Metabolic changes in alveolar type II cells after exposure to hydrogen peroxide

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LACAGNIN, L. B., L. BOWMAN, J. Y. C. MA, AND P. R. MILES. *Metabolic changes in alveolar type II cells after exposure to hydrogen peroxide.* Am. J. Physiol. 259 (Lung Cell. Mol. Physiol. 3): L57–L65, 1990.—Since oxygen metabolites may play an important role in pulmonary oxidant injury, the effects of hydrogen peroxide (H_2O_2) on energy metabolism in alveolar type II cells isolated from rats were studied. The major effect of H_2O_2 is a rapid and dramatic reduction in the steady-state level of cellular ATP; e.g., ATP levels are reduced by 77 ± 3% after only 5 min of exposure to H_2O_2 (0.5 mM). Cellular oxygen consumption is affected in a similar manner, suggesting that ATP synthesis is impaired. Experiments with isolated lung mitochondria demonstrate that exposure to 0.5 mM H_2O_2 for 5 min inhibits the rate of mitochondrial ATP synthesis by 51 ± 3%. The site of mitochondrial ATP synthesis inhibition by H_2O_2 appears to be the adenosinetriphosphatase-synthase enzyme complex which phosphorylates ADP to ATP. Mitochondrial electron transport is unaffected. The association of 3-*O*-methylglucose with type II cells and glycolytic metabolism, measured as lactate production, are reduced by 25–35% by H_2O_2. The data also show that the cells are capable of recovery following exposure to H_2O_2, at least at lower exposure levels. These results indicate that exposure of type II cells to H_2O_2 alters the energy state of the cells by decreasing ATP synthesis. In turn, other important cellular functions may be impaired.

oxidant injury; adenosine 5′-triphosphate

PULMONARY OXIDANT INJURY can occur as the result of various insults. For example, it can be induced following exposure to oxidant gases, such as ozone, nitrogen dioxide, or oxygen, during postischemic reperfusion, and as a consequence of inflammatory reactions. Regardless of the cause, however, the mechanism common to all types of oxidant damage is thought to involve the generation of reactive oxygen metabolites, such as hydrogen peroxide (H_2O_2), superoxide anion radicals, singlet oxygen, and hydroxyl radicals (13). H_2O_2, in particular, may play an important role in oxidant-mediated cell injury. It is produced in cells as a result of normal cellular metabolism and this basal rate of production is stimulated during exposure to high concentrations of oxidant gases (3, 12, 13). In addition, H_2O_2 is found at sites of inflammation when it is released into the extracellular environment by alveolar macrophages and neutrophils (34, 35). It could therefore exert its toxic effects at both intracellular and extracellular sites. In addition, this oxygen metabolite is relatively long lived and may therefore diffuse large distances. Its small size and lack of charge facilitate its movement across cell membranes. Taken together, these properties may contribute to the potentially toxic nature of H_2O_2. In fact, it has been shown to cause alterations in cell morphology and loss of viability in a variety of cell types (21, 27, 32). It may therefore be important to study the effects of H_2O_2 as they relate to pulmonary oxidant injury.

The alveolar region of the lungs appears to be particularly sensitive to oxidant injury. For example, exposure of animals or humans to high concentrations of nitrogen dioxide or ozone results in the development of pulmonary edema and atelectasis (20, 31). One cell type that is located in the alveolar region of the lungs and may be exposed to various types of oxidants is the alveolar type II cell. These cells are responsible for the synthesis and secretion of pulmonary surfactant (7, 25), regeneration of the alveolar epithelium after lung injury (23), and metabolism of foreign substances (2). Some studies have been done in which the effects of oxidants on type II cells have been investigated. For example, exposure of isolated type II cells to oxidant gases, including oxygen or ozone, results in a decrease in the synthesis of the phospholipid components of surfactant (16, 17). These results indicate that type II cell function can be affected by oxidants.

Alveolar type II cells play a critical role in maintaining normal lung function, and they are located in a region of the lungs susceptible to oxidant injury. H_2O_2 has been implicated as a mediator of oxidant-induced injury and is known to be toxic to other cell types. Therefore the objective of this investigation was to study the effects of H_2O_2 on energy metabolism in alveolar type II cells isolated from rat lungs. Energy metabolism was chosen because it is a basic cellular function providing the energy required for other cellular processes. Since type II cells depend on mitochondrial metabolism for energy production (10), we examined the effects of H_2O_2 on cellular and mitochondrial ATP production and oxygen consumption. In addition, the effects on glucose uptake and metabolism were also assessed. A preliminary report of these results has appeared previously (26).

METHODS

Isolation of alveolar type II cells. Alveolar type II cells were isolated by elastase digestion and purified by cen-

trifugal elutriation as described previously (22, 28). Briefly, male Sprague-Dawley rats (250–300 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 tracheal lavage with phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 5.0 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 incubated at 37°C for 30 min to free lung cells. After enzymatic digestion, lungs were minced with a McIlwain tissue chopper (slice thickness of 0.5 mm; Mickle Engineering, Gomshall, Surrey, UK). The minced lungs were placed in phosphate-buffered medium containing 25% fetal calf serum and 0.006% DNase (to help prevent cell clumping) and incubated for 10 min at 37°C to arrest digestion. The cell suspension was strained through nylon mesh and the type II fraction was obtained by centrifugal elutriation. The cells were washed once 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 performed ~1 h after the cell isolation procedure.

Cell number was determined with a Coulter model Z_B electronic cell counter (Coulter Instrument, Hialeah, FL). The purity of the type II cell-enriched fraction was routinely estimated with the fluorescent dye, phosphine 3R, as we have previously reported (28). In the experiments reported in this paper, we obtained $2.98 \pm 0.02 \times 10^7$ cells per rat in the type II cell-enriched fraction with a purity of 90 ± 2% (means ± SE for 10 experiments). Contamination was due to polymorphonuclear leukocytes and alveolar macrophages. Membrane integrity and cell volume were assessed in the presence or absence of H_2O_2 by measuring trypan blue exclusion and mean cell volume. The percentage of cells that excluded trypan blue dye was determined as previously described (29). Mean cell volume was measured with a Coulter Channelizer attachment calibrated with latex particles of known diameter. In some experiments the rate of disappearance of H_2O_2 from the cell suspensions was assessed by measuring H_2O_2 levels at various incubation times by the method of Root et al. (30). This fluorometric method is based on the oxidation of reduced scopoletin by a horseradish peroxidase-H_2O_2 complex. With this technique, nanomolar amounts of H_2O_2 can be measured in a very reproducible manner.

Measurement of cellular ATP levels and oxygen consumption. To determine the effects of H_2O_2 on energy metabolism, alveolar type II cell adenosine triphosphate (ATP) levels and oxygen consumption were measured. ATP content was determined by the firefly luciferase assay adapted from the method described by Wulff and Doppen (36). Cells (2×10^6/ml) were incubated (37°C) for various times in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA in the presence or absence of H_2O_2 (0.05–0.5 mM). After incubation, cells were centrifuged at 1,000 *g* for 10 min and the incubation medium removed by aspiration. The cells were washed once and then resuspended so there were 2×10^6 cells in 0.125 ml of 0.5 M tris(hydroxymethyl)amino methane (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 thoroughly by vortexing for 10 s and then was immediately analyzed for ATP content. The concentration of ATP was determined by measuring the emission of light produced 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 using a Lumi-Aggregometer (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.

Cellular 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 concentrations until saturation occurred. Cells (2×10^6/ml) were incubated at 37°C for times ranging from 10 to 60 min in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA, with and without H_2O_2 (0.05–0.5 mM). After incubation, the cell suspension was centrifuged at 1,000 *g* for 5 min, and the supernatant was removed by aspiration. The cells were washed once in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA and then resuspended in the same medium at a concentration of 2×10^6 cells/ml. The rate of oxygen consumption in 1.65 ml of this suspension was then measured for ten minutes at a constant temperature of 37°C. In some experiments, sodium cyanide (1 mM), which inhibits mitochondrial respiration, was added to the cell suspension to determine the amount of oxygen consumption attributable to mitochondrial respiration. In other experiments, the effects of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma), an uncoupler of oxidative phosphorylation, on oxygen consumption in untreated or H_2O_2-treated cells were examined by including 10 μM CCCP in the incubation medium.

Measurement of glucose influx and lactate production. To estimate glucose uptake, the association of cells with 3-*O*-methylglucose, an analogue of glucose that is taken up by the glucose transport carrier but not further metabolized (24), was measured using a radioactive tracer technique. Cells (2×10^6/ml) were preincubated at 37°C in phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, BSA, and glucose (5 mM). Preincubations were carried out in the presence or absence of 0.5 mM H_2O_2 for 10 min, a time during which cellular ATP levels are significantly depleted. In some experiments, sodium cyanide (1 mM) was included in the medium during preincubations to determine the effects of ATP depletion. After preincubation, the cell suspensions were concentrated by centrifugation and resuspended in the same supernatant so that the final cell concentration was 2×10^7/ml. Unlabeled 3-*O*-methylglucose (final concn 5 mM) and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose (final concn, 1.0 μCi/ml;

sp act, 79.0 Ci/mmol; labeled in the 3 position; Du Pont-New England Nuclear, Boston, MA) were added to the cell suspension to initiate measurement of uptake. After 1, 5, and 10 min of incubation, 0.5-ml samples of the suspension were taken. The samples were centrifuged for 5 s at 12,800 *g* with an Eppendorf microcentrifuge model 5412 (Brinkmann Instrument, Westbury, NY). The supernatants were removed, and the cell pellets were washed three times with phosphate-buffered medium containing Ca^{2+}, Mg^{2+}, and BSA by alternate resuspension and centrifugation. After the final wash, the cell pellets were resuspended in 0.1 ml Protosol (Du Pont-New England Nuclear) and the samples were incubated for 30 min at 55°C to digest the protein. Each sample was then placed in 9.4 ml Aquasol (Du Pont-New England Nuclear) containing 0.1 ml 1 N HCl, and radioactivity was measured using a Beckman LS 9000 liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). Radioactivity was also determined in an aliquot (0.1 ml) of the cell suspension. The amount of 3-*O*-methylglucose associated with the cells was calculated from the disintegrations per minute and specific activity and expressed as nanomoles of 3-*O*-methylglucose per 10^7 cells.

The metabolism of glucose via glycolysis was estimated by measuring the formation of lactate, the major end product of glycolysis. Type II cells (2×10^6/ml) were incubated for 10 min at 37°C in the presence or absence of H_2O_2 (0.5 mM). In some experiments, sodium cyanide (1 mM) was included in the medium to determine the effects of ATP depletion. After incubation, an aliquot (0.3 ml) of the cell suspension was added to perchloric acid (final concn of 3%) to precipitate cellular protein. The samples were mixed thoroughly and centrifuged in an Eppendorf microcentrifuge for 2 min at 12,800 *g*. Lactate concentrations in the perchloric acid extracts were assayed spectrophotometrically at 340 nm with enzymatic methods (15). Results were expressed as nanomoles of lactate per 10^6 cells per 10 min.

Isolation of lung mitochondria and measurement of ATP synthesis. To determine the effects of H_2O_2 on mitochondrial function, lung mitochondria were prepared as described by Spear and Lumeng (33). Lungs were perfused with 0.9% NaCl to remove blood cells, dissected free of the trachea, bronchi, and connective tissue, and then finely minced by chopping four times with a McIlwain tissue chopper that had been set for 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 tissue in the homogenate was 100 mg per milliliter. The crude homogenate was centrifuged at 2,000 *g* for 5 min, and the pellet was discarded. The mitochondrial fraction was isolated by centrifuging 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 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris·HCl, 0.1 mM EDTA, and 1.0% fatty acid-poor BSA (pH 7.2) for use in all experiments. The yield obtained with this method was 1.7 ± 0.3 mg mitochondrial protein/g lung (means ± SE for 10 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 following its (ADP) expenditure (4, 8), was determined. The RCR was 2.5 ± 0.1 (means ± SE for 4 experiments), which is comparable to that reported by others for rat lung mitochondria (11) and indicates that the mitochondrial membranes were intact and the rate of respiration acceptable.

The effects of H_2O_2 on mitochondrial ATP synthesis were determined. Mitochondria were resuspended in the incubation medium at a final concentration of 0.15 mg protein/ml for all experiments. This concentration was used because it is the approximate level of mitochondrial protein found in 2×10^6 type II cells (5), the amount used in all of our intact cell experiments. The mitochondrial suspensions were incubated at 37°C for 5 min in the presence or absence of H_2O_2 (0.1–0.5 mM). A 5-min exposure to H_2O_2 was used because longer incubation periods result in a reduced mitochondrial RCR. After this incubation, the samples were centrifuged for 15 s at 12,800 *g* with 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 (0.15 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 centrifuged in an Eppendorf microcentrifuge and ATP levels in the supernatant were determined with the firefly luciferase technique as described previously. No ATP was detectable in the mitochondrial pellet.

Some experiments were done to attempt to determine the mechanism by which H_2O_2 may affect mitochondria. The effects of H_2O_2 on mitochondrial electron transport and on the adenosinetriphosphatase (ATPase)-synthase enzyme complex, which catalyzes the phosphorylation of ADP to ATP, were studied. To assess H_2O_2 effects on electron transport, we measured the rate of oxygen consumption in the presence of succinate (5 mM) alone or in the presence of succinate and the uncoupling agent CCCP (10 μM). After exposure to H_2O_2 (0.1–0.5 mM) for 5 min, the mitochondria were centrifuged and resuspended in fresh medium as described above. Oxygen consumption was then measured at 30°C for 10 min in the presence of succinate or succinate and CCCP. Mitochondrial oxygen consumption in the presence of succinate (5 mM) and ADP (0.3 mM) is dependent on both electron transport and the ATPase-synthase complex. We therefore measured oxygen consumption in the presence of these two substrates to ascertain whether H_2O_2 affects ATPase-synthase activity.

RESULTS

Membrane integrity and mean cell volume. The effects of H_2O_2 on the general condition of type II cells were studied by measuring the exclusion of trypan blue dye to assess membrane integrity and mean cell volume to

determine whether a change in cell size occurs. The results are shown in Table 1. When cells are exposed for 2 h to H_2O_2 in concentrations up to 0.5 mM, the percentage of cells that exclude trypan blue is not different from that in control cells. In addition, the mean cell volume of H_2O_2-treated cells does not change much compared with untreated cells; i.e., there is slight swelling of the cells by <10% of control. Similarly, the release of lactate dehydrogenase from the cells is not affected at these exposure levels (data not presented). Therefore these data indicate that exposure of type II cells to 0.5 mM H_2O_2 for up to 2 h does not cause any gross damage; i.e., the cells do not shrink or swell appreciably, and the membranes appear to be intact.

Cellular ATP levels and oxygen consumption. Alveolar type II cells depend on mitochondrial (aerobic) metabolism for energy generation (10). Therefore the effects of H_2O_2 on type II cell ATP content were assessed. The ATP levels in untreated cells are ~0.6 nmol/10^6 cells. If one uses the value for type II cell water that we reported previously (22), the calculated intracellular ATP concentration is ~3 mM, a value similar to that reported by Fisher et al. (10) for type II cells in primary culture. After exposure to H_2O_2, cellular ATP levels are reduced, and the time course for these effects is shown in Fig. 1*A*. When cells are incubated with 0.5 mM H_2O_2 for up to 30 min, a rapid and substantial loss of intracellular ATP is observed. After 2 min, ATP levels are reduced by ~60% compared with untreated cells and by ~80% after 5 min. The effects of H_2O_2 are also concentration dependent, as illustrated in Fig. 1*B*. Incubation of cells for 1 h in the presence of 0.5 mM H_2O_2 results in a reduction of intracellular ATP by >90%. The concentration of H_2O_2 that causes a 50% decline in ATP after a 1-h incubation is 0.11 ± 0.02 mM. The decline in ATP is not due to a direct effect of H_2O_2 on the ATP molecule itself, since incubation of ATP with 0.5 mM H_2O_2 for 1 h does not alter the ATP level. These data indicate that H_2O_2 causes a dramatic reduction in the steady-state level of ATP in alveolar type II cells and that the effects are time and concentration dependent. The reduction in ATP may be due to a decline in its rate of synthesis, an enhancement of its rate of degradation and/or utilization, or both. The results reported in the remainder of this paper pertain to the study of H_2O_2 effects on ATP synthesis.

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 exposed to H_2O_2. The time course for the effects of 0.5 mM H_2O_2 on the rate of oxygen consumption is shown in Fig. 2. Oxygen consumption is reduced to a value that is ~55% of control after only 10 min of exposure to H_2O_2. This

TABLE 1. *Effects of H_2O_2 on exclusion of trypan blue dye and mean cell volume in alveolar type II cells*

Treatment (Concn)	Trypan Blue Exclusion, %	Mean Cell Volume, % of control
Control	95±1	100
H_2O_2 (0.5 mM)	93±1	109±2

Values are means ± SE for 4 experiments. Alveolar type II cells (2 × 10^6/ml) were incubated for 2 h in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and BSA in the presence or absence of H_2O_2. After incubation, trypan blue exclusion and mean cell volume were determined as described in METHODS. Data are reported as percentage of cells that exclude trypan blue dye or cell volume as a percentage of that in untreated cells. Mean cell volume determined with electronic cell sizing in untreated cells was 636 ± 19 μm^3.

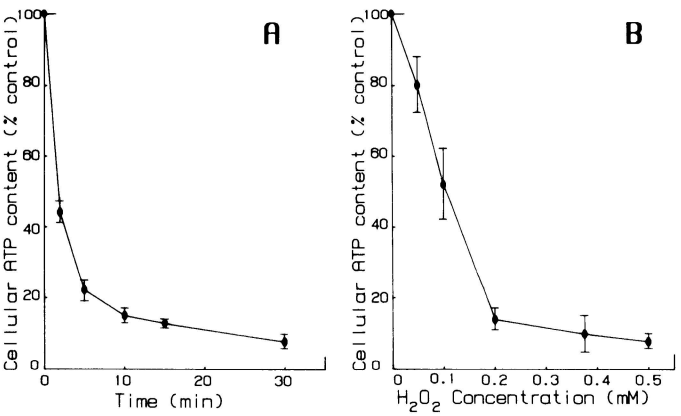

FIG. 1. *A*: time course for effects of H_2O_2 on ATP content of alveolar type II cells. Cells (2 × 10^6/ml) were incubated for various times in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and BSA in the presence or absence of 0.5 mM H_2O_2. After the appropriate incubation time, cell suspension was centrifuged, washed once, and then resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to cell suspensions to disrupt cell membranes. Measurements were made as described in METHODS. Data are reported as percentage of control at each time point. ATP levels in untreated cells are 0.60 ± 0.03 nmol/10^6 cells and do not change during incubation period. *B*: ATP content of alveolar type II cells as a function of H_2O_2 concentration. Cells were incubated for 1 h in the presence or absence of various concentrations of H_2O_2 and prepared for ATP measurements as described above. Results are expressed as percentage of ATP levels in untreated cells. Amount in untreated cells is 0.60 ± 0.03 nmol/10^6 cells. Points are mean values for 4 experiments; bars represent SE.

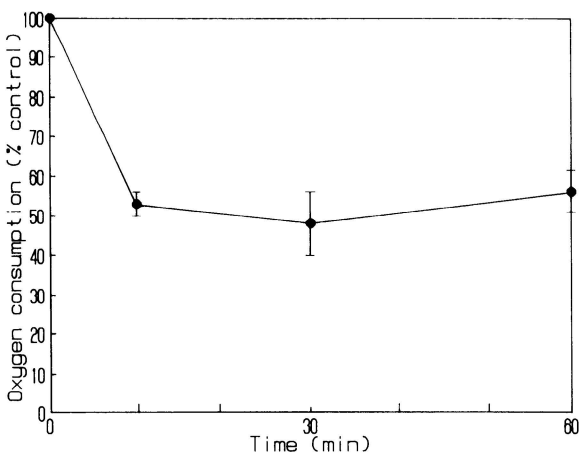

FIG. 2. Time course for effects of H_2O_2 on oxygen consumption by alveolar type II cells. Cells (2 × 10^6/ml) were incubated for various times in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and BSA in the presence or absence of 0.5 mM H_2O_2. After appropriate incubation time, cell suspension was centrifuged, washed once, and resuspended in phosphate-buffered medium containing Ca^{2+} and Mg^{2+}. Oxygen consumption was measured over 10 min. Data are reported as percentage of oxygen consumption in untreated cells vs. time of incubation with H_2O_2. Rate of oxygen consumption in untreated cells is 98 ± 9 nmol·10^6 cells^{-1}·h^{-1}. Points are mean values for 4 experiments; bars represent SE.

time course is similar to that for H_2O_2-induced effects on cellular ATP levels in that most of the effect occurs during the first 10 min of incubation. The effects of H_2O_2 on oxygen consumption are also concentration dependent (data not shown). In other experiments (data not shown), we found that addition of sodium cyanide (1 mM) completely inhibits cellular oxygen consumption, indicating that mitochondrial respiration probably accounts for all measurable oxygen consumed. Thus these results suggest that H_2O_2 interferes with ATP synthesis in alveolar type II cells and that this may account, at least in part, for the reduction in the steady-state level of type II cell ATP.

ATP synthesis in isolated lung mitochondria. Since ATP synthesis occurs primarily in mitochondria, we studied the effects of H_2O_2 on ATP production by isolated mitochondria. In these experiments, untreated and H_2O_2-treated mitochondria isolated from whole lungs were incubated in the presence of the exogenous substrates, succinate and ADP, and the formation of ATP was measured. Exposure to H_2O_2 was carried out for 5 min before ATP measurements. The concentration dependence for the effects is shown in Fig. 3. Mitochondrial ATP synthesis is inhibited by H_2O_2 in a concentration-dependent manner. At 0.5 mM H_2O_2, the maximal exposure condition used in our experiments, ATP synthesis is reduced by ~50%. We also performed experiments with mitochondria isolated from type II cells and obtained similar results (data not presented). Although a direct quantitative comparison between isolated mitochondria and intact cells cannot be made, these experiments, like those performed with intact type II cells, indicate that mitochondrial ATP synthesis is inhibited by ~50% in the presence of 0.5 mM H_2O_2 for 5 min.

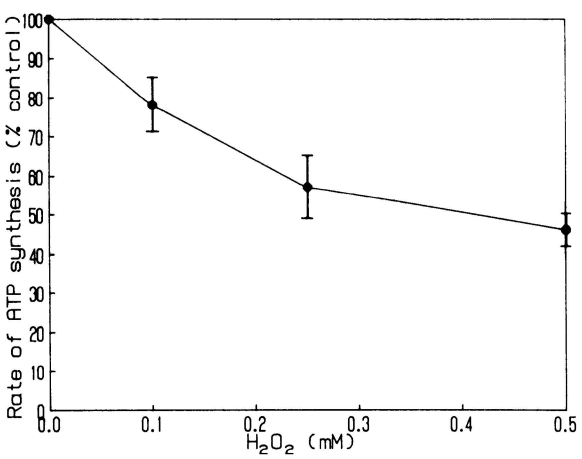

FIG. 3. Concentration dependence for effects of H_2O_2 on rate of ATP synthesis by isolated lung mitochondria. Mitochondria (0.15 mg protein/ml) were preincubated for 5 min in medium consisting of 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris·HCl, 0.1 mM EDTA, and 1.0% of fatty acid-poor BSA in the presence or absence of various concentrations of H_2O_2. After preincubation, mitochondria were washed once and resuspended in incubation medium containing 10 μM ADP and 5 mM succinate to initiate ATP synthesis. After incubation at 30°C for 5 min, mitochondria were centrifuged and supernatant was immediately removed to stop reaction. ATP levels were then determined in supernatant. Results are expressed as percentage of control. ATP synthesis in untreated mitochondrial is 185 ± 19 nmol·min^{-1}·mg protein^{-1}. Points are mean values for 4 experiments; bars represent SE.

Other experiments were performed in an attempt to determine the mechanism by which mitochondrial ATP formation is inhibited by H_2O_2. The inhibitory effect is not due to gross membrane damage, since exogenous NADH, which does not penetrate intact mitochondrial membranes, does not stimulate mitochondrial oxygen consumption (data not shown). ATP synthesis in isolated mitochondria is primarily dependent on two processes, i.e., the electron transport system and the ATPase-synthase enzyme complex, which catalyzes the phosphorylation of ADP to ATP. To study the effects of H_2O_2 on both processes, mitochondrial oxygen consumption was measured in the presence of exogenous succinate (a measure of electron transport, i.e., uncoupled respiration) or exogenous succinate and ADP (a measure of both electron transport and the activity of the ATPase-synthase enzyme complex) (4, 8). The results are shown in Table 2. Mitochondrial oxygen consumption in the presence of succinate and ADP, which is a measure of both electron transport and activity of the ATPase-synthase enzyme complex, is reduced in a concentration-dependent manner. However, there is no effect of H_2O_2 on oxygen consumption in the presence of succinate alone, i.e., electron transport. Taken together, these data suggest that the H_2O_2-induced reduction in mitochondrial ATP synthesis is due to inhibition of the enzyme complex responsible for the phosphorylation of ADP to form ATP.

Another way to determine whether electron transport is inhibited by H_2O_2 is to use an uncoupler of oxidative phosphorylation, such as CCCP. In the presence of an uncoupling agent, electron transport, and thus oxygen consumption in the presence of succinate, is stimulated. We therefore studied the effects of CCCP in untreated and in H_2O_2-treated mitochondria (in the presence of succinate). The results are shown in Table 3. When CCCP is added to the untreated mitochondrial suspension, oxygen consumption in the presence of succinate is stimulated by ~40%. The effects of CCCP in H_2O_2-treated mitochondria are similar. These results indicate that H_2O_2 has no effect on the mitochondrial electron transport system. Similar results are obtained with intact alveolar type II cells (Table 3). This result demonstrates

TABLE 2. *Effects of H_2O_2 on ATP production and electron transport chain in isolated lung mitochondria*

Treatment (Concn)	Oxygen Consumption, % of control	
	ADP + succinate	Succinate
Control	100	100
H_2O_2 (0.1 mM)	91±5	106±5
H_2O_2 (0.25 mM)	79±2	100±1
H_2O_2 (0.5 mM)	72±2	104±2

Values are means ± SE for 5 experiments. Isolated lung mitochondria (0.15 mg protein/ml) were incubated with and without H_2O_2 for 5 min in incubation medium consisting of 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris-HCl, 0.1 mM EDTA, and 1% fatty acid-poor BSA. After the incubation period, mitochondria were washed once and resuspended in incubation medium. Oxygen consumption was measured for 10 min in the presence of 0.3 mM ADP and 5 mM succinate or in the presence of succinate alone. Data are reported as percentage of control. Oxygen consumption for untreated mitochondria in the presence of ADP and succinate was 60 ± 9 nmol·min^{-1}·mg protein^{-1} and in the presence of succinate alone was 28 ± 4 nmol·min^{-1}·mg protein^{-1}.

TABLE 3. *Effects of H_2O_2 on oxygen consumption by lung mitochondria and alveolar type II cells in presence of CCCP*

Treatment (Concn)	Oxygen Consumption, % of control	
	Mitochondria	Type II cells
Control	100	100
CCCP (10 μM)	142±3	131±2
CCCP + H_2O_2 (0.5 mM)	140±5	141±5

Values are means ± SE for 5 experiments. Isolated lung mitochondria (0.15 mg protein/ml) were incubated with and without H_2O_2 for 5 min in medium consisting of 105 mM KCl, 2 mM KH_2PO_4, 30 mM Tris·HCl, 0.1 mM EDTA, and 1% fatty acid-poor BSA. After this preincubation, mitochondria were washed once and resuspended in the incubation medium, and oxygen consumption was measured in the presence of 5 mM succinate (control) or in the presence of succinate and 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation. Alveolar type II cells (2×10^6/ml) were incubated with and without H_2O_2 for 10 min in phosphate-buffered medium containing 1.8 mM $CaCl_2$ and 1.0 mM $MgCl_2$. After this preincubation, the cells were centrifuged, washed once, and resuspended in incubation medium (without H_2O_2) at a concentration of 4×10^6 cells/ml. Oxygen consumption was measured over 10 min in the presence and absence of 10 μM CCCP. All results are expressed as percentage of control. Rates of oxygen consumption in control mitochondria and type II cells were 28 ± 6 nmol·min^{-1}·mg protein^{-1} and 88 ± 6 nmol·10^6 cells^{-1}·h^{-1}, respectively.

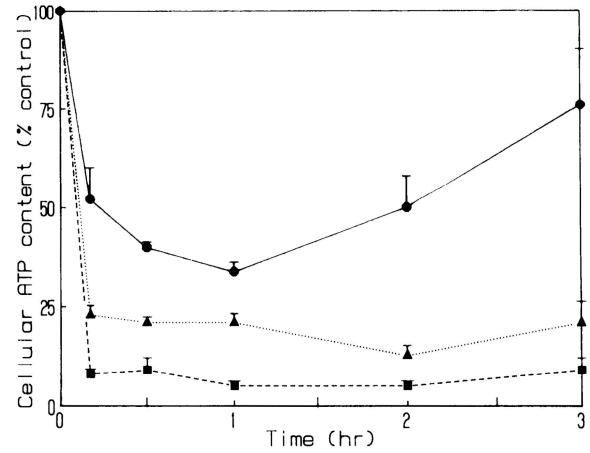

FIG. 4. Time courses for effects of H_2O_2 on ATP content of alveolar type II cells. Cells (2×10^6/ml) were incubated for 3 h in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and BSA in the presence or absence of H_2O_2 (circles, 0.1 mM; triangles, 0.2 mM; squares, 0.5 mM). After appropriate incubation time, cell suspension was centrifuged and washed once, and cells were resuspended in Tris-acetate buffer. Immediately before measurement of ATP, Triton X-100 was added to cell suspensions to disrupt cell membranes. Measurements were made as described in METHODS. Data are reported as a percentage of control at each time point. ATP levels in untreated cells are 0.52 ± 0.04 nmoles/10^6 cells and do not change with time. All points are mean values for 5 experiments; bars represent SE.

that H_2O_2-induced effects in type II cells and in whole lung mitochondria are similar. Taken together, all of these data indicate that exposure to H_2O_2 reduces mitochondrial ATP synthesis and that this effect is probably due to inhibition of the enzyme complex responsible for the phosphorylation of ADP to ATP.

Reversibility of H_2O_2-induced reduction of cellular ATP levels. Our data demonstrate that the steady-state level of type II cell ATP is reduced by H_2O_2 in a concentration-dependent manner during a 1-h exposure (Fig. 1*B*). To determine whether this effect is reversible, we incubated type II cells with different concentrations of H_2O_2 for longer periods of time and measured cellular ATP levels. The results are shown in Fig. 4. In the presence of 0.1 mM H_2O_2, ATP levels are reduced by ~65% in 1 h. However, after 1 h, cellular ATP levels begin to recover toward normal and, after 3 h, are reduced by only 25% of that in untreated cells. At higher concentrations of H_2O_2 (i.e., 0.2 and 0.5 mM), the ATP levels do not recover, at least over a 3-h incubation period. These data indicate that the effects of H_2O_2 are reversible, at least at lower exposure levels. The reversibility of these effects may be related to the rate at which H_2O_2 is destroyed by the cell suspension. (No H_2O_2 is lost from the medium in the absence of cells; i.e., there is no volatilization.) In another series of experiments, we measured the rate at which H_2O_2 disappears from the medium during incubation with type II cells. No H_2O_2 is detectable after 15 min when cells (2×10^6/ml) are incubated with 0.1 mM H_2O_2. However, when the same number of cells is exposed to 0.5 mM H_2O_2, the oxygen metabolite is still present after 30 min of incubation. The rate of H_2O_2 disappearance is also dependent on cell number (data not shown). The type II cell defense mechanisms against H_2O_2 probably include catalase, glutathione peroxidase, and ascorbate (12). The balance between the rate at which H_2O_2 is consumed by reaction with antioxidants or cellular components may determine whether the reduction in ATP levels is reversible and therefore the extent of damage.

Glucose uptake and metabolism. Our data indicate that the H_2O_2-induced reduction in type II cell ATP content is due to a direct effect of the oxygen metabolite on mitochondrial function. The evidence for this is derived from experiments performed with isolated mitochondria supplied with exogenous substrates. However, H_2O_2 could also affect glucose uptake and/or its metabolism so that substrates available to mitochondria, e.g., pyruvate, may be reduced. We therefore studied the effects of H_2O_2 on glucose uptake and its metabolism by alveolar type II cells. The effects of H_2O_2 on glucose uptake were estimated by measuring the association of 3-*O*-methylglucose, an analogue of glucose that is taken up by the glucose transport carrier but not further metabolized, with the cells. The time courses for association of 3-*O*-methylglucose with untreated cells and cells that had been pretreated with H_2O_2 are shown in Fig. 5. Exposure of the cells to 0.5 mM H_2O_2 for 10 min, a concentration and time of exposure that produces significant effects on cellular ATP levels and oxygen consumption, produces a 25% inhibition in the association of 3-*O*-methylglucose, with cells at all time points over a 10-min period. Therefore these results suggest that the ability of glucose to enter type II cells is slightly reduced after exposure of the cells to H_2O_2.

The metabolism of glucose via glycolysis was studied by measuring the production of lactate by type II cells. After treatment of the cells with 0.5 mM H_2O_2 for 10 min, the rate of lactate production is reduced by ~35% from levels in untreated cells (Table 4). These results suggest that both glucose uptake and glycolysis are inhibited by H_2O_2. In an attempt to determine whether

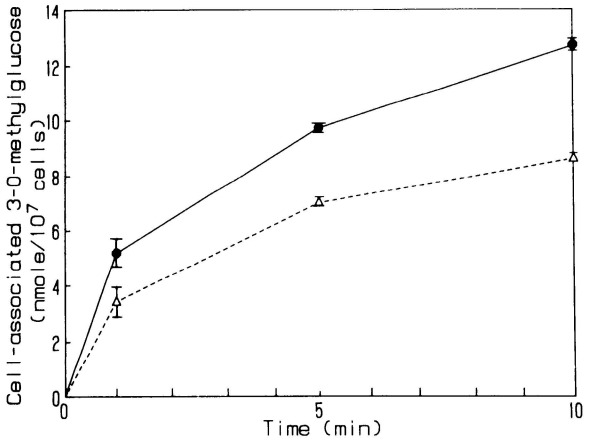

FIG. 5. Time courses for association of 3-*O*-methylglucose with untreated (circles) or H_2O_2-treated (triangles) alveolar type II cells. Cells (2×10^6/ml) were preincubated for 10 min in phosphate-buffered medium that contained 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.5% BSA, and 5 mM glucose in the presence or absence of 0.5 mM H_2O_2. After preincubation, cells were centrifuged and resuspended in same supernatant at a concentration of 2×10^7 cells/ml. Unlabeled 3-*O*-methylglucose (final concn 5 mM) and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose were added to cell suspension to initiate uptake. After various times of incubation, samples (0.5 ml) were taken and centrifuged, cell pellet was washed 3 times, and radioactivity in the cell pellet was determined. Association of 3-*O*-methylglucose with cells was calculated from disintegrations per minute and specific activity. Points are mean values for 5 experiments; bars represent SE.

TABLE 4. *Effects of H_2O_2 and cyanide on 3-O-methylglucose uptake and lactate production in alveolar type II cells*

Treatment (Concn)	ATP Content, % of control	3-*O*-Methylglucose Uptake, % of control	Lactate Production, % of control
Control	100	100	100
H_2O_2 (0.5 mM)	10±2	75±5	64±3
NaCN (1.0 mM)	24±4	84±2	87±8

Values are means ± SE for 4 experiments. Alveolar type II cells (2×10^6/ml) were incubated for 10 min in phosphate-buffered medium containing 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and BSA with and without H_2O_2 (0.5 mM) or NaCN (1.0 mM). After this preincubation period, cell suspensions were divided so that 3 different measurements were made, i.e., ATP levels, lactate formation, and association of 3-*O*-methylglucose with the cells. Some cells were washed once, resuspended in Tris-acetate buffer, and disrupted with Triton X-100, and ATP was measured as described in METHODS. To measure lactate, an aliquot (0.3 ml) of cell suspension was added to perchloric acid and the perchloric acid extract was assayed spectrophotometrically as described in METHODS. The association of 3-*O*-methylglucose with the cells was measured by washing the cells and resuspending them at a concentration of 2×10^7/ml. Measurements were made after a 10-min incubation during which 3-*O*-methylglucose became associated with the cells. Data are reported as a percentage of that in untreated cells. Values obtained in untreated cells are 0.56 ± 0.02 nmol ATP/10^6 cells, 12 ± 3 nmol 3-*O*-methylglucose·10^7 cells^{-1}·10 min^{-1}, and 2.5 ± 0.5 nmol lactate·10^6 cells^{-1}·10 min^{-1}.

these effects are secondary to the reduction in cellular ATP levels, we measured the association of 3-*O*-methylglucose with cells and cellular lactate production in the presence of sodium cyanide. The results are shown in Table 4. The level of sodium cyanide was adjusted so that the reduction in ATP levels was similar, but not identical, to that obtained during treatment of the cells with H_2O_2; i.e., ATP levels are reduced by 90 and 75% with H_2O_2 and cyanide, respectively. This concentration of cyanide does not cause gross membrane damage in these cells. Both 3-*O*-methylglucose uptake and lactate production are reduced in cyanide-treated cells but to a lesser extent than in H_2O_2-treated cells. However, the ATP levels are reduced to a lesser extent by cyanide. These results suggest that glucose uptake and its metabolism may depend, to some small degree, on ATP and that the inhibition of both these processes by H_2O_2 may be secondary to the reduced cellular ATP content. However, more careful kinetic and dose-response measurements are necessary to determine whether these effects are due directly to H_2O_2 or to a secondary effect on ATP.

DISCUSSION

The major effect of H_2O_2 on alveolar type II cell metabolism is a rapid and dramatic reduction in the cellular ATP content. A reduced steady-state level of ATP could be due to a decrease in its rate of synthesis or to an increase in its rate of degradation and/or utilization. In this paper, we studied only H_2O_2 effects on ATP synthesis. Cellular ATP synthesis, measured indirectly as the rate of cyanide-sensitive oxygen consumption, is decreased following exposure of type II cells to H_2O_2 (Fig. 2). Furthermore, H_2O_2 inhibits ATP synthesis in isolated mitochondria (Fig. 3). Therefore these data indicate that the H_2O_2-induced reduction in cellular ATP content is due, at least in part, to inhibition of ATP synthesis in mitochondria, the principal site of ATP production. In addition, the uptake of glucose and its glycolytic metabolism are also impaired in H_2O_2-treated type II cells (Table 4). This may further contribute to the reduction in cellular ATP content via a decline in the substrates available to mitochondria, e.g., pyruvate. Our data also show that type II cells are capable of recovery after exposure to H_2O_2; i.e., the decrease in cellular ATP content is reversible, at least at lower exposure levels.

Mitochondrial ATP synthesis is dependent on at least two processes within the organelles, i.e., electron transport and the ATPase-synthase enzyme complex. There are two lines of evidence to suggest that mitochondrial electron transport, which was assessed by measuring mitochondrial oxygen consumption, is not inhibited: *1*) succinate-stimulated mitochondrial oxygen consumption is unaffected by H_2O_2 (Table 2) and *2*) CCCP-stimulated oxygen consumption in H_2O_2-treated organelles is not different from that in untreated mitochondria (Table 3). When these data are taken together with the fact that mitochondrial oxygen consumption stimulated by succinate and ADP is reduced by H_2O_2 (Table 2), it suggests that the reduction in mitochondrial ATP synthesis is due to inhibition of the ATPase-synthase enzyme complex which catalyzes the phosphorylation of ADP to ATP. Furthermore, the finding that uncoupling respiration in type II cells stimulates oxygen consumption in both treated and untreated cells suggests that inhibition of this enzyme complex is at least partially responsible for the reduction in cellular ATP content. A similar conclusion was reached by Hyslop et al. (21) to explain

the H_2O_2-induced decline in intracellular ATP in $P388D_1$ cells.

It is somewhat surprising to find a dramatic effect on the energy state of alveolar type II cells but no measurable effect on membrane integrity or cell viability. There are several lines of evidence to indicate that the cell membranes are intact following exposure to H_2O_2: there is *1*) no effect on exclusion of trypan blue dye, *2*) no change in the mean cell volume of the cells, *3*) no effect on lactate dehydrogenase release, and *4*) no stimulation of cellular oxygen consumption in the presence of exogenous succinate (data not presented). It appears from these studies that alveolar type II cells can withstand severe conditions without showing any overt signs of cell injury or death.

It has yet to be determined whether loss of intracellular ATP is a critical determinant compromising cell viability. It has been reported that hepatocytes tolerate ATP levels which are reduced by >80% (9). Alveolar type II cells are also relatively tolerant to reduced ATP levels. Exposure to 0.5 mM H_2O_2 for up to 2 h does not cause cell death even though intracellular ATP content is <10% of control after 30 min. It may be that cells which contain relatively large quantities of mitochondria may be more capable of compensating for a loss of ATP. Support for this hypothesis comes from the fact that hepatocytes and alveolar type II cells are both highly metabolically active and therefore would be expected to contain relatively large quantities of mitochondria. It is perhaps more likely that ATP loss is just one event of many oxidant-induced biochemical alterations which lead to cell death.

One important function of type II cells that could be affected by exposure to H_2O_2 is the synthesis of pulmonary surfactant phospholipids. For example, there have been reports which demonstrate that oxidant injury produced by exposure of animals to high levels of oxygen results in reduced amounts of pulmonary surfactant (14, 19). Furthermore, two recent reports provide evidence that exposure of type II cells to oxygen radicals, including H_2O_2, in vitro leads to a decline in the rate of surfactant phospholipid synthesis (1, 6). Our current study began as an attempt to study the effects of H_2O_2 on phospholipid synthesis in type II cells. We found that incorporation of labeled choline, palmitate, and glycerol into disaturated phosphatidylcholines is inhibited by H_2O_2. However, none of the individual enzymes involved in the synthetic pathway, such as choline kinase, cholinephosphotransferase, choline-phosphate cytidylyltransferase, glycerophosphate acyltransferase, phospholipase A_2, and lysolecithin acyltransferase, are affected by H_2O_2. Since ATP serves as a cofactor for some of these enzymes, we conclude that the H_2O_2-induced reduction in in vitro surfactant phospholipid synthesis may be due to a reduction in the cellular ATP level. Although the events may differ in vivo, it is possible that this mechanism is operative in oxidant-induced lung injury.

It is not possible to determine from our results whether the H_2O_2-induced reduction in cellular ATP is due to a direct effect of H_2O_2 itself or to some other oxygen metabolite. It is known that hydroxyl radical is produced by the reaction of H_2O_2 with iron, which is found in the intracellular space (18). We therefore studied the effects of a hydroxyl radical scavenger, dimethylthiourea (DMTU), and two iron chelators, deferoxamine and phenanthroline, on the ability of H_2O_2 to reduce cellular ATP (data not presented). DMTU has no effect, although this could be due to its inability to penetrate the cell membrane. On the other hand, both deferoxamine and phenanthroline reverse the H_2O_2-induced effects in a concentration-dependent manner. The maximal reversal is 50–60%. The fact that reversal is incomplete may also be due to a reduced ability of these chelators to penetrate the cell membrane. These results suggest that the effects of H_2O_2 may be due, at least in part, to intracellular production of hydroxyl radicals.

In summary, the results presented in this paper demonstrate that the energy state of alveolar type II cells is altered after their exposure to H_2O_2 in vitro. The major effect is a reduction in cellular ATP content. The data indicate that a portion of the decline in intracellular ATP is due to H_2O_2-induced damage to the mitochondria. The functional integrity of the mitochondrial enzyme complex responsible for phosphorylation of ADP to ATP may be compromised, but electron transport is not affected. Glucose uptake and its metabolism via glycolysis are also inhibited, possibly as a result of the reduced cellular ATP levels. It is possible that important type II cell functions, such as pulmonary surfactant synthesis, may be impaired after oxidant injury as a result of H_2O_2-induced depletion of cellular ATP.

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