eNOS. The amount of ·NO formed is much less than that produced by eNOS in other cells, i.e., alveolar type II cells and endothelial cells. Some properties of the alveolar macrophage eNOS are similar to and some are different from the eNOS in these other cell types. Alveolar macrophage ·NO levels do not seem to be related to cellular metabolism. ·NO production is increased approximately threefold in the presence of DPPC vesicles or lung surfactant. However, this increase in ·NO formation is not related to surfactant or cellular energy metabolism. It is possible that the DPPC/lung surfactant-induced increase in alveolar macrophage constitutive ·NO levels is important in maintaining an elevated anti-inflammatory state in vivo.

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