Alveolar type II cell cNOS activity and ATP levels are increased by lung surfactant or DPPC vesicles

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Miles, P. R., L. Bowman, A. Rengasamy, and L. Huffman. Alveolar type II cell cNOS activity and ATP levels are increased by lung surfactant or DPPC vesicles. *Am. J. Physiol.* 273 (*Lung Cell. Mol. Physiol.* 17): L339–L346, 1997.—In a previous study, we reported that nitric oxide (·NO) affects surfactant synthesis and ATP levels in alveolar type II cells and suggested that there is constitutive nitric oxide synthase (cNOS) activity in the cells. In the present study, we performed experiments to confirm further the presence of cNOS and to determine the effects of lung surfactant on type II cell ·NO and ATP levels. The supernatant from freshly isolated cells contains ·NO (0.26 ± 0.08 nmol/10^6 cells). During incubation, the cells produce additional ·NO at a rate of ~0.3 nmol·10^6 $cells^{-1}\cdot h^{-1}$. ·NO formation is inhibited by 28–46% by three inhibitors of cNOS and inducible NOS (iNOS), N^G-monomethyl-L-arginine (L-NMMA), L-N^5-(1-iminoethyl)ornithine hydrochloride, and N^G-nitro-L-arginine methyl ester, but a specific inhibitor of iNOS, aminoguanidine, has no effect. The production of ·NO is reduced in Ca^{2+}-free medium, is stimulated by the Ca^{2+} ionophore A-23187, and is independent of extracellular L-arginine. One known type of cNOS, endothelial NOS (eNOS), can be detected in the cells by using Western blot analysis. Incubation of the cells with lung surfactant leads to a relatively rapid (~15 min), concentration-dependent increase in ·NO formation that reaches levels as high as 238 ± 14% of control. The surfactant effects appear to be caused by its major component, dipalmitoyl phosphatidylcholine (DPPC). Exposure of type II cells to DPPC results in maximal increases in ·NO formation, ATP content, and O_2 consumption, which are 268 ± 32, 234 ± 24, and 131 ± 6% of control, respectively. The DPPC-induced increases in ·NO, ATP, and O_2 consumption are inhibited by L-NMMA. These results confirm the presence of type II cell cNOS and suggest that it may have a role in the cellular processing of lung surfactant.

surfactant metabolism; adenosine 5′-triphosphate; constitutive nitric oxide synthase; dipalmitoyl phosphatidylcholine

ALVEOLAR TYPE II CELLS play a very important role in the metabolism of pulmonary surfactant, a complex mixture of lipids and proteins that lines the alveoli and prevents their collapse by lowering surface tension forces. The major component of surfactant is dipalmitoyl phosphatidylcholine (DPPC; see Ref. 12). It is well known that surfactant is synthesized in type II cells, stored in lamellar bodies, and then released on to the alveolar surface (23, 24). After synthesis, release, and use, it is thought that much of the surfactant phospholipid (PL) is recycled through these same type II cells. Chander et al. (3) first reported that isolated alveolar type II cells are capable of internalizing surfactant lipids. Results from several studies suggest that these internalized lipids may be either recycled intact or degraded (see Ref. 23 for a review). A recent report by Horowitz et al. (10) suggests that some surfactant proteins can influence the uptake of lipids by the cells. Thus type II cells appear to be involved in the synthesis, release, and recycling of lung surfactant.

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 ·NO 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 Ca^{2+}, and the other is an inducible form (iNOS), which is inducible by cytokines and/or endotoxin and is transcriptionally regulated (5). It has been reported by several investigators that type II cells can synthesize ·NO and that this synthesis is due to iNOS activity. For example, Punjabi et al. (21) demonstrated that iNOS expression in type II cells is increased after the inhalation exposure of rats to ozone. Gutierrez et al. (8) have shown that there is enhanced expression of iNOS after exposure of isolated type II cells to cytokines or lipopolysaccharide. Thus there is ample evidence that iNOS activity exists in these cells.

Recently, we reported that ·NO levels in alveolar type II cells affect cellular PL and energy metabolism and suggested that this may be due to cNOS activity (17). If there is cNOS activity present, it raises the question as to what extent it may be involved in the regulation of cellular function(s). One very important type II cell function is metabolism of lung surfactant. Because we have already shown that ·NO alters surfactant PL synthesis and ATP levels in type II cells (17), we wondered what effects exposure of type II cells to pulmonary surfactant, i.e., the initial event involved in surfactant reuptake and/or recycling by the cells, may have on cellular ·NO levels. Therefore, the objectives of these experiments were *1*) to confirm further that there is cNOS activity in isolated alveolar type II cells and *2*) to determine the effects of lung surfactant and some of its components on type II cell ·NO and ATP levels.

METHODS

Isolation of alveolar type II cells. Male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, PA), weighing 250–350 g, were used for all experiments. Alveolar type II cells were isolated by elastase digestion and were purified by centrifugal elutriation as we described previously (11, 17). The animals were anesthetized with pentobarbital sodium (150 mg/kg body wt), and the heart and lungs were removed rapidly. Lungs were perfused with 0.16 M NaCl to remove blood cells, and free alveolar macrophages were removed by tracheal lavage with phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5 mM

glucose, pH 7.4). The lungs were then filled with phosphate-buffered medium containing elastase (40 U/ml, type I; US Biochemical, Cleveland, OH) and deoxyribonuclease (DNase, 0.006%; Sigma Chemical, St. Louis, MO) and were incubated at 37°C for 30 min to free lung cells. After enzymatic digestion, the lungs were minced with a McIwain tissue chopper (Mickle Engineering; Gomshall, Surrey, UK) set at a slice thickness of 0.5 mm. This mince was placed in phosphate-buffered medium containing 25% fetal calf serum and 0.006% DNase (to prevent cell clumping) and was incubated for 10 min at 37°C to arrest digestion. The resultant cell suspension was strained through nylon mesh, and the type II cell fraction was obtained by centrifugal elutriation. Cells in the type II cell fraction were washed one time in and then 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. All studies were begun <30 min after the cell isolation procedure was complete.

Cell number was determined with a Coulter model Z_B electronic cell counter (Coulter Instrument, Hialeah, FL). The fluorescent dye, phosphine 3R, was routinely used to estimate the purity of the type II cell-enriched fraction as we have reported previously (11). In the experiments reported in this paper, we obtained $8.9 \pm 0.4 \times 10^6$ cells/rat in the type II cell fraction with a purity of 92 ± 1% (means ± SE for 12 experiments). The major contaminating cell type was alveolar macrophages.

Isolation of pulmonary surfactant. Pulmonary surfactant was obtained from a separate set of rats. The animals were anesthetized with pentobarbital sodium, and the heart and lungs were removed rapidly. A concentrated form of alveolar lavage materials was obtained by bronchoalveolar lavage of the right lung with 5 ml of phosphate-buffered medium followed by lavage of the left lung using the same 5 ml of fluid. Alveolar macrophages were removed from the lavage fluid by centrifugation at 300 *g* for 5 min and then washed three times by alternate centrifugation and resuspension in phosphate-buffered medium. All of the washings were spun at 15,000 *g* for 10 min. The pellet obtained from these washings was added back to the remaining cell-free alveolar lavage materials because a significant amount of surfactant PLs is recovered in this pellet (18).

Purified lung surfactant was then obtained according to the method of King and Clements (13). Briefly, the cell-free lavage materials were spun at 100,000 *g* for 2 h. The resultant pellet was resuspended in phosphate-buffered medium and was applied to a linear sodium bromide density gradient (density range: 1.028–1.100 g/ml). The gradient was then spun at 81,500 *g* for 15 h in a SW 27 swinging bucket rotor (Beckman Instrument, Fullerton, CA). The band containing the surfactant (density = 1.050 ± 0.003 g/ml; $n = 6$) was removed and was spun at 66,000 *g* for 1 h. This pellet was washed two times with phosphate-buffered medium and then resuspended in the same medium for use as the surfactant preparation in the experiments. In a separate set of experiments, we determined from osmolarity measurements that most of the NaBr was removed from the lung surfactant during the wash procedure. PLs were measured as the phosphorus present in lipid extracts of surfactant (1), and content, in milligrams, was obtained by multiplying the lipid phosphorus values by 25 (19). Surfactant disaturated phosphatidylcholines (DSPC) were isolated according to the method of Mason et al. (15), and content (in mg) was measured as described above for PL.

Measurement of ·NO production. The amounts of ·NO produced by alveolar type II cells 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. Some experiments were done in the absence of extracellular Ca^{2+} and/or Mg^{2+}. After the incubation, cells were removed by centrifugation, and the supernatants were saved for analysis. In some cases, the samples were frozen at −20°C and were stored overnight before analysis. All samples were first incubated with *Escherichia coli* reductase to convert the nitrate to nitrite. ·NO production was then measured by using the Greiss reaction (6). 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, a Ca^{2+} ionophore, lung surfactant, DPPC vesicles, and surfactant protein A (SP-A) on ·NO production by type II cells were determined. Four different NOS inhibitors, N^G-monomethyl-L-arginine acetate (L-NMMA), L-N^5-(1-iminoethyl)ornithine hydrochloride (L-NIO), N^G-nitro-L-arginine methyl ester (L-NAME), or aminoguanidine, were included in some incubations with type II cells. The final concentration of each inhibitor was 1 mM. In some experiments, the effects of the Ca^{2+} ionophore 4-bromo-A-23187 on cellular ·NO production were determined. A-23187 was dissolved in dimethyl sulfoxide (DMSO) and was added to the incubation mixture so that the final concentration was 1 mM. The amount of DMSO in the final incubation mixture was only 1 μl/ml, a level that has no adverse effects. Experiments with lung surfactant were carried out by adding various amounts of the purified surfactant preparation directly to the incubation mixture. In some instances, type II cells were incubated with DPPC vesicles, which were formed as described previously (14). Briefly, DPPC 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 type II cell incubation mixtures. The amount of ethanol used has no effect on type II cell function. When incubated at 37°C, the vesicles formed in this manner are mostly single bilayer vesicles of ~30–40 nm in diameter (14). During each experiment, we measured nitrate and nitrite in the lung surfactant and DPPC vesicle preparations and found none. SP-A was a gift from Dr. Jo Rae Wright (Duke University) and was purified from bronchoalveolar lavage materials from human alveolar proteinosis patients.

Detection of cNOS. To determine if cNOS protein could be detected in type II cells, Western blot analysis was used. The type II cells were first sonicated to disrupt cell membranes. Sodium dodecyl sulfate-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 were used, i.e., mouse [immunoglobulin (Ig) G_1] monoclonal anti-endothelial NOS (eNOS) and mouse (IgG_{2a}) monoclonal anti-brain NOS (bNOS; Transduction Laboratories, 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 a 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) and rat pituitary cell lysate (for bNOS; Transduction Laboratories).

Measurement of cellular ATP levels and oxygen consumption. The efffects of DPPC vesicles on type II cell ATP levels were determined. ATP content was measured with the firefly luciferase assay adapted from the method of Wulff and Doppen (25). Cells (1×10^6/ml) were incubated (37°C) for 1 h in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA in the absence (control) and presence of varying amounts of DPPC vesicles. After incubation, the cells were spun at 1,000 *g* for 10 min, and the incubation medium was removed by aspiration. The cells were washed one time and then resuspended so 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 sample was mixed by vortexing for 10 s and then was analyzed immediately for ATP content. The ATP concentration was determined by measuring the emission of light when 0.05 ml of the sample were mixed with 0.05 ml of firefly lantern extract (Sigma) in 0.4 ml Tris-acetate. Light emission was recorded with a Lumiaggregometer (model 400; Chrono-Log, Havertown, PA). Cellular ATP content was calculated from a standard curve of ATP standard solutions (Sigma) and was expressed as nanomoles per 10^6 cells.

Type II cell oxygen consumption was measured with a Gilson K-IC oxygraph fitted with a Clark electrode (Gilson Medical Instruments, Middletown, WI). The oxygraph was calibrated by measuring the levels of oxygen in aliquots of phophate-buffered medium that had been bubbled with gases of known oxygen concentration until saturation occurred. The cells (1×10^6/ml) were incubated (37°C) for 25 min in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA in the absence (control) and presence of DPPC vesicles. After this incubation, the cell suspension was transferred to the oxygraph, and oxygen consumption was measured for 10 min at 37°C. In some experiments, sodium cyanide (1 mM) was added to the cell suspension to inhibit mitochondrial respiration and thus to determine the amount of oxygen consumption that is attributable to mitochondrial respiration. All type II cellular oxygen consumption appears to be due to mitochondrial respiration. The results of these experiments were expressed as nanomols of oxygen consumed per 10^6 cells per hour.

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

·NO production: time course, L-arginine dependence, and inhibitor effects. There is some endogenous ·NO in unincubated, freshly isolated alveolar type II cells, i.e., the nitrate and nitrite in supernatant from these cells is 0.26 ± 0.08 nmol/10^6 cells. The time course for production of additional ·NO by the cells is shown in Fig. 1. ·NO production proceeds along an approximately linear time course for 4 h. During this time, the rate of ·NO formation is ~0.3 nmol·10^6 cells^{-1}·h^{-1}. The relationship between cellular ·NO production and extracellular L-arginine over the 4-h incubation period is shown in Fig. 2. Addition of L-arginine, at concentrations between 10 and 100 μM, has no effect on ·NO formation by alveolar type II cells. The effects of four different NOS inhibitors on type II cell ·NO production are shown in Table 1. L-NMMA, L-NIO, and L-NAME, inhibitors of both cNOS and iNOS (16, 22), inhibit ·NO

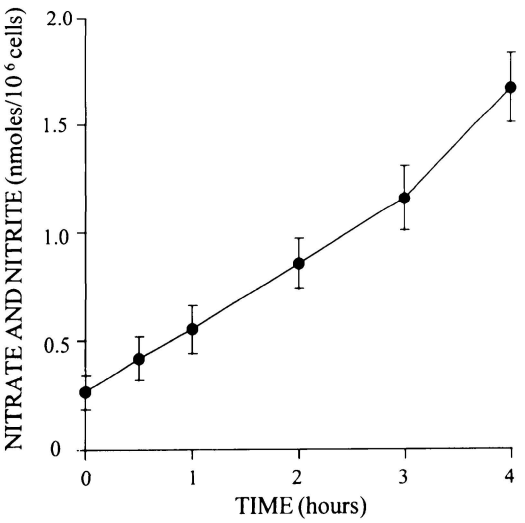

Fig. 1. Time course for production of nitric oxide by alveolar type II cells. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and bovine serum albumin (BSA, 0.5%) for varying times up to 4 h. After the appropriate incubation period, cells were centrifuged, and 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.

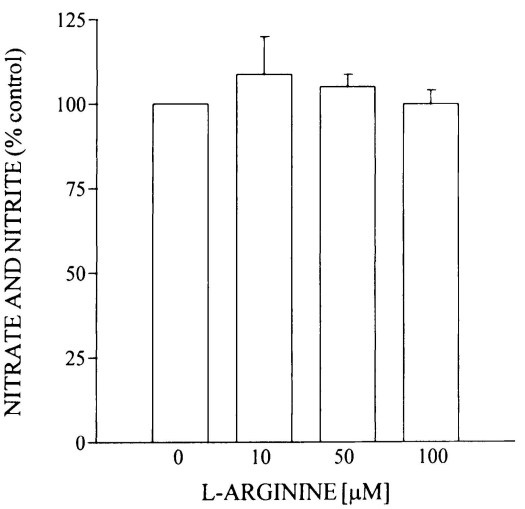

Fig. 2. Effects of extracellular L-arginine on nitric oxide production by alveolar type II cells. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and BSA (0.5%) for 4 h in the absence (control) and presence of various concentrations of L-arginine. After the incubation period, cells were centrifuged, and supernatants were saved for analysis. Nitrate and nitrite were measured as described in METHODS. Nitrate and nitrite production by control cells was 1.47 ± 0.14 nmol/10^6 cells. Values are means + SE for 6 experiments.

Table 1. *Effects of L-NMMA, L-NIO, L-NAME, and aminoguanidine on nitric oxide production by alveolar type II cells*

Treatment (concn)	Nitrate + Nitrite, %control
Control	100
L-NMMA (1 mM)	54 ± 7*
L-NIO (1 mM)	58 ± 7*
L-NAME (1 mM)	72 ± 4*

Values are means ± SE for 6 experiments. Type II cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and bovine serum albumin (BSA, 0.5%) for 4 h in the absence (control) and presence of N^G-monomethyl-L-arginine acetate (L-NMMA), L-N^5-(1-iminoethyl)ornithine hydrochloride (L-NIO), N^G-nitro-L-arginine methyl ester (L-NAME), or aminoguanidine. After the incubation period, cells were centrifuged, and supernatants were saved for analysis. Nitrate + nitrite production by control cells was 0.73 ± 0.09 nmol/10^6 cells. *Values are significantly different from control ($P < 0.05$).

formation to varying degrees. ·NO production is inhibited by 42–46% in the presence of L-NMMA or L-NIO, but incubation with L-NAME results in only 28% inhibition. On the other hand, aminoguanidine, a specific inhibitor of iNOS (7), has no effect on cell ·NO formation. The results of these experiments demonstrate that ·NO is produced by type II cells, and the process is not dependent on extracellular L-arginine. Furthermore, the experiments with inhibitors suggest that there is cNOS activity in the cells.

Ca^{2+} dependence of ·NO formation. Because cNOS activity is dependent on Ca^{2+} (5), the effects of altering extracellular Ca^{2+} levels on type II cell ·NO production were determined. At the same time, effects of extracellular Mg^{2+} were also studied, and the results are shown in Table 2. ·NO formation is maximal when the external concentrations of Ca^{2+} and Mg^{2+} are 1.8 and 1.0 mM, respectively. Elimination of extracellular Ca^{2+} leads to a 35% reduction in ·NO production. When both external Ca^{2+} and Mg^{2+} are removed, there is a 23% inhibition of ·NO formation, a result that is not significantly different from removal of Ca^{2+} alone. Elimination of extracellular Mg^{2+} alone does not affect ·NO production. To increase intracellular Ca^{2+} levels, the cells were exposed to the Ca^{2+} ionophore A-23187.

Table 2. *Effects of extracellular Ca^{2+} and Mg^{2+} on nitric oxide production by alveolar type II cells*

Extracellular Ca^{2+} Concn, mM	Extracellular Mg^{2+} Concn, mM	Nitrate + Nitrite, %control
1.8 (control)	1.0 (control)	100
0	1.0	65 ± 8*
0	0	77 ± 5*
1.8	0	104 ± 8

Values are means ± SE for 6 experiments. Type II cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing BSA (0.5%) for 4 h in the absence and presence of $CaCl_2$ and $MgCl_2$ in concentrations shown above. After the incubation period, cells were centrifuged, and supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells (i.e., medium containing 1.8 mM Ca^{2+} and 1.0 mM Mg^{2+}) was 0.80 ± 0.09 nmol/10^6 cells. *Values are significantly different from control ($P < 0.05$).

Table 3. *Effects of the Ca^{2+} ionophore A-23187 on nitric oxide production by alveolar type II cells*

Treatment (concn)	Nitrate + Nitrite, %control
Control	100
A-23187 (1 mM)	225 ± 38*

Values are means ± SE for 6 experiments. Type II cells (4×10^6/ml) were incubated in phosphate-buffered medium containing Ca^{2+} (1.8 (control) and presence of A-23187. After incubation period, cells were centrifuged, and supernatants were saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite production by control cells was 0.24 ± 0.02 nmol/10^6 cells. *Value is significantly different from control ($P < 0.05$).

Incubation of type II cells with A-23187 (1 mM) leads to a greater than twofold increase in ·NO formation (Table 3). All of these results demonstrate that type II cell ·NO production is Ca^{2+} dependent and further suggest that there is cNOS activity.

Immunochemical detection of cNOS. To more directly assess whether or not there is cNOS in type II cells, Western blot analysis was used. Antibodies against two known types of cNOS, i.e., eNOS and bNOS, were employed. The results, which are shown in Fig. 3, demonstrate the presence of a type II cell protein that reacts with a monoclonal anti-eNOS antibody (Fig. 3, *lane 3*). The molecular weight of this protein corresponds to that of an eNOS standard, which was obtained from human endothelial cell lysate (Fig. 3, *lane 2*). However, no bNOS could be detected with the monoclonal anti-bNOS antibody. To further confirm that no bNOS exists in these cells, a rabbit polyclonal anti-bNOS antibody (Transduction Laboratories) was

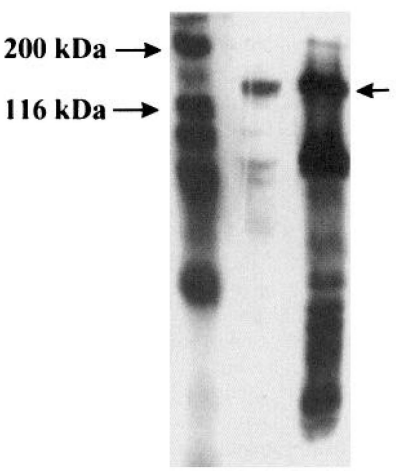

Fig. 3. Western blot analysis of type II cell proteins with anti-endothelial nitric oxide synthase (eNOS) antibody. Cells were sonicated to disrupt cell membranes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to fractionate 100-μg aliquots of cell sonicate protein. Proteins were 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 eNOS at a molecular mass of ~140 kDa. Blot is representative of results obtained from 7 different type II cell preparations.

also used, but no bNOS was detected. The results of these experiments show that there is an endothelial type of cNOS in alveolar type II cells.

Lung surfactant and DPPC effects on ·NO production. The effects of incubating alveolar type II cells with lung surfactant on cellular ·NO production were determined. Our results demonstrate that this leads to an increase in cellular ·NO formation. A time course for the effects produced during incubation of the cells with ~300 μg surfactant PL/ml, an amount that seems to produce a maximal effect, is shown in Fig. 4. The surfactant-induced increase in ·NO production is relatively rapid, i.e., effects are seen as rapidly as 15 min. This increase continues to become greater up to 1 h of incubation. After 1 h, the surfactant-induced response remains relatively constant during the next 3 h. The effects of using different amounts of pulmonary surfactant on cell ·NO production were determined, and the results are shown in Fig. 5. A 2-h incubation was used for these experiments because that is a time during which the surfactant-induced effects appear to be maximal. Surfactant-induced increases in ·NO formation occur at levels as low as 10 μg PL/ml and become greater up to ~200 μg PL/ml, where the effects seem to be maximal and ·NO formation is 238 ± 14% of control. These results show that alveolar type II cell ·NO production is increased in a time- and concentration-dependent manner after incubation of the cells with surfactant.

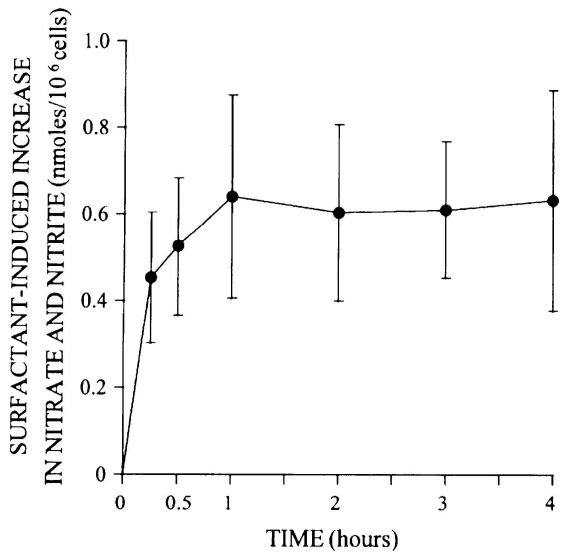

Fig. 4. Time course for surfactant-induced increase in nitric oxide production by alveolar type II cells. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and BSA (0.5%) for varying times up to 4 h in the absence and presence of lung surfactant [298 ± 15 μg phospholipid (PL)/ml], which was obtained as described in METHODS. After the appropriate incubation period, cells were centrifuged, and the nitrate and nitrite in the supernatants were measured as described in METHODS. Points in this figure were obtained by subtracting ·NO production by control cells from that by surfactant-exposed cells at each time point. Values are means ± SE for 5 different experiments. Statistical analysis of these data was made by using a split-plot analysis of variance appropriate to the repeated-measures nature of these experiments. Because of variability in response between subgroups of cells, data were transformed to a log scale for purposes of analysis. Responses at all time points in the presence of surfactant were significantly different from control ($P < 0.05$).

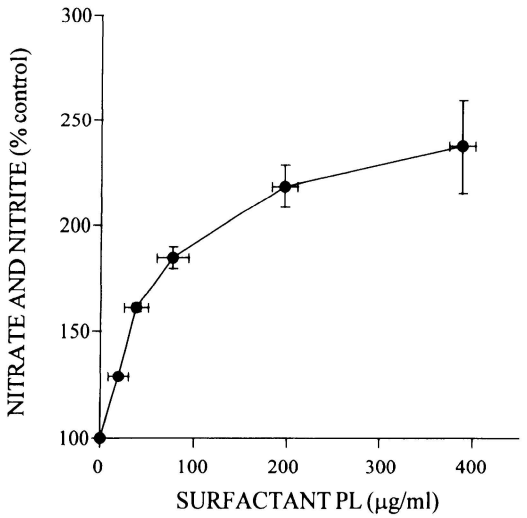

Fig. 5. Effects of pulmonary surfactant on nitric oxide production by alveolar type II cells. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and BSA (0.5%) for 2 h in the absence (control) and presence of lung surfactant, which was obtained as described in METHODS. Amount of surfactant used was expressed as μg PL/ml. After the incubation period, cells were centrifuged, and the nitrate and nitrite in the supernatants were measured as described in METHODS. Nitrate and nitrite production by control cells was 0.54 ± 0.16 nmol/10^6 cells. Values are means ± SE for 6 experiments.

The effects of SP-A and DPPC vesicles were assessed in an attempt to determine which component(s) of lung surfactant is responsible for the enhanced production of ·NO by type II cells. Incubation of the cells with SP-A at concentrations up to 40 μg/ml has no effect on cellular ·NO formation (data not shown). However, the effects of lung surfactant seem to be duplicated by its major component, DPPC. Incubation of the cells with DPPC vesicles leads to increased production of ·NO along a time course similar to that obtained with lung surfactant. The effects of different concentrations of DPPC are shown in Fig. 6. Increased ·NO formation is seen at concentrations as low as 10 μg/ml. This effect increases with increasing concentrations of DPPC up to ~100 μg/ml, where the effect seems to be maximal and ·NO production is 268 ± 32% of control. Also shown in Fig. 6 are the effects of lung surfactant plotted as the surfactant DSPC level. The concentration-effect curves for lung surfactant DSPC and DPPC vesicles are similar and are not significantly different from each other at any concentration. The effects of the two NOS inhibitors, L-NMMA and aminoguanidine, on the DPPC-induced increase in type II cell ·NO production were determined (Table 4). L-NMMA, an inhibitor of cNOS and iNOS, inhibits the response by ~40%, but aminoguanidine, an iNOS inhibitor, has no effect. All of these results show that exposure of type II cells to lung surfactant leads to increased cellular production of ·NO. The response appears to be caused by DPPC, the major component of surfactant, and appears to be due to increased activity of a cNOS.

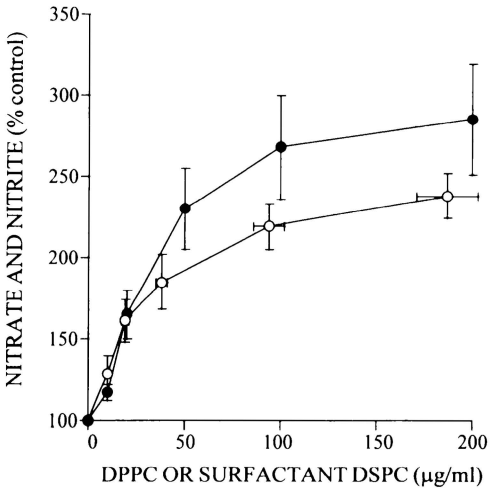

Fig. 6. Effects of dipalmitoyl phosphatidycholine (DPPC) vesicles (●) and of pulmonary surfactant (○), expressed as μg of disaturated phosphatidylcholines (DSPC), on nitric oxide production by alveolar type II cells. Cells (4×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and BSA (0.5%) for 2 h in the absence (control) or presence of DPPC vesicles or lung surfactant, which was obtained as described in METHODS. After the incubation period, cells were centrifuged, and the nitrate and nitrite in the supernatants were measured as described in METHODS. Values for nitrate and nitrite production by control cells were 0.65 ± 0.18 and 0.54 ± 0.16 nmol/10^6 cells for experiments done with DPPC vesicles or with lung surfactant, respectively. Values are means ± SE for 6 experiments.

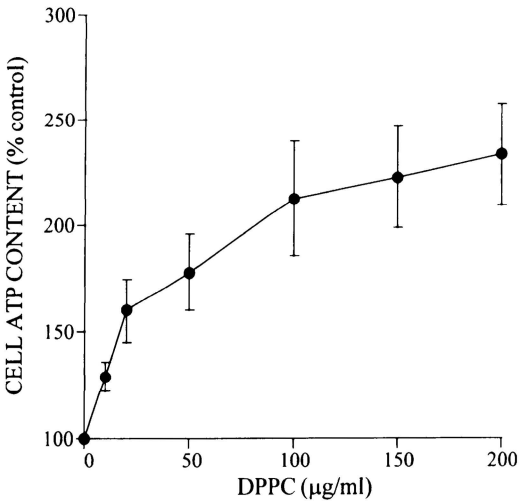

Fig. 7. Effects of DPPC vesicles on type II cell ATP levels. Cells (1×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA for 1 h in the absence and presence of varying amounts of DPPC vesicles. After the incubation period, cells were centrifuged, washed one time, and resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to the cell suspensions to disrupt cell membranes. ATP content was then measured with the firefly luciferase assay as described in METHODS. ATP content in control cells was 0.56 ± 0.07 nmol/10^6 cells. Points are mean values for 6 experiments, and bars represent SE.

DPPC effects on cell ATP content and oxygen consumption. We have shown previously that ·NO can alter type II cell ATP content and oxygen consumption (17). Therefore, we studied the effects of incubating type II cells with DPPC on these two parameters. Exposure of the cells to DPPC leads to a concentration-dependent increase in type II cell ATP levels (Fig. 7). The effect is maximal at 100–150 μg DPPC/ml, where the cell ATP content is 234 ± 24% of control. The DPPC-induced elevation of cell ATP levels is blocked by the NOS inhibitor, L-NMMA (Table 5), suggesting that increased ·NO production may be involved in the ATP response.

Synthesis of ATP cannot be measured directly in intact cells. However, since the process is tightly coupled to cellular respiration, the rate at which oxygen is consumed provides an indirect assessment of cellular ATP synthesis. Exposure of type II cells to DPPC leads to a 31 ± 6% increase in cellular oxygen consumption (Table 6). As is the case with cell ATP levels, the DPPC-induced increase in oxygen consumption is blocked by L-NMMA, suggesting that ·NO may be involved. Thus these results suggest that the DPPC-induced increase in type II cell ATP content is due to an increase in cellular ATP synthesis, perhaps caused by increased ·NO formation.

Table 4. *Effects of L-NMMA or aminoguanidine on DPPC-induced nitric oxide production by alveolar type II cells*

Treatment (concn)	Nitrate + Nitrite, %DPPC-induced response
DPPC (100 μg/ml)	100
DPPC + L-NMMA (1 mM)	63 ± 4*
DPPC + aminoguanidine (1 mM)	97 ± 5

Values are means ± SE for 5 experiments. Type II cells (4×10^6/ml) were incubated in phosphate-buffered medium containing Ca^{2+} (1.8 mM), Mg^{2+} (1.0 mM), and BSA (0.5%) for 2 h in the presence of dipalmitoyl phosphatidylcholine (DPPC), DPPC and L-NMMA, or DPPC and aminoguanidine. After the incubation period, cells were centrifuged, and supernatants were saved for analysis of nitrate + nitrite as described in METHODS. Nitrate + nitrite production by cells exposed to DPPC was 1.34 ± 0.37 nmol/10^6 cells. Nitrate + nitrite production by untreated cells (i.e., in the absence of DPPC) was 0.59 ± 0.19 nmol/10^6 cells. *Value is significantly different from DPPC alone ($P < 0.05$).

DISCUSSION

The results of these experiments confirm the suggestion from our previous paper (17) and provide additional evidence that there is cNOS activity in alveolar type II cells. One result that supports this notion is that

Table 5. *Effects of L-NMMA on type II cell ATP content in the absence or presence of DPPC*

Treatment (concn)	Cell ATP Content, %control
Control	100
L-NMMA (1 mM)	105 ± 9
DPPC (150 μg/ml)	223 ± 24*
DPPC + L-NMMA	87 ± 17

Values are means ± SE for 6 experiments. Type II cells (1×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA for 1 h in the absence or presence of L-NMMA, DPPC, or DPPC and L-NMMA. After the incubation period, cells were centrifuged, washed one time, and resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to cell suspensions to disrupt cell membranes. ATP content was then measured as described in METHODS. ATP content in control cells was 0.56 ± 0.07 nmol/10^6 cells. *Value is significantly different from control ($P < 0.05$).

Table 6. *Effects of DPPC or DPPC and L-NMMA on oxygen consumption by alveolar type II cells*

Treatment (concn)	Cellular Oxygen Consumption, %control
Control	100
DPPC (150 μg/ml)	131 ± 6*
DPPC + L-NMMA (1 mM)	99 ± 7

Values are means ± SE for 6 experiments. Type II cells (1×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA for 25 min in the absence (control) or presence of DPPC or DPPC and L-NMMA. After this incubation period, cell suspension was transferred to oxygraph, and cellular oxygen consumption was measured for 10 min at 37°C. Oxygen consumption by control cells was 50 ± 6 nmol/10^6 cells^{-1}·h^{-1}. *Value is significantly different from control ($P < 0.05$).

some endogenous ·NO can be measured in the supernatant from unstimulated, unincubated cells. Furthermore, during incubation of the cells, the rate of additional ·NO formation appears to remain rather constant at 0.3 nmol·10^6 cells^{-1}·h^{-1}. If this rate is calculated on the basis of type II cell protein per minute, the value is ~50 pmol·mg protein^{-1}·min^{-1}. This rate is slightly greater than that of 37 pmol·mg protein^{-1}·min^{-1} reported for bovine aortic endothelial cells (2), where cNOS has been shown to be important to cellular function. The observation that ·NO formation is independent of extracellular L-arginine may also be important. Partially purified cNOS from bovine aortic endothelial cells has been reported to have a low requirement for L-arginine (4, 20). Therefore, it is thought that the endogenous levels of the substrate are sufficient to support cNOS activity in these cells. Perhaps this is also the case in type II cells. Also, if the endogenous level of L-arginine is sufficient to support cNOS activity, this may account for the fact that NOS inhibitors, which are arginine analogs, do not cause complete inhibition of ·NO formation. It may be that the addition of relatively large amounts of the inhibitors leads to some reduction in ·NO formation, but competition from the endogenous substrate prevents greater levels of inhibition.

Experiments in which cNOS inhibitors were used and in which the Ca^{2+} dependence of ·NO formation was studied, along with Western blot analysis, provide further evidence for the involvement of a cNOS. Three inhibitors of both iNOS and cNOS, L-NMMA, L-NIO, and L-NAME (16, 22), inhibit production of ·NO by 28–46%. On the other hand, a specific inhibitor of iNOS, aminoguanidine (7), has no effect on type II cell ·NO formation. There are two lines of evidence to show that the type II cell NOS activity is Ca^{2+} dependent, which is characteristic of cNOS activity (5). First, cellular ·NO production is reduced by 35% when the cells are incubated in Ca^{2+}-free medium. Second, ·NO formation is increased by more than twofold when the intracellular Ca^{2+} concentration is increased by incubating the cells with the Ca^{2+} ionophore A-23187. Finally, Western blot analysis provides more direct evidence for type II cell cNOS and suggests that it is similar to the human eNOS isoform. Thus all of these current results taken together with the observations from our previous paper (17) indicate that there is cNOS activity in alveolar type II cells and suggest that it is an eNOS.

To determine if this cNOS may be involved in alveolar type II cell metabolism of lung surfactant, we studied the effects of incubating the cells with a lung surfactant preparation. We did this because reuptake of surfactant is the initial step in recycling of the material. The results show that cellular ·NO levels increase rather quickly (within 15 min) after exposure to surfactant and that the response is concentration dependent, at least up to 200 μg surfactant PL/ml. Furthermore, the surfactant-induced increase in ·NO formation appears to be caused by its major component, DPPC. Exposure of the cells to DPPC also leads to increases in cellular ATP levels. The fact that incubation of type II cells with DPPC also leads to an increase in oxygen consumption suggests that there is an increase in the rate of ATP synthesis. It is possible that the increase in ATP synthesis and the subsequent elevation in ATP levels is caused by the increased cellular production of ·NO.

There is some evidence to suggest that the surfactant-induced elevation of type II cell ·NO levels is due to DPPC. First, the time courses for the surfactant and DPPC effects are similar. Second, the concentration-effect curves for the DPPC- and surfactant DSPC-induced increases in cell ·NO levels are similar. In addition, there is evidence that the DPPC-induced response is due to a cNOS, i.e., the DPPC-induced increase in ·NO production is inhibited by L-NMMA but not by aminoguanidine. Although these results suggest that the surfactant-induced increase in cellular ·NO levels is due to DPPC, we did not look at other components of surfactant except for SPA, which has no effect.

It is possible that the DPPC-induced increase in ·NO production leads to an increase in the rate of alveolar type II cell ATP synthesis and a subsequent elevation of cell ATP levels. The fact that the DPPC-induced increase in cellular oxygen consumption, and thus the rate of ATP synthesis, is blocked by L-NMMA suggests that ·NO formation is required for this effect. There are two additional lines of evidence to support a link between the DPPC-induced increases in ·NO production and ATP levels. First, the DPPC concentration-effect curves for increased amounts of ·NO and ATP are similar. In fact, if one calculates from double-reciprocal plots the DPPC concentration that causes 50% of the maximal response, the range of values is similar for both the ·NO and ATP effects (8–16 μg/ml). Second, the DPPC-induced increase in cell ATP levels is inhibited by the NOS inhibitor L-NMMA. This latter result suggests that the increase in cellular ·NO production occurs first and is required for the elevation of ATP.

There are some possible discrepancies that arise when one compares our current results with those obtained by us and others in previous studies (9, 17). For example, it has been reported that exposure of type II cells to exogenous ·NO, by using the ·NO generator *S*-nitroso-*N*-acetylpenicillamine, leads to a decrease in type II cell ATP levels (9, 17). On the other hand, we now report that, when endogenous ·NO levels are

increased by exposing the cells to DPPC, the type II cell ATP levels are increased. This may mean that widely different effects can be obtained, depending on whether the source of ·NO is endogenous or exogenous. Another possible explanation is that peroxynitrite may be formed after exposure of the cells to exogenous ·NO and that it is the peroxynitrite that leads to the exogenous ·NO-induced decrease in ATP levels. Another possible discrepancy involves the effects of manipulating endogenous ·NO levels with NOS inhibitors. We reported previously that L-NAME causes a decrease in endogenous ·NO levels and an increase in the amounts of cell ATP (17). In our current study, the DPPC-induced increase in endogenous ·NO leads to increased cellular ATP levels. Although this is difficult to explain, it may have something to do with the effects of L-NAME relative to some other NOS inhibitors. For example, L-NAME is not as potent at inhibiting ·NO production by type II cells as two other inhibitors, L-NMMA and L-NIO, which were used in our current study. Furthermore, although exposure to L-NAME leads to increases in cell ATP levels (17), incubating cells with L-NMMA has no effect on type II cell ATP levels, i.e., incubating cells with L-NMMA (1 mM) leads to cell ATP levels that are 105 ± 9% of control (Table 5). Therefore, it may be that there are some different isoforms of NOS in type II cells that respond differently to different inhibitors and that may have different effects on cell ATP levels.

In summary, we have shown that exposure of type II cells to lung surfactant or DPPC vesicles leads to increased production of ·NO and increases in the rate of ATP synthesis and cell ATP levels. It is known that there may be several events involved in surfactant recycling, including reuptake, repackaging, and/or degradation and resynthesis of these materials (23). Chander et al. (3) have shown that uptake of DPPC vesicles by type II cells is not inhibited by uncouplers of oxidative phosphorylation. This result suggests that large amounts of ATP are not required for reuptake. Perhaps the increased levels of ATP are required for repackaging and/or degradation and resynthesis of surfactant. Furthermore, the events involved in making increased amounts of ATP available to type II cells for these processes may be set into motion by DPPC-induced ·NO formation.

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