Nitric oxide alters metabolism in isolated alveolar type II cells

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Miles, P. R., L. Bowman, and L. Huffman. Nitric oxide alters metabolism in isolated alveolar type II cells. *Am. J. Physiol.* 271 (*Lung Cell. Mol. Physiol.* 15): L23–L30, 1996.—Alveolar type II cells may be exposed to nitric oxide (·NO) from external sources, and these cells can also generate ·NO. Therefore we studied the effects of altering ·NO levels on various type II cell metabolic processes. Incubation of cells with the ·NO generator, *S*-nitroso-*N*-acetylpenicillamine (SNAP; 1 mM), leads to reductions of 60–70% in the synthesis of disaturated phosphatidylcholines (DSPC) and cell ATP levels. Cellular oxygen consumption, an indirect measure of cell ATP synthesis, is also reduced by SNAP. There is no direct effect of SNAP on lung mitochondrial ATP synthesis, suggesting that ·NO does not directly inhibit this process. On the other hand, incubation of cells with N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), the enzyme responsible for ·NO synthesis, results in increases in DSPC synthesis, cell ATP content, and cellular oxygen consumption. The L-NAME effects are reversed by addition of L-arginine, the substrate for NOS. Production of ·NO by type II cells is inhibited by L-NAME, a better inhibitor of constitutive NOS (cNOS) than inducible NOS (iNOS), and is reduced in the absence of external calcium. Aminoguanidine, a specific inhibitor of iNOS, has no effect on cell ATP content or on ·NO production. These results indicate that alveolar type II cell lipid and energy metabolism can be affected by ·NO and suggest that there may be cNOS activity in these cells.

lung surfactant; adenosine 5′ triphosphate

NITRIC OXIDE (·NO) is a free radical which is produced by a variety of cell types in the lungs. The synthesis of ·NO from L-arginine is catalyzed by the enzyme 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 (8). Nitric oxide generated by cNOS in the lungs is found in endothelial cells (5) and airway neurons (1) and seems to be important in the regulation of vascular and bronchial smooth muscle activity. On the other hand, it is known that iNOS is involved in ·NO generation by other lung cell types. For example, exposure of alveolar macrophages to diverse stimuli of inflammation, such as cytokines, lipopolysaccharide (LPS), and interferon-γ, leads to the generation of large amounts of ·NO for prolonged periods of time (17, 25). iNOS has also been reported in fibroblasts (19), pulmonary artery smooth muscle cells (26), and neutrophils (34). Thus both cNOS and iNOS are found in various cell types in the lungs.

Alveolar type II cells play a critical role in maintaining normal lung function. Although they perform a variety of important functions, one major role is the synthesis of lung surfactant, a mixture of lipids and proteins that lines the alveolar surface and prevents its collapse by reducing surface tension forces at the air-liquid interface. The major surface active component is dipalmitoyl phosphatidylcholine (DPPC) (20). Because type II cells are located on the alveolar surface, they may be affected by substances, such as ·NO or oxygen radicals, released from other cells in their vicinity or by substances released into the alveolar lining layer. For example, it is known that exposure to H_2O_2, which may be released from alveolar macrophages or neutrophils during the inflammatory process, can lead to metabolic changes in type II cells (21).

It is also known that ·NO can be generated by alveolar type II cells themselves. Punjabi et al. (28) have shown that type II cells synthesize ·NO and that iNOS expression in these cells is increased after inhalation of ozone. Gutierrez et al. (11) demonstrated that there is enhanced expression of iNOS in type II cells after exposure of the cells to cytokines or LPS. Thus these results suggest that there is iNOS activity in alveolar type II cells. On the other hand, to the best of our knowledge, there are no reports of cNOS activity in these cells. Because these cells may be exposed to ·NO and because they have iNOS activity, it may be important to determine the effects of exposing these cells to ·NO and of altering the endogenous synthesis of ·NO on some aspects of type II cell function and metabolism. In the experiments reported in this paper, we generated extracellular nitric oxide by using *S*-nitroso-*N*-acetylpenicillamine (SNAP) and inhibited cellular ·NO synthesis with N^G-nitro-L-arginine methyl ester (L-NAME). In some experiments, we used another NOS inhibitor, aminoguanidine. The objectives of this investigation were to study the effects of SNAP and L-NAME on *1*) synthesis of lung surfactant DPPC, a major function of type II cells, and *2*) some basic processes involved in energy metabolism, such as cellular ATP levels, cellular oxygen consumption, and mitochondrial ATP formation. In addition, we measured the effects of some NOS inhibitors and external calcium on cellular ·NO production.

METHODS

Isolation of alveolar type II cells. Alveolar type II cells were obtained according to methods we described previously (18, 21). Briefly, specific pathogen-free male Sprague-Dawley rats (200–250 g; Hilltop Laboratories, Scottdale, PA) were anesthetized with pentobarbital sodium (150 mg/kg body wt), and the

heart and lungs were rapidly removed. Lungs were perfused with 0.9% NaCl to remove blood cells, and free alveolar macrophages were removed by bronchoalveolar lavage with phosphate-buffered medium (in mM: 145 NaCl, 5 KCl, 9.35 Na_2HPO_4, 1.9 NaH_2PO_4, 5 glucose, pH 7.4). To disperse lung cells, the lungs were filled with phosphate-buffered medium containing elastase (40 U/ml, type I; U.S. Biochemical, Cleveland, OH) and deoxyribonuclease (DNase, 0.006%; Sigma Chemical, St. Louis, MO) and incubated at 37°C for 30 min. After enzymatic digestion, lungs were minced with a McIlwain tissue chopper (Mickle Engineering, Gomshall, Surrey, UK) set at a slice thickness of 0.5 mm. The minced lungs were then placed in phosphate-buffered medium containing 25% fetal calf serum and 0.006% DNase (to prevent cell clumping) and incubated at 37°C for 10 min to arrest digestion. The cell suspension was then strained through nylon mesh, and the type II cell fraction was obtained by centrifugal elutriation (21). The type II cells were washed once and 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.

The cell number was determined with a Coulter model Z_B electronic cell counter (Coulter Instrument, Hialeah, FL), and purity of the type II cell fraction was routinely estimated with the fluorescent dye, phosphine 3R, as we have reported previously (18). In the experiments reported in this paper, we obtained 5.8 ± 0.3 × 10^6 cells per rat in the type II cell-enriched fraction with a purity of 91 ± 2% (means ± SE for 10 experiments). Contamination was due to alveolar macrophages. Membrane integrity was assessed by measuring the exclusion of trypan blue dye (27) and the release of lactate dehydrogenase (LDH; 32) from the cells.

Measurement of incorporation of [^{3}H]choline into DSPC. To assess synthesis of lung surfactant disaturated phosphatidylcholines (DSPC), we measured the incorporation of [^{3}H]choline into DSPC in alveolar type II cells. The cells (1 × 10^6 per ml) were suspended in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, choline chloride (0.05 mM), palmitic acid (0.1 mM, complexed with BSA), glycerol (0.1 mM), and [*methyl*-^{3}H]-choline (1 μCi/ml; sp act 80 Ci/mmol; New England Nuclear, Boston, MA), and incubated at 37°C for 2 h in the absence (control) or presence of substances which may affect nitric oxide levels and are listed below. Incorporation of choline into DSPC is linear over this 2-h period. After the incubations, a 0.5-ml aliquot of the cell suspension was taken for analysis. The lipids were extracted according to the method of Folch et al. (7). Then DSPC was isolated from the lipids, using neutral alumina columns according to the method of Mason et al. (22). After isolation of the DSPC, solvents were evaporated, and Aquasol (10 ml; New England Nuclear) was added to each sample which was counted in a liquid-scintillation counter. The results were expressed as nanomoles choline incorporated into DSPC per 10^6 cells.

Some substances were added to the incubation medium to determine their effects on incorporation of choline into DSPC and/or other cellular functions. All of them are water soluble, so they were dissolved in the same phosphate-buffered medium used for incubation of the cells. All of the concentrations shown in this paper are final concentrations in the incubation mixtures. SNAP is a generator of nitric oxide and was used to determine the effects of ·NO exposure (16). L-NAME·HCl is an inhibitor of both cNOS and iNOS and was used to determine the effects of NOS inhibition in type II cells (24, 29). Aminoguanidine hemisulfate is a specific inhibitor of iNOS (3, 10). All of the substances listed above were obtained from Research Biochemicals International (Natick, MA). Superoxide dismutase (SOD; Sigma) and catalase (Sigma) were used to scavenge superoxide anion and H_2O_2, respectively, from the incubation mixture. Finally, $NaNO_3$ and $NaNO_2$, two degradation products of nitric oxide, were used to determine whether the degradation products were responsible for any of the observed effects.

Measurement of cellular ATP levels and oxygen consumption. The effects of SNAP, L-NAME, aminoguanidine, SOD, $NaNO_3$, and $NaNO_2$ on type II cell ATP levels were determined. ATP content was determined by the firefly luciferase assay adapted from the method of Wulff and Doppen (33). Cells (1 × 10^6 per ml) were incubated (37°C) for 1 h in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA in the absence (control) or presence of the substances listed above. 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 once and then resuspended so there were 1 × 10^6 cells in 0.125 ml of 0.5 M tris(hydroxymethyl)aminomethane (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 immediately analyzed 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 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 (Sigma) standard solutions and 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 phosphate-buffered medium that had been bubbled with gases of known oxygen concentration until saturation occurred. The cells (1 × 10^6 per ml) were incubated (37°C) for 25 min in phosphate-buffered medium containing Ca^{2+}, Mg^{2-}, and BSA in the absence (control) or presence of SNAP or L-NAME. After this incubation, the cell suspension was transferred to the oxygraph, and oxygen consumption was measured for 10 min at 37°C. In separate experiments, we determined that neither the SNAP nor the L-NAME in the cell suspensions affects the measurement of oxygen consumption. 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 nanomoles of oxygen consumed per 10^6 cells per hour.

Measurement of nitric oxide production. The amounts of nitric oxide generated by SNAP were measured over the different time periods and in the various incubation media used in our experiments. The ·NO released into the medium was measured with an ISO-NO meter (World Precision Instruments, Sarasota, FL) connected to a chart recorder. Amounts of ·NO produced by isolated alveolar type II cells were measured as the stable oxidation products of ·NO, nitrite (NO_2^-) and nitrate (NO_3^-). Alveolar type II cells (2 × 10^6) were incubated for 2 h in 0.5 ml of phosphate-buffered medium with Ca^{2+}, Mg^{2+}, and BSA. Some experiments with cells were done in the absence of extracellular calcium. The NOS inhibitors, L-NAME (1 mM) and aminoguanidine (1 mM), were included in some incubations with type II cells. After the incubation, cells were removed by centrifugation,

and the supernatants were saved for analysis. All samples were first incubated with *Escherichia coli* nitrate reductase to convert the NO_3^- to NO_2^-. The net ·NO production was then measured by the Greiss reaction (9). The amount of nitrate and nitrite in the samples was calculated from a standard curve which was constructed from $NaNO_2$ standards. Conversion of nitrate to nitrite was checked in each assay by using standard $NaNO_3$ preparations.

Isolation of lung mitochondria and measurement of ATP synthesis. To determine the effects of nitric oxide on mitochondrial function, lung mitochondria were prepared as described by Spear and Lumeng (30). Lungs were perfused with 0.9% NaCl, dissected free of the trachea, bronchi, and connective tissue, and finely minced by chopping four times with a McIlwain tissue chopper set for a slice thickness of 0.5 mm. The mince was homogenized in cold isolation medium (0.25 M sucrose, 2 mM EDTA, 5 mM Tris·HCl, and 1% fatty acid-poor BSA; pH 7.4), using a Teflon-glass Potter-Elvehjem homogenizer. Homogenization was performed by three strokes of the pestle at 1,000 rpm. The concentration of lung tissue in the homogenate was 100 mg per ml. The crude homogenate was centrifuged at 2,000 *g* for 5 min, and the pellet was discarded. The mitochondrial fraction was isolated by spinning the supernatant at 17,800 *g* for 5 min. The mitochondrial pellet was washed twice with cold isolation medium and resuspended in an incubation medium (containing in mM: 105 KCl, 2 KH_2PO_4, 30 Tris·HCl, 0.1 EDTA, and 1.0% BSA, pH 7.2) for use in all experiments. To assess the condition of the isolated mitochondria, the respiratory control ratio (RCR), defined as the ratio of the rate of oxygen consumption in the presence of added ADP (0.3 mM) to the rate obtained after ADP expenditure (2), was determined. The RCR was 2.6 ± 0.1, which is comparable to that reported by others for rat lung mitochondria (6) and indicates that the mitochondrial membranes were intact and the rate of respiration acceptable.

The effects of SNAP on mitochondrial ATP synthesis were determined. Mitochondria were resuspended in the incubation medium at a final concentration of 0.075 mg protein/ml for all experiments. This concentration was used because it is the approximate level of mitochondrial protein found in 1×10^6 type II cells (4), the amount used in all of our intact cell experiments. The mitochondrial suspensions were incubated at 37°C for 5 min in the absence (control) or presence of 1 mM SNAP. A 5-min exposure was used because longer incubation periods result in a reduced mitochondrial RCR. After this incubation, the samples were spun for 15 s at 12,800 *g* in an Eppendorf microcentrifuge. The supernatants were removed, and the mitochondrial pellets were resuspended in fresh incubation medium. To determine the rate of ATP synthesis, these mitochondria (0.075 mg protein/ml) were incubated at 30°C for 5 min in the presence of succinate (5 mM) and ADP (10 μM). After incubation, the mitochondrial suspension was spun in an Eppendorf microcentrifuge, and ATP levels in the supernatants were measured with the firefly luciferase assay as described previously. No ATP was detectable in the mitochondrial pellets.

Statistical analyses. All comparisons of statistical significance were made by using an analysis of variance to assess whether or not significant differences exist between the treatment groups and using the Student's *t*-test to compare each treatment group with control. In addition, the Dunnett method was used when different treatments were compared with a common control. $P < 0.05$ was taken as the limit to indicate significance.

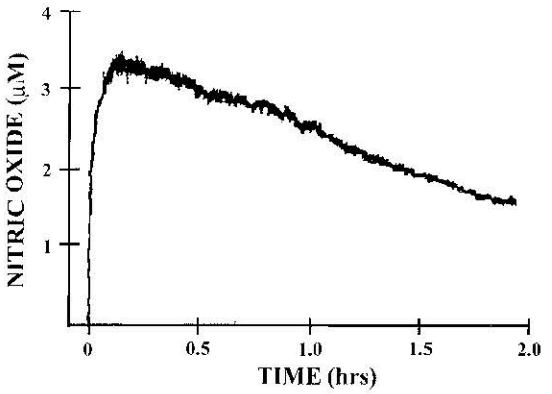

Fig. 1. Time course of nitric oxide produced during the incubation of 1 mM *S*-nitroso-*N*-acetylpenicillamine (SNAP) for 2 h in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and bovine serum albumin (BSA). ·NO was measured with an ISO-NO meter. This result is a typical experiment which was reproduced 4 times.

RESULTS

SNAP-induced nitric oxide formation and membrane integrity. The time course for nitric oxide production by SNAP (1 mM; 2 h) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA is shown in Fig. 1. ·NO formation peaks at ~3.5 μM in 7 min and then declines to ~75 and 40% of the peak value in 1 and 2 h, respectively. Inclusion of the substrates for surfactant synthesis, i.e., palmitate, choline, and glycerol, in the medium has no effect on the SNAP-induced ·NO production. To assess the effects of nitric oxide exposure on the general condition of type II cells, the ability of the cells to exclude trypan blue dye and retain LDH in the absence (control) and presence of SNAP (1 mM; 2 h) was determined. The amount of ·NO produced by SNAP during this incubation period was measured as nitrate and nitrite production and was 14 ± 1 μM (mean ± SE for 3 determinations). The percentage excluding trypan blue was 94 ± 1 and 93 ± 2 for control and SNAP-treated cells, respectively. LDH release from SNAP-treated cells was 97 ± 4% of that from control cells (mean ± SE for 5 experiments). These results suggest that exposure of type II cells to SNAP does not result in gross membrane damage.

Incorporation of [^{3}H]choline into DSPC. The effects of incubating alveolar type II cells with the nitric oxide generator, SNAP, on the incorporation of [^{3}H]choline into DSPC were determined. Exposure of the cells to SNAP leads to inhibition of DSPC synthesis, and the dose-response relationship for these effects is shown in Fig. 2. The SNAP-induced inhibition occurs at concentrations as low as 0.01 mM and is maximal at 1.0 mM. Therefore, for other experiments reported in this paper, 1.0 mM SNAP was used. To determine if the effects of SNAP involve superoxide anion or H_2O_2, both of which can be released from type II cells (31), or to the ·NO degradation products, nitrate and/or nitrite, additional experiments were performed (Table 1). Neither SOD, which eliminates superoxide anion, nor catalase, which destroys H_2O_2, has any effect on the SNAP-induced inhibition of DSPC synthesis. Because peroxynitrite is

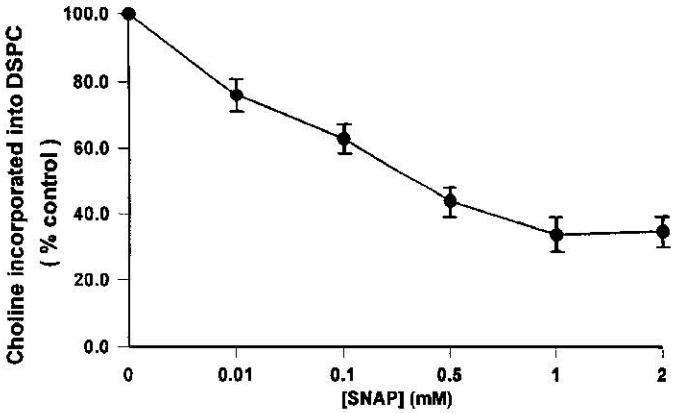

Fig. 2. Concentration dependence for effects of SNAP on incorporation of [^{3}H]choline into disaturated phosphatidylcholine (DSPC) in type II cells. The cells (1×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, BSA, choline chloride, glycerol, palmitate, and [^{3}H]choline for 2 h in the absence and presence of various concentrations of SNAP. After the incubation period, lipids were extracted from the cell suspensions, DSPC was isolated, and the choline incorporated into DSPC was measured as described in METHODS. Results are expressed as %control. Choline incorporated into DPSC in control cells is 0.161 ± 0.014 nmol per 10^6 cells. ●, Mean values for 6 experiments; bars represent SE.

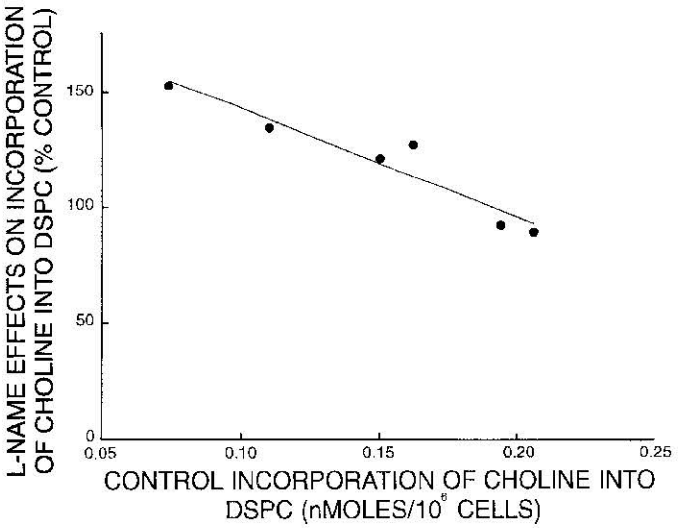

Fig. 3. N^G-nitro-L-arginine methyl ester (L-NAME) effects on incorporation of [^{3}H]choline into DSPC in type II cells. The cells (1×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, BSA, choline chloride, glycerol, palmitate, and [^{3}H]choline for 2 h in absence and presence of L-NAME (0.5 mM). After incubation period, lipids were extracted from cell suspensions, DSPC was isolated, and choline incorporated into DSPC was measured as described in METHODS. Results from 6 individual experiments are shown. ●, Relationship between control level of choline incorporated into DSPC and effect of L-NAME on that level. Line in figure was constructed by using linear regression analysis. Correlation coefficient is 0.955.

formed from ·NO and superoxide, these results also suggest that peroxynitrite is not involved. Furthermore, neither nitrate nor nitrite inhibits DSPC synthesis. In fact, nitrite appears to stimulate the process for reasons we don't understand. We also measured DSPC synthesis in the presence of another ·NO generator, spermine-nitric oxide complex (1 mM; 2 h), which results in ·NO production that peaks at ~8.7 μM in 7 min and then declines to ~73% of the peak value in 2 h. DSPC synthesis is reduced to 40 ± 8% of control (mean ± SE for 4 experiments) in the presence of the spermine-nitric oxide complex. All of these results indicate that incorporation of [^{3}H]choline into DSPC in type II cells is inhibited after exposure of the cells to nitric oxide.

Table 1. *Effects of SNAP, superoxide dismutase, catalase, nitrate, and nitrite on incorporation of [^{3}H]choline into disaturated phosphatidylcholines in alveolar type II cells*

Treatment (conc.)	Incorporation of [^{3}H]choline into DSPC (% control)
Control	100
SNAP (1 mM)	41 ± 5*
SOD (0.4 mg/ml)	121 ± 6*
Catalase (4 mg/ml)	110 ± 12
SNAP – SOD	31 ± 3*
SNAP – catalase	45 ± 4*
$NaNO_3$ (1 mM)	96 ± 7
$NaNO_2$ (1 mM)	130 ± 5*

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+}, bovine serum albumin (BSA), choline chloride, glycerol, palmitate, and [^{3}H]choline for 2 h in absence (control) and presence of substances shown above. SNAP, *S*-nitroso-*N*-acetylpenicillamine; SOD, superoxide dismutase; and DSPC, disaturated phosphatidylcholine. After incubation period, lipids were extracted from cell suspensions, DSPC was isolated, and choline incorporated into DSPC was measured. Choline incorporated into DSPC in control cells is 0.166 ± 0.023 nmol per 10^6 cells. *Values are significantly different from control ($P < 0.05$).

To study the effects of altering the endogenous level of ·NO, L-NAME, an inhibitor of NOS, was employed. The effects of 0.5 mM L-NAME on the incorporation of [^{3}H]choline into DSPC seem to depend on the control levels of DSPC synthesis (Fig. 3). When control values for incorporation of [^{3}H]choline into DSPC are relatively high, L-NAME has no effect. On the other hand, if the control levels are relatively low, L-NAME appears to stimulate DSPC synthesis by as much as 50%. In fact, the relationship between control rates of [^{3}H]choline incorporation into DSPC and stimulation of this process by L-NAME is reasonably linear; i.e., the correlation coefficient for the best-fit line (obtained with linear regression analysis) in Fig. 2 is 0.955. Thus these results suggest that inhibition of ·NO production by type II cells leads to elevated levels of incorporation of [^{3}H]choline into DSPC, if only when baseline amounts of DSPC synthesis are relatively low.

Cellular ATP levels and oxygen consumption. One possibility is that the effects of ·NO on DSPC synthesis may be due to the ability of ·NO to alter type II cell ATP levels. In fact, we have shown previously that exposure of the cells to H_2O_2 results in these responses (21). Therefore, the effects of SNAP on cellular ATP levels were determined, and the results are shown in Table 2. Exposure to 1 mM SNAP leads to a 72% reduction in cell ATP content. This inhibition is probably not due to the production of extracellular peroxynitrite because SOD has no effect on the SNAP-induced response. Also, neither nitrate nor nitrite, two degradation products of

Table 2. *Effects of SNAP, SOD, nitrate, and nitrite on alveolar type II cell ATP content*

Treatment (conc.)	Cellular ATP Content (% control)
Control	100
SNAP (1 mM)	28 ± 7*
SOD (0.4 mg/ml)	97 ± 8
SNAP – SOD	28 ± 7*
$NaNO_3$ (1 mM)	97 ± 4
$NaNO_2$ (1 mM)	90 ± 1*

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 hour in absence (control) and presence of substances shown above. After incubation period, cells were centrifuged, washed once, and resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to cell suspensions to disrupt membranes. ATP content was measured with firefly luciferase assay as described in METHODS. ATP levels in control cells are 0.63 ± 0.09 nmol per 10^6 cells. *Values are significantly different from control ($P < 0.05$).

Table 3. *Effect of L-NAME, L-arginine, and aminoguanidine on alveolar type II cell ATP content*

Treatment (conc.)	Cellular ATP content (% control)
Control	100
L-NAME (0.5 mM)	143 ± 7*
L-NAME (1.0 mM)	192 ± 17*
L-Arginine (10 mM)	98 ± 8
L-NAME (0.5 mM) and L-Arginine (10 mM)	99 ± 4†
Aminoguanidine (0.5 mM)	94 ± 3
Aminoguanidine (1.0 mM)	97 ± 5

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 hour in absence (control) and presence of substances shown above. After incubation period, cells were centrifuged, washed once, and resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to cell suspensions to disrupt membranes. ATP content was measured with the firefly luciferase assay as described in METHODS. ATP levels in control cells are 0.60 ± 0.07 nmol per 10^6 cells. *Values are significantly different from control ($P < 0.05$). †Value is significantly different compared with L-NAME (0.5 mM) alone ($P < 0.05$).

nitric oxide, appears to be involved in the inhibition. In other experiments (data not shown), we also found that the decline in ATP is not due to a direct effect of ·NO on the ATP molecule itself; i.e., incubation of ATP with SNAP does not alter the ATP level.

Incubation of type II cells with L-NAME leads to increased ATP levels. Like the L-NAME effects on DSPC synthesis, these alterations are dependent on control levels of ATP (Fig. 4). When control values for cell ATP content are relatively high, the L-NAME-induced increases in ATP are relatively low, i.e., 30% increase. However, when control levels are relatively low, L-NAME appears to increase cell ATP content by as much as 240%. The relationship between control levels of cell ATP and the L-NAME-induced increase is linear; i.e., the correlation coefficient for the best-line fit (obtained with linear regression analysis) is 0.985.

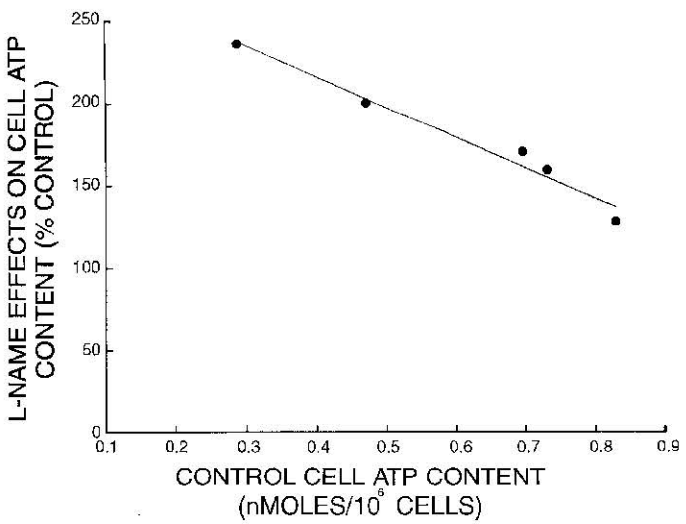

Fig. 4. L-NAME effects on type II cell ATP content. Cells (1×10^6/ml) were incubated (37°C) in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA for 1 h in absence and presence of L-NAME (0.5 mM). After incubation period, cells were centrifuged, washed once, and resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to cell suspensions to disrupt cell membranes. ATP content was measured with firefly luciferase assay, as described in METHODS. Results from 5 individual experiments are shown. ●, Relationship between control ATP content and effect of L-NAME on that content. Line was constructed by using linear regression analysis. Correlation coefficient is 0.985.

The effects of L-NAME, an inhibitor of both iNOS and cNOS (24, 29), and aminoguanidine, a specific inhibitor of iNOS (3, 10), on type II cell ATP levels are summarized in Table 3. There appears to be a dose-dependent effect of L-NAME; i.e., treatment with 0.5 mM inhibitor results in a 43% increase in ATP, whereas exposure to 1.0 mM L-NAME leads to a 92% increase. In addition, we incubated the cells with L-arginine, the substrate for NOS. L-arginine (10 mM) alone does not affect cell ATP levels, but it does reverse the stimulation produced by 0.5 mM L-NAME. Aminoguanidine has no effect on cell ATP content. The results of all of these experiments show that exposure of type II cells to nitric oxide results in reduced levels of ATP, whereas incubation of the cells with L-NAME leads to elevated levels of ATP, and aminoguanidine has no effect. This latter result suggests that cNOS may be involved in determining type II cell ATP levels.

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. Therefore, oxygen consumption was measured in untreated alveolar type II cells and in cells treated with SNAP or L-NAME. The results are shown in Table 4. SNAP inhibits cellular oxygen consumption, and L-NAME stimulates the process. We have shown previously (21) and confirmed in another set of experiments (data not shown) that sodium cyanide completely inhibits type II cell oxygen consumption, indicating that mitochondrial respiration accounts for all measurable oxygen consumed. Thus these results suggest that SNAP interferes with ATP synthesis, whereas L-NAME stimulates ATP synthesis. These effects may account, at least in part, for the changes in the steady-state levels of type II cell ATP.

Table 4. *Effects of SNAP and L-NAME on oxygen consumption in alveolar type II cells*

Treatment (conc.)	Cellular Oxygen Consumption (% control)
Control	100
SNAP (1 mM)	81 ± 2*
L-NAME (1 mM)	129 ± 6*

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 minutes in absence (control) or presence of SNAP or L-NAME. After this incubation period, cell suspension was transferred to oxygraph, and cellular oxygen consumption was measured for 10 min at 37°C. Oxygen consumption in control cells is 56 ± 5 nmol·10^6 cells $^{-1}$·h $^{-1}$. *Values are significantly different from control ($P < 0.05$).

ATP synthesis in isolated lung mitochondria. Exposure of type II cells to SNAP leads to a reduction in the steady-state ATP content. One possible reason for this is that the rate of ATP synthesis, which occurs primarily in mitochondria, is diminished. Therefore we studied the effects of SNAP on ATP production by isolated lung mitochondria. ·NO formation by SNAP in the mitochondrial buffer system increased rapidly during the 5-min incubation period and reached a value of ~3 μM. The results, which are shown in Table 5, indicate that SNAP has no effect on mitochondrial ATP synthesis. These results suggest that the SNAP-induced reduction in cellular ATP content is probably not due to a direct effect of ·NO on mitochondrial ATP synthesis.

Nitric oxide production. To determine whether the L-NAME effects on cell metabolism are due to inhibition of cellular ·NO production, we measured the production of nitrate and nitrite, the stable oxidation products of ·NO, by type II cells. The results are shown in Table 6. Basal production of ·NO is 0.80 ± 0.08) nmol of NO_2^- and NO_3^- per 10^6 cells in 2 h. Interestingly, this compares favorably with a rate reported by Punjabi et al. (28) for unstimulated type II cells in culture. L-NAME, a better inhibitor of cNOS than iNOS, inhibits ·NO production. The effect of L-NAME is maximal at 1 mM. However, aminoguanidine, a specific inhibitor of iNOS, has no effect. Furthermore, the cellular production of ·NO is reduced when calcium is removed from the medium, suggesting that the ·NO production is Ca^{2+}-dependent. These results demonstrate that the L-NAME effects on cell metabolism may be due to inhibition of ·NO production and that the ·NO produced may be due to cNOS activity in the type II cells.

Table 5. *Effects of SNAP on ATP formation in isolated lung mitochondria*

Treatment (conc.)	ATP Formation (% control)
Control	100
SNAP (1 mM)	108 + 5

Values are means ± SE for 7 experiments. Isolated lung mitochondria (0.075 mg protein/ml) were incubated (37°C) for 5 minutes in absence (control) or presence of 1 mM SNAP. After this incubation, samples were spun for 15 s at 12,800 *g*, supernatants were removed, and mitochondrial pellets were resuspended in fresh incubation medium (105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris·HCl, 0.1 mM EDTA, and 1% BSA, pH 7.2). These mitochondria were incubated (30°C) for 5 min in presence of succinate (5 mM) and ADP (10 μM) to measure ATP formation. After this incubation, mitochondria were spun at 12,800 *g* for 15 s, and ATP in supernatants was measured with the firefly luciferase assay as described in METHODS. ATP levels formed by control mitochondria are 45 ± 3 nmol/mg mitochondrial protein.

Table 6. *Effects of L-NAME, aminoguanidine, and extracellular calcium on nitric oxide production by alveolar type II cells*

Treatment (conc.)	Nitrate + Nitrite (% control)
Control	100
L-NAME (1 mM)	77 ± 2*
Aminoguanidine (1 mM)	108 ± 6
Ca^{2+}-free medium	82 ± 2*

Values are means ± SE for 6 experiments. Type II cells (2×10^6/0.5 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 absence (control) and presence of L-NAME or aminoguanidine. To determine effects of extracellular calcium, some cells were incubated in same medium shown above except that calcium was omitted (Ca^{2+}-free medium). After incubation period, cells were centrifuged and supernatants saved for analysis. Nitrate + nitrite was measured as described in METHODS. Nitrate + nitrite in control cells was 0.80 ± 0.08 nmol/10^6 cells. *Values are significantly different from control ($P < 0.05$).

DISCUSSION

The results of our experiments show that exposure of alveolar type II cells to extracellular nitric oxide does not cause gross membrane damage, but such exposure does lead to inhibition of DSPC synthesis and other detrimental effects on cellular metabolism. In addition to causing inhibition of choline incorporation into DSPC, incubation of the cells with a ·NO generator (SNAP) leads to a decrease in the steady-state level of ATP. Recently, Haddad et al. (12) reported similar results in abstract form. A reduction in the steady-state level of ATP could be due to a decrease in its rate of synthesis or an increase in its rate of degradation and/or utilization. In this paper, we measured only ATP synthesis indirectly. Cellular ATP synthesis, measured indirectly as the rate of cyanide-sensitive oxygen consumption, is reduced after exposure of the cells to ·NO. However, there is no effect of ·NO on lung mitochondrial ATP synthesis. Therefore, the reduction in the steady-state level of ATP appears to be due, at least in part, to a decrease in cellular ATP synthesis which is not the result of a direct effect of ·NO on mitochondria.

There are some potential problems with the interpretation of the lack of SNAP effects on mitochondrial ATP synthesis. First, mitochondria were exposed to SNAP for only 5 min, but cells were exposed for 2 h. We have measured the effects of SNAP on cell ATP levels after only 5 min of exposure and found a 27 ± 8% (mean ± SE for 5 experiments) decrease. Since it seems reasonable to assume that it would take longer for the SNAP-generated ·NO to affect mitochondria inside the cells than to affect isolated mitochondria, these results suggest that a direct effect of ·NO on mitochondrial

ATP synthesis is not the mechanism by which cell ATP synthesis is diminished. Another potential problem is the use of mitochondria isolated from whole lungs rather than those isolated from type II cells. In this regard, we have shown previously that at least the responses of both mitochondrial preparations to H_2O_2 are identical (21). In addition, mitochondria isolated from whole lungs must contain some type II cell mitochondria. If only type II cell mitochondrial ATP formation is inhibited by SNAP, one should see at least a small inhibition of ATP formation in lung mitochondria. However, this is not the case. Finally, it is possible that the use of a substrate other than succinate, e.g., another substrate more dependent on the tricarboxylic acid cycle, may have caused mitochondrial ATP synthesis to be more sensitive to ·NO. However, when one takes into account all of these factors, especially the fact that cell ATP levels are reduced after only 5 min of SNAP exposure, it seems likely that the SNAP-induced decreases in cell ATP levels are not due to a direct effect of ·NO on mitochondria.

Mitochondrial ATP synthesis is not directly inhibited by SNAP. Why then is mitochondrial respiration in the cells (i.e., cellular oxygen consumption) reduced by SNAP? Although the answer to this question is not known for certain, there is at least one possibility. Nitric oxide may inhibit glucose uptake and/or its glycolytic metabolism, as H_2O_2 does in type II cells (21). This could contribute to a decrease in ATP synthesis due to a decline in the substrates available to mitochondria. In this regard, it has been shown that ·NO inhibits glyceraldehyde-3-phosphate dehydrogenase, an enzyme involved in glycolysis, in peritoneal macrophages (23). It is also known that ·NO can bind to iron-sulfur groups in enzymes, which can result in enzyme inhibition in cytotoxic-activated macrophages and in L10 hepatoma cells (14).

It is possible that the inhibitory effects of SNAP on type II cell ATP levels are due to ·NO itself or to increased levels of intracellular peroxynitrite; i.e., it may be that exposure of the cells to SNAP leads to increased levels of intracellular peroxynitrite which then inhibits cellular ATP synthesis. Our experiments do show that extracellular peroxynitrite and the stable decomposition products of ·NO, nitrate or nitrite, are probably not involved in the reduction of cell ATP levels. However, we did not inhibit intracellular superoxide anion production. Thus it is possible that the ·NO generated extracellularly penetrates the membrane and reacts with superoxide anion to form peroxynitrite inside type II cells. Peroxynitrite could then lead to a decrease in cell ATP levels, either by affecting mitochondria or via some other mechanism. It has been shown that peroxynitrite can cause nitration of tyrosine residues, which could lead to inhibition of enzyme activity (13). Furthermore, Hu et al. (15) have shown that exposure of type II cells to peroxynitrite leads to a reduction in the rate of cellular oxygen consumption.

Some of the most interesting findings from our experiments are the effects of altering endogenous levels of nitric oxide in type II cells. When the cells are incubated with L-NAME, an inhibitor of NOS, there are increases in DSPC synthesis and the steady-state level of ATP. One reason for the increase in cellular ATP levels may be the increase in the rate of cellular ATP synthesis, measured indirectly as the rate of type II cell oxygen consumption. The mechanism by which alterations in the endogenous ·NO levels affect type II cell metabolism is not known. However, once again, these effects may be due to changes in intracellular amounts of peroxynitrite. Because there are intracellular sources of superoxide anion, changes in intracellular ·NO levels may lead to similar changes in peroxynitrite levels. Peroxynitrite may then affect cellular metabolism.

The results obtained with NOS inhibitors and results obtained by using Ca^{2+}-free medium suggest that there is NOS activity in the type II cells and that there may be a constitutive form of the enzyme (cNOS) present. The fact that incubation of the cells with L-NAME results in increases in DSPC synthesis and cellular metabolism and that this effect can be reversed by L-arginine, the substrate for NOS, supports the involvement of a NOS enzyme in these processes. There is additional evidence to support the involvement of a cNOS in these effects. First, L-NAME is known to be a more effective inhibitor of cNOS than of iNOS, at least in endothelial and phagocytic cells (24, 29), and it has stimulatory effects on type II cell metabolism and blocks cell ·NO production. Second, aminoguanidine, which is a specific inhibitor of iNOS (3, 10), has no effect on either type II cell metabolism or cellular ·NO production. Our results also suggest that type II cell ·NO production is dependent on external calcium, a finding which is consistent with cNOS activity (8). Finally, other investigators, who have focused their studies on the regulation of iNOS activity in type II cells (11, 28), report detectable levels of nitrate and nitrite in unstimulated type II cell preparations. All of this evidence taken together suggests that there may be cNOS activity in type II cells.

It is possible that cNOS activity is important as a regulatory mechanism in alveolar type II cells. That may be the reason for the correlations between the L-NAME effects on DSPC synthesis and cellular ATP levels and the basal (control) levels of each. For example, when basal levels of DSPC synthesis and cell ATP content are relatively low, there may be relatively high cNOS activity in the cells. If this is the case, one would expect the effect of L-NAME to be more apparent. On the other hand, when basal levels of cellular metabolism are relatively high, the cell cNOS activity may be relatively low. In this case, one may expect L-NAME to be less effective.

In summary, the results of our experiments demonstrate that the nitric oxide levels in alveolar type II cells can affect lipid and energy metabolism. Exposure of the cells to ·NO from an external source leads to reductions in DSPC synthesis, cell ATP levels, and cellular oxygen consumption. On the other hand, inhibition of endogenous ·NO levels induced by the NOS inhibitor, L-NAME, leads to increases in DSPC synthesis, cell ATP levels, and cellular oxygen consumption.

Our results also suggest that at least some of the endogenous ·NO may be generated by cNOS activity. However, the precise regulatory role, if any, for NOS activity in these cells remains to be determined.

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