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ATP Accelerates Respiration of Mitochondria From Rat Lung and Suppresses Their Release of Hydrogen Peroxide

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Lung mitochondria were isolated by differential centrifugation from pentobarbital-anesthetized male rats. One to three millimolar Mg^{2+}-ATP increased the consumption of oxygen of lung mitochondria oxidizing 10 mM succinate >fourfold ($P < 0.01$) whereas ATP increased the respiration of liver mitochondria by <35%. ATP also hyperpolarized partially uncoupled lung mitochondria in the presence of the mitochondria-specific antagonist, oligomycin. However, only 20% of the ATPase activity in the lung mitochondria was blocked by oligomycin compared to a blockade of 91% for liver mitochondria. We investigated the effect of reducing the non-mitochondrial ATPase activity in the lung preparation. A purer suspension of lung mitochondria from a Percoll gradient was inhibited 95% by oligomycin. The volume fraction identified as mitochondria by electron microscopy in this suspension ($73.6 \pm 3.5\%$) did not differ from that for liver mitochondria ($69.1 \pm 4.9\%$). ATP reduced the mean area of the mitochondrial profiles in this Percoll fraction by 15% ($P < 0.01$) and increased its state 3 respiration with succinate as substrate by 1.5-fold ($P < 0.01$) with no change in the state 4 respiration measured after carboxyatractyloside. Hence, ATP increased the respiratory control ratio (state 3/state 4, $P < 0.01$). In contrast, state 3 respiration with the complex 1-selective substrates, glutamate and malate, did not change with addition of ATP. The acceleration of respiration by ATP was accompanied by decreased production of H_2O_2. Thus ATP-dependent processes that increase respiration appear to improve lung mitochondrial function while minimizing the release of reactive oxygen species.

KEY WORDS: Fluoride-sensitive ATPase; mitochondrial ultrastructure; reactive oxygen species; safranin.

INTRODUCTION

Mitochondria are important intracellular sources of reactive oxygen species (ROS) and have been implicated in the pulmonary cellular damage caused by hyperoxic exposuse (Sanders *et al*., 1993; Buccellato *et al*., 2004; Pagano *et al*., 2004). Superoxide anion of mitochondrial origin is converted by superoxide dismutase (SOD) to hydrogen peroxide to facilitate its diffusion into the cytoplasm (Turrens, 1997). Because the mitochondrial formation of superoxide increases linearly with the local oxygen tension (Freeman and Crapo, 1981), organs such as the lung in which the tissue oxygen tension is high may have particularly well-developed mechanisms to defend against the mitochondrial formation of ROS. An increase in mitochondrial SOD is recognized as one such defense (Powell and Jackson, 2003) and has been observed in response to systemic hyperoxia (Vincent *et al*., 1994; Chang *et al*., 1995).

Mitochondrial ROS release is also dependent on the oxidation state of the electron transport chain. Superoxide anion is formed at the intermediate complexes (1 and 3) in the chain, and its formation is greatest when

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these complexes are in the reduced state (Turrens, 1997; Votyakova and Reynolds, 2001; Starkov *et al*., 2002). The production of superoxide decreases when the intermediate complexes are oxidized during states of accelerated electron transport. Thus, mechanisms that increase electron transport and the associated consumption of oxygen reduce the formation of ROS. Agents that accelerate respiration include uncoupling proteins (UCP) that increase the permeability of the mitochondria to hydrogen ions. These proteins have been implicated in the generation of heat by brown adipose tissue (Jezek *et al*., 2004) and have been shown to be protective in experimental models of traumatic brain injury (Sullivan *et al*., 2004; Conti *et al*., 2005). Furthermore, the lung is rich in mRNA for UCP2 (Pecqueur *et al*., 2001). However, recent results from UCP2 knockout animals suggest that UCP2 does not play a significant protective role in the normal lung (Couplan *et al*., 2002).

State 3 respiration occurs when there is sufficient ADP and metabolic substrate to maximize the mitochondrial synthesis of ATP. Rapid electron transport then maintains the oxidation of the intermediate complexes and minimizes the formation of superoxide. In the lung the relative abundance of ADP appears to be high since recent evidence indicates that energy charge $\{([ATP] + 0.5^*[ADP])/([ATP] + [ADP] + [AMP])\}$ is less in lung than in several other tissues (Keller *et al*., 2003). The low energy charge in the lung is surprising given the availability of oxygen and the high rate of blood flow to provide adequate metabolic substrates. Accordingly, we have tested the hypothesis that mitochondria from the lung have intrinsic ATPase activity that maintains an accelerated rate of electron transport in the presence of ATP. Our results indicate that the addition of ATP to lung mitochondrial fractions accelerates respiration more than twofold with or without the addition of ADP. This increase in respiration is accompanied by a reduction in the mitochondrial release of H_2O_2 and appears to involve both the hydrolysis of ATP to maintain a supply of ADP substrate and a possible phosphorylation step that increases the oxidation of metabolic substrates by complex 2 during state 3 respiration.

MATERIALS AND METHODS

Experimental protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. Sprague-Dawley male rats weighing between 300 and 400 g were housed two to three per cage and kept in a 12-h light/dark cycle with ad libitum access to food and water. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.). To obtain liver mitochondria the liver was removed through an abdominal incision. To obtain lung mitochondria the thorax was opened and the left atrial appendage was severed. Fifty milliliters of normal saline at 4°C was infused into the right cardiac ventricle to flush blood from the lungs. Harvested organs were immediately placed in the appropriate homogenization buffer at 4°C.

Isolation of Liver Mitochondria

Liver was homogenized in 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 0.5 mg/mL fatty acid-free BSA, and 1 mM EGTA, pH 7.4 at 4°C based on (Holland *et al*., 1973). The organ was rinsed three to four times with this buffer before it was minced with scissors. The pieces were homogenized with four strokes in a Potter-Elvejehm homogenizer and centrifuged in a Sorvall SS34 rotor for 3 min at $1698 \times g$. Excess fat was removed with cotton swabs from the supernatant that was then centrifuged 12 min at $8884 \times g$. The pellets were resuspended in buffer before being centrifuged for 12 min at $14{,}763 \times g$. The pellets were resuspended, washed in the same buffer but without EGTA, and concentrated by centrifugation for 12 min at $14{,}763 \times g$. The final suspension was in buffer without EGTA at 4°C until use.

Isolation of Lung Mitochondria

Based on the method of (Fisher *et al*., 1973) lungs were rinsed three to four times in 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1% fatty acid-free BSA, and 2 mM EGTA, pH 7.4 at 4°C. They were then minced with scissors, macerated with a Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK) at speed 1 for 15 s, and homogenized with three slow up-and-down passes of a Potter-Elvejehm homogenizer. The homogenate was spun for 5 or 10 min at $1960 \times g$ in a Sorvall SS34 rotor. The supernatant was filtered through nylon mesh and centrifuged 10 min at $16{,}984 \times g$. The pellet was resuspended, rinsed in buffer but with 0.1% BSA and no EGTA, and centrifuged for 10 min at $16{,}984 \times g$. The pellet was resuspended and kept at 4°C.

Separation of Lung Mitochondria on Percoll Gradients

Lungs from five or six rats were required to obtain a sufficient yield of mitochondria from Percoll gradients.

The above protocol for lung mitochondria was followed through the first centifugation at 16,984 × *g*. Percoll was diluted with lung homogenation buffer to 40, 25, and 15%. The raw mitochondrial pellet was suspended in 2 mL of 15% Percoll. Each of two 9-mm test tubes was layered first with 1.5 mL 40% Percoll, then 5 mL 25% Percoll, and finally 1 mL of mitochondrial suspension in 15% Percoll. The sample was centrifuged 6 min at 41,000 × *g* in a Sorvall SS34 rotor with a slow-stop setting. Each band was washed with 10 mL isolation buffer without EGTA containing 0.1% BSA and centrifuged for 10 min at 30,000 × *g*. Washing was repeated as needed to obtain a hard and distinct pellet. Each pellet was resuspended and kept at 4°C.

Measurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was measured with a Clark-type oxygen electrode (Hansatech, Norfolk, UK) at 30°C. The protein content of the mitochondrial suspensions was measured using the biuret reagent. Mitochondria were suspended at 0.25–1 mg of protein/mL in 125 mM KCl, 2 mM K_2HPO_4, 20 mM HEPES (pH 7.0), 1 mM MgCl, 0.4 mg/mL BSA, and 0.25 mM EGTA with substrates and added Mg^{2+}-ATP as indicated in Results section. To determine the state 3 rate of respiration, 0.1 or 0.2 mM K-ADP was added. Measurement continued until the rate of respiration declined indicating the depletion of ADP. Then 2 μM carboxyatractyloside was added to block the exchange of ADP external to the mitochondrial matrix with newly formed ATP preventing ATP synthesis and causing a state 4 rate of respiration.

Assessment of Mitochondrial Membrane Potential

Qualitative measurements of lung mitochondrial membrane potential were done at 30°C in the same buffer used to measure oxygen consumption with 5 μM safranin O at excitation and emission wavelengths of 495 and 586 nm, respectively (Votyakova and Reynolds, 2001) with a mitochondrial concentration of protein of 0.25 mg/mL and 10 mM succinate as substrate.

Measurement of ATPase Activity Using NADH Oxidation Method

Mitochondrial samples were assayed after a freeze–thaw cycle at −70°C and diluted with water to the appropriate protein concentration (0.4 mg/mL for fraction 3 of the Percoll gradient, 0.2 mg/mL for all other samples). Additional sonication was not found to affect the measured ATPase activity. Ten microliters of the mitochondrial sample or water was added to each well of a 96-well plate followed by 20 μL of 1 U/μL lactate dehydrogenase and 35 μL of 8.5 mM NADH. Then 135 μL of an activation mix consisting of 33 mM Tris, 83 mM sucrose, 10 mM MgCl, 1 mM KCN, 1 mM EDTA, 2.22 mM PEP, and 2 mM ATP was added. The change in absorbance over 10 min was read every 30 s at both 340 and 385 nm. Inhibitors tested in this assay were 1 μg/mL oligomycin, 10 mM sodium azide, 10 mM sodium fluoride, 10 mM sodium tartrate, 10 mM sodium (meta)vanadate, 10 mM furosemide, 10 and 1 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), and 1 mM suramin.

Measurement of ATPase Activity Using Malachite Green Assay

Ten microliters of mitochondrial sample prepared as described for the NADH oxidation method or water was added to a well in a 96-well plate, followed by 14 μL of water or inhibitor and 116 μL of an activation mix consisting of 33 mM Tris, 83 mM sucrose, 10 mM MgCl, 1 mM EDTA, 1.56 mM KCN, and 3.12 mM ATP. The reaction was incubated 30 min at 22°C with gentle rocking. It was stopped with 50 μL of 0.6 M perchloric acid, and 20 μL of the reaction was transferred to a well in another plate containing 25 μL of 1.4 mM malachite green and 180 μL of 21 mM ammonium molybdate in 2.5 N HCl. After shaking for 30 s, the absorbance was read at 650 nm. Inhibitors tested in this assay were 1 μg/mL oligomycin, 10 and 0.1 mM sodium azide, 10 mM sodium fluoride, 10 mM sodium tartrate, and 10 mM sodium (meta)vanadate. The percent inhibition of each agent was calculated from the difference between the activity in the presence of the inhibitor and in the presence of a vehicle control.

Measurement of H_2O_2 Release From Mitochondria

Mitochondrial H_2O_2 release at 37°C was measured using 1 μM Amplex red reagent with 1 U/mL horseradish peroxidase at excitation and emission wavelengths of 550 and 585 nm, respectively (Zhou *et al*., 1997). Mitochondria were suspended at 0.1–0.25 mg/mL of protein in the same buffer used to measure the consumption of oxygen with 10 mM succinate as substrate and Mg^{2+}-ATP added as indicated in Results section. The release of H_2O_2 was determined during the same respiratory states for which oxygen consumption was measured.

Electron Microscopy

Liver mitochondria and Percoll fractions of lung mitochondria were pelleted by centrifugation and fixed in 4% glutaraldehyde with 0.1 M cacodylate followed by 2% osmium tetroxide in cacodylate buffer. Fixed pellets were dehydrated in graded ethanols and embedded in Epon-Araldite. Ultrathin sections (silver-gray in appearance) were cut on a Sorvall MT5000 ultramicrotome, picked up on 300 mesh grids and stained with uranyl acetate followed by lead citrate. Care was taken to sample from the middle of each pellet.

Areas containing mitochondria were chosen at low magnification (1500×) from a series of contiguous grid squares (1 area/square), and photographed at 15,000×. Analyses of micrographs were performed with NIH Image 1.63, using macros written by DWP. For stereology, micrographs covering 31.6 μ^2 each were digitized and overlain with a grid of 225 equally spaced test points. Points were counted as lying over mitochondria, lamellar bodies, other membrane-bound structures, or cilia. We calculated a volume percentage of mitochondria as (number of points overlying mitochondria)/(number of points overlying mitochondria + the other structures). We counted three independent regions each from Percoll fractions 2 and 3 of the lung preparation and six regions from the liver preparation.

To assess size differences among respiring mitochondria, selected mitochondrial fractions were incubated with brisk stirring at 30°C in 0.7 mL of respiration buffer with and without 3 mM Mg^{2+}-ATP, using10 mM succinate as substrate. After 6 min, an equal volume of 8% glutaraldehyde in 0.2 M cacodylate was added. The fixed mitochondria were prepared for electron microscopy and random areas containing mitochondria were photographed at 15,000× as described above. Mitochondrial profiles were traced by hand from digital prints (enlarged 2.5×) of the micrographs. Traced profiles were digitized and their enclosed areas determined with NIH Image.

Statistical Analysis

Differences in means for buffers with and without ATP were tested with analysis of variance (ANOVA) corrected for repeated measures in the same mitochondrial preparation (Winer, 1971). Differences in variables determined with electron microscopy were tested with one (stereology) or two-factor (area of profiles) ANOVA with no correction for repeated measures. When more than two means were tested with ANOVA, the simple main effects were determined using the Newman-Keuls procedure (Winer, 1971).

Chemicals

Chemicals and reagents were obtained from Sigma-Aldrich (Saint Louis, MO) with the exception of carboxyatractyloside (Calbiochem, La Jolla, CA) and Amplex Red substrate (Molecular Probes, Eugene, OR).

RESULTS

ATP Accelerates the Respiration of Lung Mitochondria in Comparison to Liver Mitochondria

Mitochondrial respiration was measured in buffers containing 0, 1, and 3 mM Mg^{2+}-ATP with 10 mM succinate as a substrate. In the liver preparation the initial consumption of oxygen before the addition of ADP was similar with or without ATP (Fig. 1), and the addition of ADP accelerated respiration in all groups to similar state 3 rates. With depletion of the added ADP, oxygen consumption returned to the initial rates. Interrupting the transport of ADP and ATP across the inner mitochondrial membrane with carboxyatractyloside reduced the respiration to state 4 rates that were similar in all groups.

In contrast to liver mitochondria, the addition of either 1 or 3 mM ATP increased the initial respiratory rate of lung mitochondria, and the subsequent addition of ADP did not accelerate respiration further (Fig. 1). Thus, the presence of ATP by itself promoted state 3 respiration. In the buffer without ATP, the rate of respiration declined with time after the addition of ADP but did not decrease to the initial baseline suggesting that the depletion of ADP in the suspension of lung mitochondria was incomplete. This decline was blunted in the presence of ATP possibly because of its partial hydroysis to ADP. As with liver mitochondria, carboxyatractyloside reduced the respiration to state 4 rates that were similar in all groups.

ATP Hyperpolarizes Partially Uncoupled Mitochondria From Lung

Membrane potential was assessed by safranin fluorescence in lung mitochondria partially uncoupled with carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) so that both depolarizing and hyperpolarizing responses could be observed. In companion experiments we found that the addition of 150 nM FCCP to respiring mitochondria in 10 mM succinate increased the baseline rate of oxygen consumption by about 2.8-fold to a rate less than state 3. This low dose of FCCP also increased the safranin fluorescence to the baseline shown by the lower

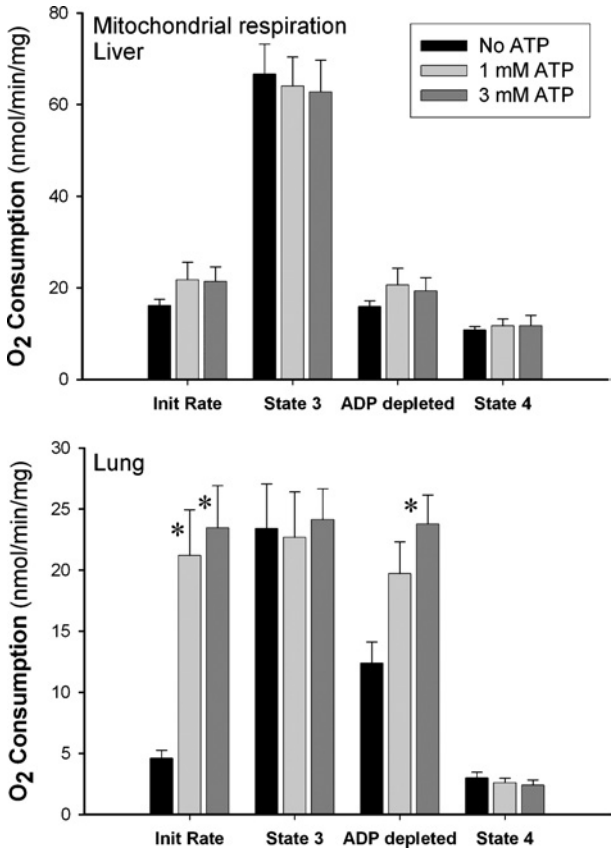

Fig. 1. Rate of respiration of liver and lung mitochondria isolated by differential centrifugation with and without 1 or 3 mM Mg^{2+}-ATP. $N = 5$ in each group. Note that the presence of ATP in the respiration buffer increases the initial rate (Init Rate) of respiration of mitochondria from lung to near the state 3 rate that was measured after the addition of ADP. Rate was then measured after depletion of ADP before addition of 1 μM carboxyatractyloside (state 4). Asterisk indicates the difference from the value with no ATP is significant, $P < 0.02$. Error bars are SEM.

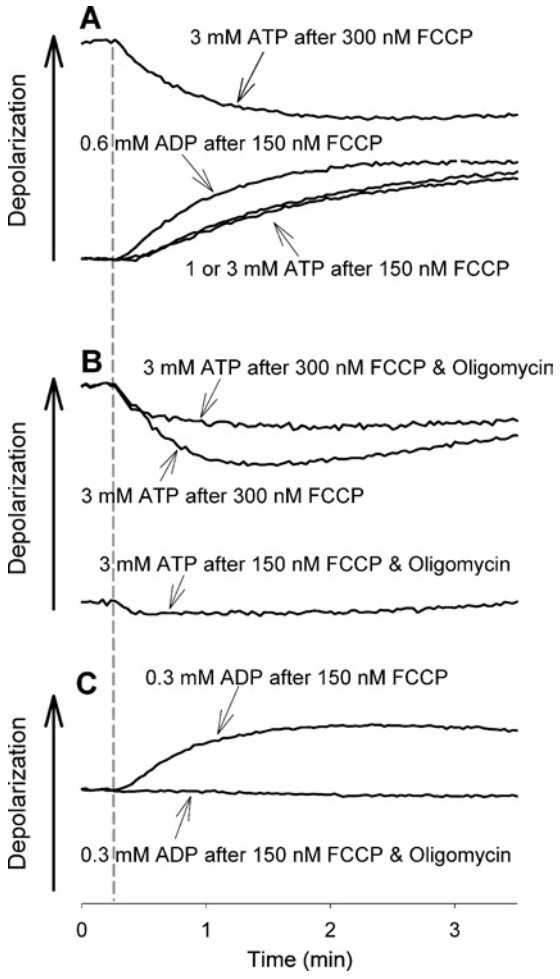

Fig. 2. Effect of ADP and Mg^{2+}-ATP on safranin fluorescence in suspensions of partially uncoupled mitochondria from lung. Traces shown are after addition of indicated doses of carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) in the presence or absence of 1 μg/mL oligomycin. Dotted vertical grey line indicates time of nucleotide injection.

traces in Fig. 2(A). Under these conditions the addition of either ADP or ATP at the dotted grey line caused additional depolarization as indicated by the increased signals (Fig. 2(A)), and consistent with increases in mitochondrial respiration to state 3 rates in the presence of this low dose of FCCP (data not shown). Although ATP and ADP were equally effective in accelerating the rate of respiration, ATP in two different concentrations elicited similar and less depolarization than did ADP. Furthermore, ATP led to hyperpolarization in the presence of 300 nM FCCP (Fig. 2(A)), top trace). To eliminate any extrusion of H^+ from the mitochondrial matrix due to the hydrolysis of ATP by the F0, F1-ATPase, we repeated this test in the presence of 1 μg/mL oligomycin. Oligomycin in the presence of 150 or 300 nM FCCP reduced the hyperpolarization in response to ATP but failed to block it completely (Fig. 2(B)). The dose of oligomycin used completely blocked depolarization in response to ADP (Fig. 1(C)).

ATPase Activity in Lung Mitochondrial Fractions

The acceleration of lung mitochondrial respiration by ATP may have resulted in part from a non-mitochondrial ATPase that converted enough ATP to ADP to support a state 3 rate of oxygen consumption. Accordingly, we assayed the ATPase activity of both the liver and the

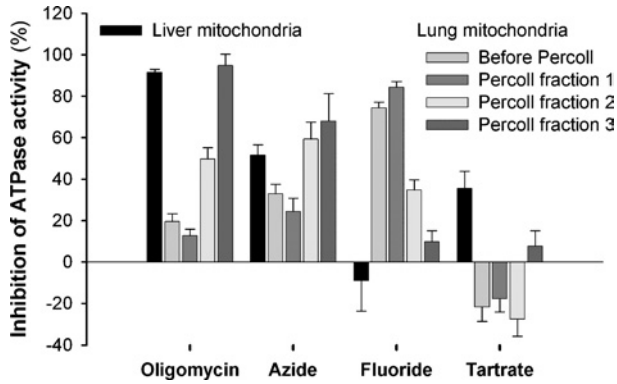

Fig. 3. Effect of various inhibitors on the ATPase activity of different suspensions of mitochondria measured with the malachite green assay. Note that Percoll fractions 2 and 3 of lung mitochondria were more sensitive to 1 μg/mL oligomycin and to 0.1 mM sodium azide than were the suspensions of lung mitochondria isolated by differential centrifugation alone (Before Percoll). Percoll fraction 3 was less sensitive to 10 mM sodium fluoride and 10 mM sodium tartrate than the other preparations from lung. Fraction 3 was as sensitive to oligomycin as the liver preparation. Similar findings for oligomycin, fluoride, and tartrate were obtained using the NADH oxidation method. $N = 6$–11 for all groups except for oligomycin with the liver preparation ($N = 4$), and for sodium azide with the liver preparation ($N = 5$) and Percoll fractions 1 ($N = 4$) and 2 ($N = 2$). Error bars are SEM.

lung mitochondria to determine if ATPases other than the expected!la F0, F1-ATPase were present. The ATPase activity of liver mitochondria was inhibited 91% by oligomycin and 52% by a low dose (0.1 mM) of sodium azide (Fig. 3). This result indicated that the predominant ATPase in the liver preparation was the F0, F1 type that functions as an ATP synthetase in respiring mitochondria. In contrast, only 20 and 43% of the ATPase activity of the lung mitochondria obtained by differential centrifugation was inhibited by oligomycin or azide, respectively, suggesting that this preparation contained a non-mitochondrial ATPase that could hydrolyze enough ATP to ADP to accelerate respiration in samples containing ATP.

We then compared the effects of other inhibitors of ATPase activity on the lung and liver preparations. Mitochondria from both organs showed similar inhibition by 10 mM sodium (meta)vanadate, 10 mM furosemide, 10 and 1 μM DIDS, and 1 mM suramin. However, there were significant differences between the liver and lung preparations in the effects of 10 mM sodium tartrate and 10 mM sodium fluoride (Fig. 3). The ATPase activity of liver mitochondria was inhibited 35% by tartrate, but this compound modestly facilitated the activity of the lung mitochondria that were isolated by differential centrifugation alone. In contrast, fluoride inhibited 74% of the activity in this lung preparation whereas fluoride's effect on the liver preparation was not significant (Fig. 3). This result suggested that the initial suspension of lung mitochondria contained a fluoride-sensitive phosphatase (Mavis *et al*., 1978) or Ca^{2+}-ATPase (Murphy and Coll, 1992). To determine the contribution of this activity to the accelerated respiration observed in the presence of ATP, we used a discontinuous Percoll gradient to separate the non-mitochondrial ATPase activity from the lung mitochondrial suspension.

Depleted and enriched mitochondrial fractions from the lung were found in the bands of the Percoll gradients. The lightest band (Percoll fraction 1, Fig. 3) remained in 15% Percoll and was enriched with fluoride-sensitive activity and depleted of oligomycin-sensitive activity. The respiratory rate of this band was less than the initial suspension of lung mitochondria before Percoll separation. Two denser bands showed greater sensitivity to oligomycin and lesser sensitivity to fluoride than did initial lung preparation. The lighter of these latter two bands (Percoll fraction 2) was located at the top of the 25% Percoll just below its interface with Percoll fraction 1. Percoll fraction 3 was found at the interface between 25 and 40% Percoll. As shown in Fig. 3 both Percoll fractions 2 and 3 were more sensitive to oligomycin and 0.1 mM azide and less sensitive to fluoride than was the initial preparation from lung. Furthermore, Percoll fraction 3 showed a profile of sensitivity to the various inhibitors that did not differ significantly from that for liver mitochondria. The heaviest fraction that pelleted through 40% Percoll had no significant ATPase or respiratory activity (data not shown).

Ultrastructure and Stereology of Mitochondrial Fractions

Electron microscopy of Percoll fractions 2 and 3 and of the liver preparation (Fig. 4) indicated that their mitochondrial content was directly related to the sensitivity of their ATPase activity to oligomycin. The volume percentage of membrane-bound structures that were identified positively as mitochondria did not differ between Percoll fraction 3 and the liver preparation (Table I). In contrast, Percoll fraction 2 showed a lower volume percentage of mitochondrial profiles along with a greater presence of lamellar bodies and other membranous structures having the characteristics of endoplasmic reticulum and cilia (Fig. 4). Both lung fractions also contained bar-like structures that were described previously in Type II pneumocytes (Shimura *et al*., 1985; Vincent and Nadeau, 1987; Risco *et al*., 1994).

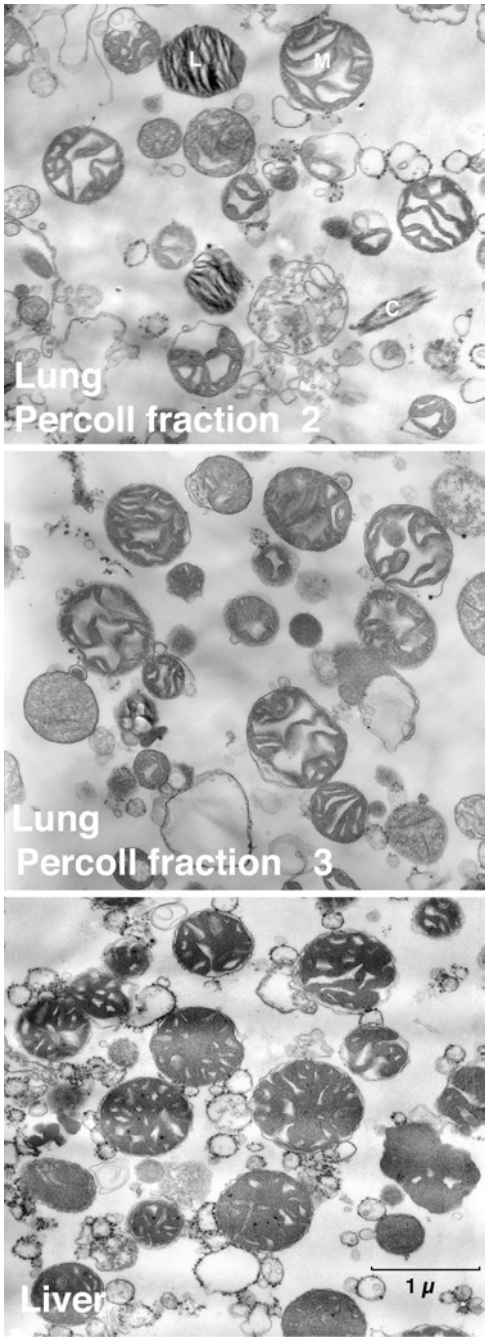

Fig. 4. Representative electron micrographs of mitochondrial suspensions obtained from Percoll fractions 2 and 3 from lung and from liver. Abbreviations: m: mitochondrion; l: lamellar body; and c: cilium.

ATP Accelerates Respiration in Percoll-Enriched Mitochondrial Fractions

The initial findings (Fig. 1) indicated that the addition of both 1 and 3 mM ATP to the lung mitochondria obtained by differential centrifugation accelerated their respiration. In subsequent experiments (Fig. 5) improved rates of respiration were obtained from lung mitochondria, and an effect of ATP on the state 3 rate of respiration became apparent. These latter results coincided with a reduction in the duration of the initial centrifugation in the isolation process from 10 to 5 min.

Table I. Characteristics of Mitochondrial Fractions

Mitochondrial fraction	% Positively identified mitochondria	% Inhibition by oligomycin
Percoll fraction 2 from lung	37.6 ± 3.4	49.7 ± 5.4
Percoll fraction 3 from lung	73.6 ± 3.5^a	94.8 ± 5.6^a
Liver	69.1 ± 4.9^a	91.4 ± 1.5^a

Note. Data are means ± standard errors.
[a]Different from Percoll Fraction 2, $P < 0.001$.

Figure 5 shows that reducing the fluoride-sensitive ATPase activity of the lung mitochondrial suspension by Percoll separation did not block the acceleration of respiration by ATP. In Percoll fraction 2 the sensitivity to fluoride was reduced by 53%. However, the addition of ATP to the buffer increased the initial rate of respiration by a factor of 3.7 as compared to a 4.4-fold increase in this rate in the initial suspension before Percoll. In Percoll fraction 3 the fluoride sensitivity was reduced by 87% yet the addition of ATP to this fraction still increased the initial rate of respiration more than 2.5-fold. The presence of ATP in the buffer had effects on the response to ADP that differed between the various fractions. In Percoll fraction 2 the initial rate with ATP did not differ significantly from the state 3 rate measured with or without ATP. Furthermore, a significant decline in the rate measured after the addition of ADP only occurred in the absence of ATP. Thus, the presence of ATP appeared to prolong the state 3 rate. In Percoll fraction 3, ATP led to a rate of respiration after the addition of ADP that was greater than the state 3 rate observed in the absence of ATP ($P < 0.01$). This augmented state 3 rate was not sustained, and it declined to a value similar to the initial rate before the addition of ADP ($P < 0.01$). The addition of carboxyatractyloside led to minimal rates of respiration in all fractions that were similar in the presence and absence of ATP. Computation of the respiratory control ratio (RCR = state 3/state 4) using the rate after carboxyatracyloside as state 4 showed RCRs of 6 or more in all but Percoll fraction 3 when it was tested in the absence of ATP (Fig. 6(A)). The RCR of the mitochondria in Percoll fraction 3 increased significantly in the presence of ATP.

The increase in the baseline respiration of the lung mitochondria in response to ATP decreased linearly

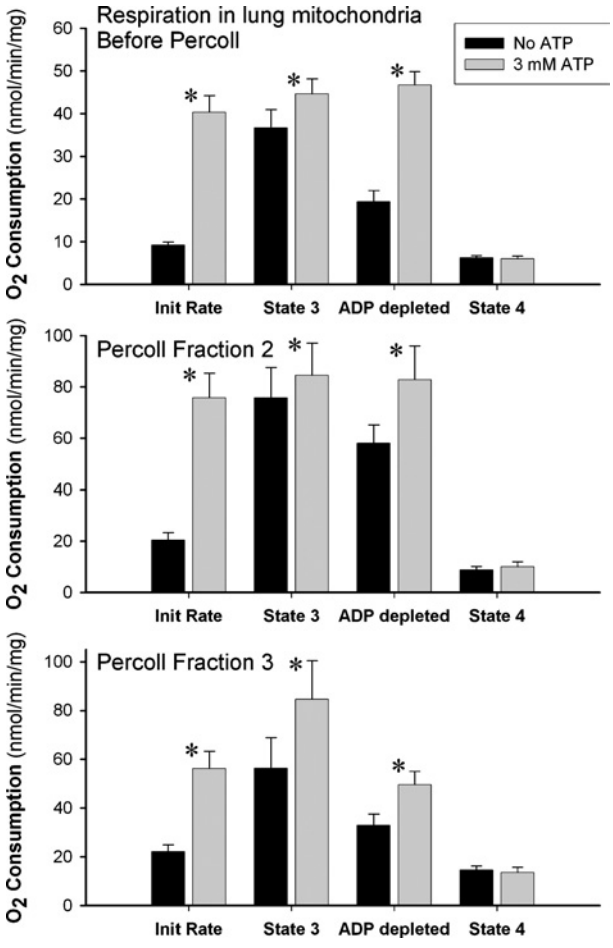

Fig. 5. Rate of respiration with 10 mM succinate as substrate in suspensions of lung mitochondria obtained before Percoll separation ($N = 7$), in Percoll fraction 2 ($N = 7$), and in Percoll fraction 3 ($N = 6$) with and without 3 mM Mg^{2+}-ATP. Initial (Init) rate occurred before the addition of ADP to cause State 3. Rate was then measured after depletion of ADP before addition of 1 μM carboxyatractyloside (state 4). Asterisk indicates the difference from the value with no ATP is significant, $P < 0.01$. Error bars are SEM.

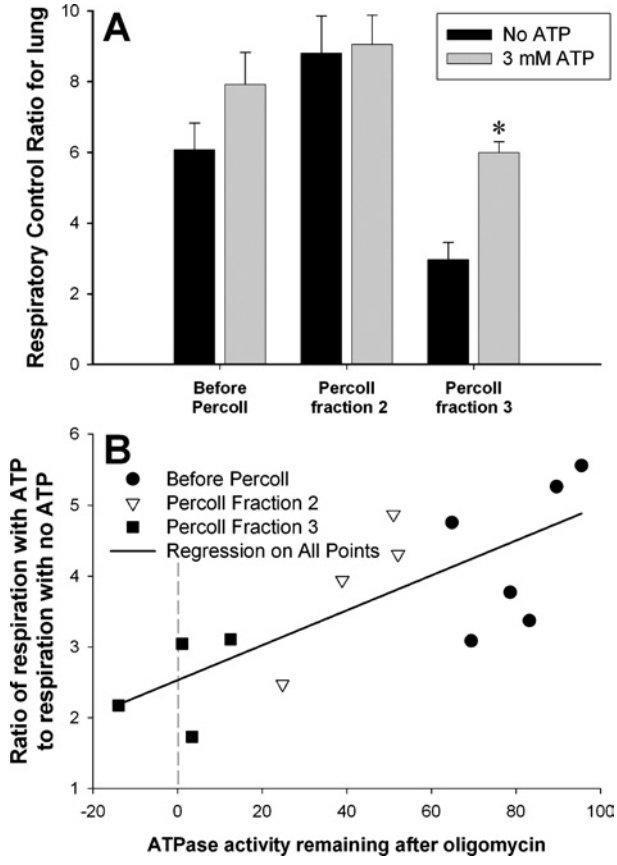

Fig. 6. (A) Respiratory control ratio (state 3/state 4) with and without 3 mM Mg^{2+}-ATP for suspensions of lung mitochondria obtained before Percoll separation and in Percoll fractions 2 and 3. *N*s as in Fig. 4. Asterisk indicates the difference from the value with no ATP is significant, $P < 0.01$. Error bars are SEM. (B) Relationship of the increase in baseline respiration in response to 3 mM Mg^{2+}-ATP to the residual ATPase activity measured with malachite green after oligomycin in all fractions of lung mitochondria for which both variables were measured. Negative value occurred when oligomycin reduced the ATPase activity below the blank measured with no sample.

as oligomycin became more effective in inhibiting the ATPase activity of the various Percoll fractions (Fig. 6(B)). However, the regression line in Fig. 6(B) has a significant positive intercept predicting that ATP would still increase the baseline respiration by a factor of 2.53 ± 0.36 in mitochondrial suspensions for which oligomycin blocks all of the detectable ATPase activity. A similar analysis (not shown) substituting the percent inhibition of ATPase activity by fluoride on the abscissa yields a significant intercept of 2.80 ± 0.46 predicting a similar effect of ATP in fractions of lung mitochondria that are insensitive to fluoride.

Effect of ATP on Mitochondrial Size

Mitochondrial fractions were incubated in respiration buffer in the presence or absence of 3 mM ATP. After 6 min, the fractions were immediately fixed and prepared for electron microscopy. The mean areas of the positively identified mitochondrial profiles are shown in Table II. In Percoll fraction 2 the mean area of the profiles was similar with or without ATP in the buffer. In contrast, ATP added to the mitochondria in Percoll fraction 3 from lung or to those from liver reduced their mean areas by 15 and 10%, respectively ($P < 0.01$). In addition the mean areas of all the fractions that were isolated from the lung were

Table II. Mean Area of Respiring Mitochondrial Profiles (μm^2)

Mitochondrial fraction	No ATP	3 mM ATP
Percoll fraction 2 from lung	0.243 ± 0.012	0.251 ± 0.015
Percoll fraction 3 from lung	0.268 ± 0.011	0.227 ± 0.010^a
Liver	0.439 ± 0.010^b	$0.396 \pm 0.009^{a,b}$

Note. Each cell is the mean of 161–360 profiles. Percoll fractions from lung were obtained from pooled homogenates from five rats. Liver fractions were obtained from three rats. Data are means ± standard errors.

[a] Different from No ATP, $P < 0.01$.

[b] Different from both Percoll fractions from lung, $P < 0.001$.

37–45% less than mean area of the mitochondria isolated from the liver ($P < 0.001$) with or without ATP in the buffer.

ATP Reduces the H_2O_2 Production From Percoll Enriched Lung Fractions

Before addition of ADP the presence of ATP in the buffer reduced the formation of hydrogen peroxide 68–74% in the various fractions of lung mitochondria (Fig. 7). In the absence of ATP the addition of ADP led to a similar reduction in peroxide release so that adding ADP in the presence of ATP caused no further reduction in the production of peroxide. In the absence of ATP peroxide release increased in all fractions as added ADP was depleted by ATP synthesis ($P < 0.05$, in each case), and this response was blunted when ATP was included in the buffer. Finally, peroxide release increased to maximal rates with the addition carboxyatractyloside. However, the presence of ATP in the buffer reduced this maximal production of peroxide significantly in lung mitochondria not separated on Percoll and in Percoll fraction 2 ($P < 0.01$, in each case). In four suspensions of mitochondria separated by differential centrifugation only, we measured both respiration and peroxide production. We normalized the maximal rate of peroxide production after administration of carboxyatractyloside dividing this rate by the maximal state 3 rate of respiration (as an index of functional electron transport chains). The resulting ratio decreased from 4.21 ± 1.17 to 2.82 ± 0.79 pmol/nmol with the addition of 3 mM ATP to the buffer.

Effect of ATP on State 3 Respiration With Complex Specific Substrates

Respiration of Percoll fractions 2 and 3 from lung was measured using a combination of substrates, 5 mM glutamate and 5 mM malate, that is selective for complex 1 in the electron transport chain. With this combination ATP caused a significant increase in the initial rate of respiration but no significant increase in the rate of state 3 respiration (Fig. 8). To determine effects specific for complex 2, 10 mM succinate was used with the complex 1 inhibitor, rotenone (1 μM). In this case, ATP led to a significant increase in the state 3 rate of Percoll fraction 3 ($P < 0.05$) that did not differ from that observed with succinate alone. RCR with succinate and rotenone increased significantly in response to ATP in both Percoll fraction 2 ($P = 0.047$, Wilcoxan signed-rank test)

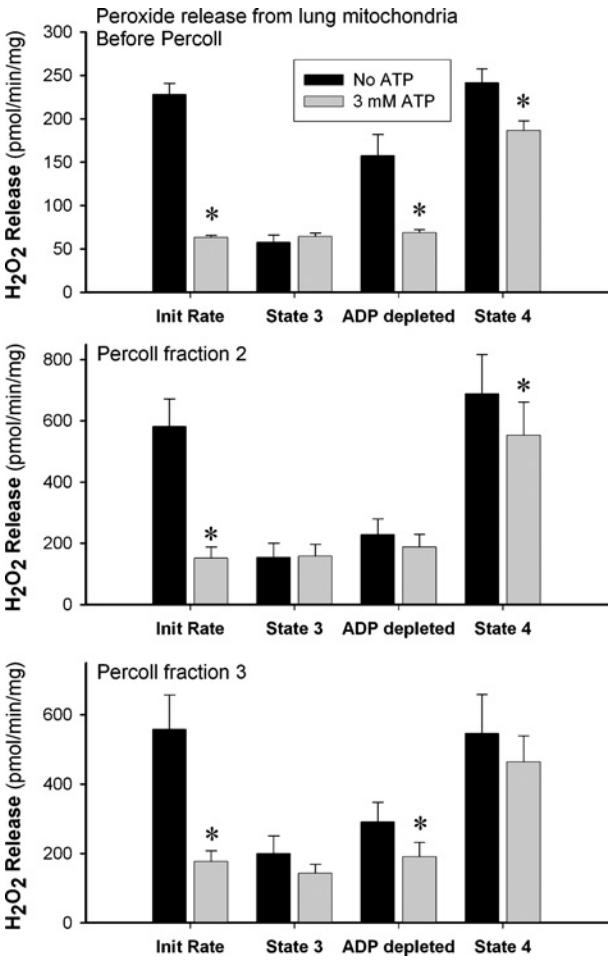

Fig. 7. Release of hydrogen peroxide in suspensions of lung mitochondria obtained before Percoll separation ($N = 10$) and mitochondria from Percoll fraction 2 ($N = 5$) and from Percoll fraction 3 ($N = 5$) with and without 3 mM Mg^{2+}-ATP. Initial (Init) rate occurred before the addition of ADP to cause State 3. Rate was then measured after depletion of ADP before addition of 1 μM carboxyatractyloside (state 4). Asterisk indicates the difference from the value with no ATP is significant, $P < 0.02$. Error bars are SEM.

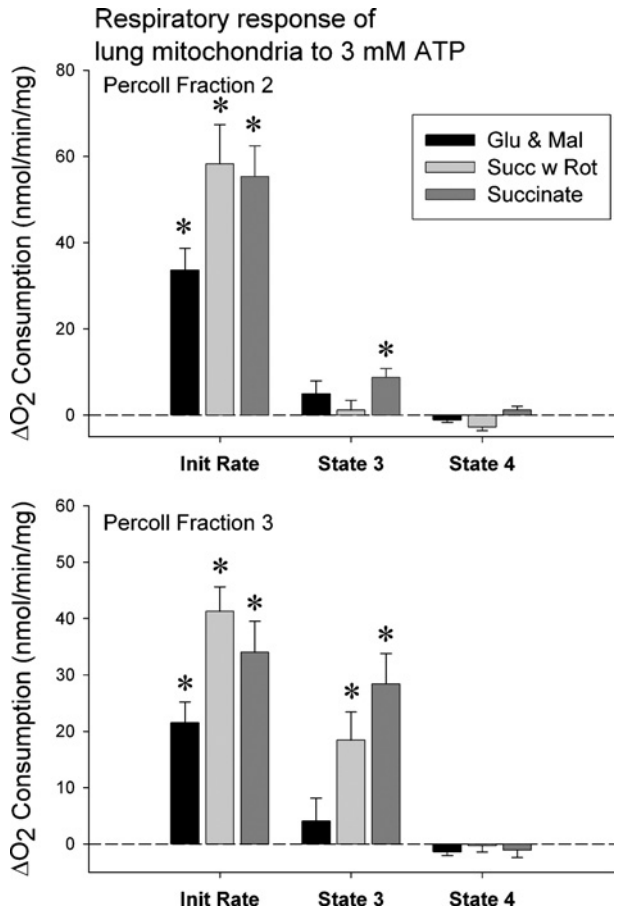

Fig. 8. Response of oxygen consumption in lung mitochondria from Percoll fractions 2 and 3 to 3 mM Mg^{2+}-ATP with 5 mM glutamate and 5 mM malate (Glu and Mal), with 10 mM succinate and 1 μM rotenone (Succ w Rot), and with 10 mM succinate alone. Initial (Init) rate occurred before the addition of ADP to cause State 3. State 4 was measured after addition of 1 μM carboxyatractyloside. $N = 5$–8 in each group. Asterisk indicates the response is different from zero, $P < 0.05$. Responses to ATP with 10 mM succinate and rotenone show no difference by ANOVA from those with succinate alone. Error bars are SEM.

and Percoll fraction 3 ($P = 0.031$, Wilcoxan signed-rank test).

DISCUSSION

The present study was prompted by the initial observation that the consumption of oxygen by mitochondria isolated by differential centrifugation from the lung but not from the liver was accelerated by cytosolic concentrations of Mg^{2+}-ATP. The lack of an effect in the liver mitochondria confirmed that the ATP used in our experiments (>99% pure according to manufacturer's specification) was relatively free of ADP that could increase respiration. In addition, the inclusion of EGTA in the incubation buffers precluded any unmeasured changes in Ca^{2+} that could account for these findings by modulating electron transport.

Our measurements of lung mitochondrial membrane potential suggested that uncoupling could not account for the acceleration of respiration by ATP. At the low dose of FCCP in Fig. 2(A), an uncoupling effect of ATP should have elicited greater depolarization than the addition of ADP because uncoupling by ATP would augment the depolarization associated with the observed acceleration of respiration. However, the membrane depolarization in response to the addition of ATP was less than that observed after ADP (Fig. 2(A)). Furthermore, ATP led to hyperpolarization of the lung mitochondria in the presence of oligomycin consistent with an ATP-dependent mechanism that improved membrane potential independently of a reversal in the normal function of the F0, F1-ATPase.

The non-mitochondrial hydrolysis of ATP to ADP remained a potential source for the accelerated respiration of the initial preparation of lung mitochondria before Percoll enrichment. Eighty percent of the ATPase activity of this preparation was not inhibited by oligomycin in agreement with an early report (Reiss, 1966). We determined further that a major component of the activity was inhibited uniquely by fluoride. Although several other inhibitors also suppressed the ATPase activity of the initial preparation from lung, they had similar effects on liver mitochondria. Thus, the initial lung preparation appeared to have a nonmitochondrial component that was rich in fluoride-sensitive ATPase (Murphy and Coll, 1992) or phosphatase (Mavis *et al*., 1978). Separation on a Percoll gradient reduced this enzymatic activity and its contribution to the acceleration of mitochondrial respiration through the extramitochondrial hydrolysis ATP to ADP.

Mitochondrial enrichment in Percoll fractions 2 and 3 was confirmed by stereology. The frequency of structures in electron micrographs that were identified positively as mitochondria increased in direct proportion to the sensitivity of the ATPase activity to oligomycin. Furthermore, these variables for Percoll fraction 3 did not differ from their values in suspensions of liver mitochondria.

The effect of ATP to increase the initial rate of respiration decreased as the fluoride-sensitive ATPase activity was reduced by Percoll separation. Nonetheless, fraction 3 whose sensitivity to fluoride did not differ from the liver preparation still showed a 2.5-fold increase in the initial rate of oxygen consumption in response to ATP. Furthermore, ATP affected not just the initial rate but also the state 3 rate measured after the addition of ADP. In both the initial preparation from lung and in Percoll fraction 2

there was a modest but significant acceleration of state 3 respiration by ATP (Fig. 5). This effect was likely obscured by the presence nonmitochondrial ATPases since it was more prominent in Percoll fraction 3 than in the less pure preparations. The addition of ATP to Percoll fraction 3 also increased its RCR suggesting that the presence of ATP improves lung mitochondrial function by reducing the permeability of the inner membrane.

The changes in the area of the mitochondrial profiles were consistent with the evidence against mitochondrial uncoupling and with the effects of ATP on RCR because none of the fractions tested swelled in response to ATP. Moreover, the mitochondria in Percoll fraction 3 from lung and those from liver shrank in response to ATP. These results were consistent with known effects of ATP on ATP-dependent K^+ channels and on UCP2. ATP-dependent K^+ channels close in response to ATP (Inoue *et al*., 1991), and ATP inhibits the activity of UCP2 (Couplan *et al*., 2002; Zackova *et al*., 2003). These or other unknown effects could reduce ionic leakage across the inner membrane to prevent swelling during the increases in lung mitochondrial respiration. However the marked decrease in mitochondrial volume found in Percoll fraction 3 together with the hyperpolarization of lung mitochondria observed upon addition of ATP suggest that there is an additional ATP-dependent mechanism for active ionic transport across the inner membrane. In the presence of ATP such a mechanism could produce ADP to accelerate respiration and at the same time counterbalance the associated increase in proton flux.

Mitochondrial release of H_2O_2 results from the partial reduction of oxygen at the intermediate complexes in the electron transport chain to form superoxide anion within the mitochondrial matrix. Superoxide dismutase then converts this product to H_2O_2 that diffuses easily through the inner and outer mitochondrial membranes into the surrounding space (Turrens, 1997). We found that this release decreased markedly in the presence of ATP and was strongly related to the acceleration of oxygen consumption. However, when the effect of ATP on respiration was blocked by carboxyatractyloside there remained a small reduction in H_2O_2 release that was statistically significant in both the raw preparation and in Percoll fraction 2. Since carboxyatractyloside prevents the entrance of ATP into the mitochondrial matrix, this result may indicate an effect of ATP on ROS release that originates from the intramembrane space. How such an effect would occur remains to be determined.

In mitochondria from brain with succinate as a substrate, a major pathway for the formation of superoxide is the reverse flow of electrons from complex 2 in the electron transport chain to complex 1 (Votyakova and Reynolds, 2001; Liu *et al*., 2002; Starkov *et al*., 2002). The partial reduction of oxygen by complex 1 also appears prominent in lung mitochondria because we have observed marked inhibition of succinate-driven H_2O_2 release by the complex 1 inhibitor rotenone in companion experiments. Furthermore, the production of H_2O_2 with complex 1 substrates is less than with succinate. Nonetheless, in other experiments where we have measured H_2O_2 release with the complex 1 substrates, glutamate and malate, ATP still reduces baseline release of H_2O_2 by about 27% and leads to modest suppression of ROS release in the presence of carboxyatractyloside. Thus, the suppression of ROS release by ATP does not appear to be specific to the use of succinate as a metabolite.

The results with succinate and rotenone suggest that the acceleration of state 3 respiration by ATP was mediated primarily through an action on the FAD-dependent complex 2 since the acceleration remained significant and was not different from that observed with succinate alone. Furthermore, the use of rotenone did not block the effect of ATP to improve RCR. In contrast, the results with the NAD-linked substrates, glutamate and malate, indicated, at best, a small effect of ATP on complex 1 since the acceleration of state 3 with these substrates was not statistically significant. Thus the cyclic AMP phosphorylation pathway that increases the respiratory activity by the stimulation of complex 1 (Papa *et al*., 2002) appears to play a small or no role in our observations. Nonetheless, the acceleration of the baseline rate of respiration occurred regardless of substrate in both Percoll fractions 2 and 3.

In summary, these results indicate that investigations of lung mitochondria obtained by differential centrifugation alone need to account for significant effects of nonmitochondrial ATPases. On the other hand, the results obtained from the Percoll fractions that were enriched with mitochondria suggest that, in the lung, these organelles have important and unique ATP-dependent processes that are independent of the F0, F1-ATPase. These processes oppose mitochondrial swelling, improve mitochondrial function as evidenced by the ATP-mediated increases in RCR and membrane potential, and may act within the intramembrane space to reduce the ROS production as evidenced by the results in the presence of carboxyatractyloside. ATP also acts at complex 2 to increase state 3 respiration. We suggest that, as a result of these ATP-dependent mechanisms, lung mitochondria hydrolyze enough newly synthesized ATP to ADP to support the state 3 consumption of oxygen. A potential benefit of this apparent inefficiency is a sustained reduction in the mitochondrial production of ROS. The physiologic importance of these properties of lung mitochondria and

their relation to pulmonary dysfunction in shock, sepsis, or other disease states warrant further investigation.

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