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Pulmonary O_2 Toxicity: Energy Metabolism and Data Analysis (40828)

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Changes in enzyme activity and oxidative phosphorylation during CNS O_2 toxicity (1–5) and pulmonary O_2 toxicity (6–8) have been reported using homogenates and mitochondrial preparations. Reports by Masoro and Porter (9), Smith and Fairhurst (10), and Patkin and Masoro (11) on liver from cold acclimatized rats have shown that lowered *P*/O ratios and decreased efficiency of respiration in homogenates returned toward normal controls after successive washings in mitochondrial preparations. Extramitochondrial regulatory factors which were present in homogenates from the cold acclimatized rats were lost in the mitochondrial isolation steps. They concluded that tissue homogenates more nearly reflect *in vivo* oxidative phosphorylation, since homogenates from the exposed rats contained extramitochondrial regulatory factors which were not present in isolated mitochondrial preparations.

In studies of pulmonary O_2 toxicity methods of expressing results using tissue homogenates or mitochondria have included:

microliters	O_2/gram (wet) lung per minute,
microliters	O_2/milligram DNA per minute,
microliters	O_2/lung per minute (or μl O_2/right lung per min or μl O_2/left lung per min).
microliters	O_2/milligram mitochondrial protein per minute, or micromoles ATP/milligram (wet wt), etc.

In studies of hyperoxia and lung respiratory activity, difficulties arise with the selection of the best means of expressing results. This paper reports the comparison of lung homogenate vs mitochondrial preparations in the study of lung respiration in pulmonary O_2 toxicity and efforts to explain the importance of the manner in which results are expressed.

Methods. Male Sprague–Dawley rats (150–200 g) were used in all experiments. Groups of animals received sham exposures (controls), 1.5 atm absolute pressure (ATA) 100% O_2 exposures for 24, 27, and 30 hr, and 1.0 ATA O_2 exposures for 1, 2, 3, 4, and 6 days. During exposures standard laboratory rat chow and water were available *ad lib*. Soda lime was placed in containers along the sides and ends of cages for CO_2 absorption. Oxygen flow through the chamber was maintained at 1 liter/min per animal. In sham exposures, air was used instead of 100% O_2. Effluent gas from the chamber had relative humidity levels ranging from 30 to 40%, and pCO_2 levels of 1 to 4 mm Hg. Temperature in the chamber was maintained at 22 ± 1°C. The cages and separators were constructed from 3/8 in. square metal cloth which permitted the free flow of O_2 (or air) throughout the chamber. In the oxygen exposures, the chambers were flushed with 100% O_2 with a volume of 20× the chamber volume prior to compression to 1.5 ATA O_2, or to being considered at 1.0 ATA O_2 (ambient pressure).

At the end of the 1.5 ATA O_2 exposure the chamber was decompressed over a 2-min interval. Each rat was rapidly removed, decapitated, the lungs removed, blotted, weighed, minced, and 5 ml of homogenizing medium (0.32 *M* sucrose, 0.001 *M* EDTA) added per gram of lung. The lung tissue was homogenized with a Potter–Elvehjem homogenizer, and 1 ml of homogenate was removed for respiratory studies. The remaining homogenate was used to isolate mitochondria by the method of Reiss (12), and the mitochondria were suspended in homogenizing medium to a volume of 1.5 ml per gram lung. An aliquot of the mitochondrial suspension was taken to determine the milligrams mitochondrial protein per gram of lung by the method of Jacobs *et al.* (13). The remaining lung mitochondrial suspension was used for studies

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on respiration and oxidative phosphorylation. Respiration studies were performed on 0.2 ml of homogenate (or mitochondria) added to 2.4 ml reaction medium (0.225 *M* sucrose, 0.01 *M* K_2HPO_4, 0.02 *M* KCl, and 0.02 *M* triethanolamine), 0.1 ml substrate (0.3 *M* α-ketoglutarate or 0.45 *M* succinate) in a temperature regulated (37 ± 0.1°) reaction vessel with a Clark oxygen electrode to record O_2 consumption by the method of Chance and Williams (14). When a basal rate was established 0.5 μmol of adenosine diphosphate (ADP) was added for determination of the maximum (ADP stimulated) respiration rate (State 3). After the exogeneous ADP had been utilized, the oxygen consumption rate returned to the basal rate (State 4) which was used in the study. From O_2 consumption curves the basal respiration rate (State 4) and the maximum respiration rate (State 3) were determined on lung homogenates and mitochondrial preparations. Lung ATP determinations were made on aliquots of frozen, pulverized lung by the firefly luminescence method (15–18). Excised lung was frozen in liquid N_2 within 10 sec of opening the thoracic cavity.

Results. The respiratory activities (States 3 and 4) and respiratory control ratios (RCR) for homogenates and for isolated mitochondrial suspensions are shown for comparison in Tables Ia and Ib, respectively. Note that the State 3 and State 4 respiration rates for the isolated mitochondria for the same lung tissue mass are markedly lower than homogenate respiration rates. This is considered to be due to losses in the mitochondrial isolation procedure. To determine the mitochondrial percentage recovery, enzyme assay techniques using the respiration rates (States 3 and 4) for succinate dehydrogenase and α-ketoglutarate dehydrogenase activities were determined on control animal lung homogenates and lung mitochondrial suspensions. It is assumed that in the controls optimal conditions exist for assays of respiration of lung homogenates and isolated lung mitochondria, i.e., there has been no inhibitory factor to respiration induced by experimental conditions imposed upon the animal. The mitochondrial percentage recovery was obtained from the ratio (mitochondria State 3 rate/homogenate State 3 rate) × 100%, and the ratio (mitochondria State 4 rate/homogenate State 4 rate) × 100%, for the two enzymes, for State 3 and State 4 rates, and for each animal in the control group. These data from State 3 and State 4 comparisons, and the 2 different enzymes which were as-

TABLE Ia. RESPIRATION RATES FOR HOMOGENATES OF LUNG FROM CONTROL RATS AND RATS EXPOSED TO 1.5 ATA O_2

1.5 ATA O_2 Exposure (hr)[a]	Microliters O_2/milligram (wet) lung per hour					
	State 4	Percentage of control	State 3	Percentage of control	R.C.R.[b]	*n*
			Succinate			
24	0.50 ± 0.04[c]	72.5	0.86 ± 0.11	56.2	1.71 ± 0.13	7
27	0.42 ± 0.04	60.8	0.72 ± 0.02	47.1	1.71 ± 0.09	7
30	0.39 ± 0.03	56.5	0.65 ± 0.05	42.5	1.69 ± 0.16	7
Control	0.69 ± 0.06		1.53 ± 0.08		2.22 ± 0.16	13
			α-Ketoglutarate			
24	0.39 ± 0.05	84.7	0.55 ± 0.03	64.7	1.40 ± 0.06	7
27	0.36 ± 0.01	78.3	0.46 ± 0.01	54.1	1.29 ± 0.04	7
30	0.32 ± 0.02	69.5	0.34 ± 0.20	40.0	1.06 ± 0.08	7
Control	0.46 ± 0.04		0.85 ± 0.56		1.84 ± 0.10	13

[a] $P < 0.01$ for all exposures to 1.5 ATA O_2. Probability of being from population by Student t test for 2 group comparison.

[b] R.C.R. = respiratory control ratio.

[c] Mean ± SEM.

TABLE Ib. RESPIRATION RATES FOR MITOCHONDRIA OF LUNG FROM CONTROL RATS AND RATS EXPOSED TO 1.5 ATA O_2

1.5 ATA O_2 Exposure (hr)[a]	Microliters O_2/milligram (wet) lung per hour					
	State 4	Percentage of control	State 3	Percentage of Control	R.C.R.[b]	*n*
			Succinate			
24	0.14 ± 0.01[c]	82.4	0.23 ± 0.02	69.6	1.57 ± 0.37	7
27	0.14 ± 0.02	60.8	0.20 ± 0.03	60.6	1.42 ± 0.22	7
30	0.13 ± 0.01	56.5	0.18 ± 0.02	54.5	1.41 ± 0.07	7
Control	0.17 ± 0.02		0.33 ± 0.02		1.94 ± 0.17	13
			α-Ketoglutarate			
24	0.115 ± 0.01	104.3	0.14 ± 0.02	82.4	1.32 ± 0.07	7
27	0.086 ± 0.005	78.2	0.11 ± 0.005	64.7	1.39 ± 0.06	7
30	0.078 ± 0.01	70.9	0.10 ± 0.01	58.8	1.37 ± 0.06	7
Control	0.11 ± 0.01		0.17 ± 0.02		1.64 ± 0.07	13

[a] $P < 0.01$ for all exposures to 1.5 ATA O_2. Probability of being from population by Student's *t* test for 2 group comparison.
[b] R.C.R. = Respiratory control ratio.
[c] Mean ± SEM.

sayed, were pooled to give a mitochondrial yield of 22.4 ± 0.3% (mean ± SEM) for the 13 control animals.

The mitochondrial protein per gram of lung for controls was 4.21 ± 0.15 (mean ± SEM) for the 13 control animals. The weights of the right lung of controls and hyperoxic exposed animals are shown in Table II along with mitochondrial protein per gram of lung. The respiration rates (State 3) of lung from animals exposed to 1.0 ATA O_2 are expressed per milligram mitochondrial protein, per gram wet weight, and per right lung and shown in Table III. The ATP levels of lung from animals exposed to 1.0 and 1.5 ATA O_2 are shown in Table IV.

Discussion. The lung respiratory activity is decreased to a greater degree in homogenates than in isolated mitochondria, as

TABLE II. MITOCHONDRIAL PROTEIN/GRAM LUNG AND LUNG WEIGHTS FOR CONTROLS AND ANIMALS EXPOSED TO 1.0 AND 1.5 ATA O_2

	Milligrams mitochondrial protein/gram lung	*n*[a]	Average weight of rt lung
1.0 ATA O_2			
1 day	4.26 ± 0.08[b]	9	0.645 ± 0.10
			P = NS[c]
2 day	4.26 ± 0.08	11	0.680 ± 0.13
			P = NS
3 day	4.08 ± 0.08	12	0.909 ± 0.10
4 day	4.30 ± 0.11	10	1.43 ± 0.14
6 day	4.47 ± 0.09	10	1.25 ± 0.11
1.5 ATA O_2			0.643 ± 0.07
24 hr	4.16 ± 0.14	7	P = NS
27 hr	4.03 ± 0.18	7	0.645 ± 0.08
			P = NS
30 hr	4.37 ± 0.20	7	0.642 ± 0.10
			P = NS
Control	4.21 ± 0.15	13	0.636 ± 0.10

[a] *n* = number of animals.
[b] Mean ± SEM.
[c] $P < 0.01$ unless shown. Probability of being from control population by Student's *t* test for two-group comparison. NS = not significant.

TABLE III. RESPIRATION RATES FOR LUNG HOMOGENATES FROM RATS EXPOSED TO 1.0 ATA O_2 α-KETOGLUTARATE

1.0 ATA O_2 Exposure (days)	Microliters O_2/milligram mitochondrial protein per min (State 3)	Percentage of control	n[a]	Microliters O_2/right lung per min (State 3)	Percentage of control	Microliters O_2/gram (wet) lung per min (State 3)	Percentage of control
1	1.84 ± 0.14[b]	63.7	12	5.06 ± 0.38	65.4	7.84 ± 0.60	64.4
2	1.84 ± 0.10	52.9	12	4.43 ± 0.29	57.2	7.84 ± 0.43	64.4
3	1.58 ± 0.13	54.7	12	5.86 ± 0.48	75.7	6.45 ± 0.53	53.0
4	1.59 ± 0.13	55	8	9.78 ± 0.51	126.4	6.84 ± 0.56	56.2
6	1.13 ± 1.17	39.1	8	6.31 ± 0.95	81.5	5.05 ± 0.76	41.5
Control	2.89 ± 0.12		16	P = NS 7.74 ± 0.32		12.17 ± 0.03	

[a] number of animals.
[b] Mean ± SEM.
[c] $P < 0.01$ unless shown. Probability of being from control population by Student's t test for two-group comparison. NS = not significant.

shown in Tables Ia and Ib for 1.5 ATA O_2 exposures. The maximum rate (State 3) is decreased using both succinate and α-ketoglutarate as substrate for isolated mitochondrial and lung homogenates, with homogenates being affected greater than isolated mitochondria. The FAD-linked substrate (succinate) lung respiration was not affected as severely as the NAD-linked substrate (α-ketogluatrate) by exposure of the rat to 1.5 ATA O_2. The NAD-linked substrate (α-ketoglutarate) respiration was inhibited greater in homogenates than in mitochondria as reflected in the respiratory control ratios at 30 hr exposure to 1.5 ATA O_2 for homogenates (1.06) when compared to mitochondria (1.37). Thus, lung homogenates after exposure to 1.5 ATA O_2 appear to contain extra mitochondrial factors inhibitory to lung respiration which were lost in the mitochondrial isolation procedure. These studies are consonant with the conclusions of Patkin and Masoro (11) and Masoro and Porter (9) that tissue homogenates more nearly reflect *in vivo* respiration and oxidative phosphorylation, and should be used in preference to isolated mitochondrial suspensions to evaluate effects of stress (hyperoxia, etc.) on lung.

When pulmonary edema is a factor it is possible that expressing results in units per milligram wet weight or per milligram dry weight could be misleading. There is a similar problem in expressing data in units per milligram DNA, since 1 ATA O_2 exposures have been shown by Gail and Masoro (8) to decrease DNA with respect to controls at 48 hr, but to increase DNA with respect to controls at the 96 hr exposure to 1.0 ATA O_2. Both exposure times showed edema by lung weight. Block (10) has reported increased DNA content per gram of lung in hyperoxic exposures. These studies indicate lung metabolic changes occurring in addition to increased H_2O contents. Thus expressing results in terms of the organelle involved in the activity being measured, allows a more clear interpretation than when results are expressed per milligram DNA.

In the 1.5 ATA O_2 exposures, a consistent interpretation results when the data are expressed as microliters O_2/lung per min-

TABLE IV. ATP CONCENTRATION OF RAT LUNG DURING 1 ATA O_2 EXPOSURE

	Micromoles ATP/mg mitochon. protein	Micromoles ATP/ right lung	Micromoles ATP/ gram (wet) lung	n[a]
1 ATA O_2				
1 day	0.25 ± 0.012[b]	0.72 ± 0.030	1.07 ± 0.050	10
2 day	0.24 ± 0.006	0.69 ± 0.017	1.02 ± 0.026	10
3 day	0.26 ± 0.003	0.95 ± 0.011	1.04 ± 0.012	18
4 day	0.23 ± 0.008	P = NS[c]	0.98 ± 0.034	10
6 day	0.22 ± 0.009	1.41 ± 0.049	1.00 ± 0.040	10
1.5 ATA O_2		1.25 ± 0.050		
24 hr	0.24 ± 0.14	—	—	11
27 hr	0.19 ± 0.005	—	—	11
30 hr	0.156 ± 0.001	—	—	7
Control	0.304 ± 0.006	0.814 ± 0.016	1.28 ± 0.025	49

[a] n = number of rats
[b] Mean ± SEM.
[c] $P < 0.01$ unless shown. Probability of being from control population by Student's t test for two group comparison. NS = not significant.

ute, microliters O_2/right lung per minute or microliters O_2/milligram mitochondrial protein per minute. Thus when there is no pulmonary edema, the three means of expressing the data will give the same relative changes. The weight of the right lung did not vary significantly for all 1.5 ATA O_2 exposures (Table II.)

Maximum respiration rates (State 3) of lung from rats exposed to 1.0 ATA O_2, as shown in Table III, showed a significant decrease down to 55 and 39.1% at Days 4 and 6, respectively, (μl O_2/mg mitochondrial protein per min) when right lung weight had increased by 224.8% (Day 4) and 196.5% (Day 6) of controls. Decreased respiratory rate was followed by decreased ATP levels/mg mitochondrial protein (Table IV) from the lung of rats exposed to 1.0 ATA O_2. The decreased ATP levels preceded lung edema as indicated by the increased weight of the right lung. The lung ATP levels from animals exposed to 1.0 ATA exposures decreased to 82.2% of control within 24 hr, and varied between 72.4 and 85.5% of control between Days 2 and 6 (Table IV). Even though the respiration rates for lung homogenates decreased during this interval, the ATP concentration remained in the range of 72.4 to 85.5% of control levels. This implies that even with the edema and other factors changing in lung tissue over this interval there was a compensatory decrease in ATP utilization by the lung, conceivably in effort to sustain cell life. In contrast when animals were exposed to 1.5 ATA O_2 for 24, 27, and 30 hr, the lung ATP concentration decreased to 78.9, 62.5, and 51.3% of control values. Thus, with the 1.5 ATA O_2 exposures the lung was unable to compensate to prevent the decrease in lung ATP concentration with the increased stress of the higher O_2 exposure level.

When respiratory rates are expressed per right lung the weight correction factor from the increased weight of the edematous lung results in right lung respiratory rates greater than control values. Similarly, when ATP concentration is expressed per right lung the level increases to greater than control values. Thus, to express results in terms of whole lung or right lung under edematous conditions can be misleading. However, when the respiration rates are expressed per unit of the functioning organelle, e.g., per milligram mitochondrial protein, the decreased respiratory activity of the 1.0 ATA O_2 lung mitochondria is seen. This decreased respiratory activity is consistent with the observed decreased ATP, Na^+, and H_2O intracellular influx, and observed lung edema.

When the respiratory activity or ATP levels of a normal 1.0-g lung and a normal 1.5-g lung are compared per gram lung, or

per milligram mitochondrial protein, they are not statistically significantly different. However, when the respiratory activities or ATP levels per total lung are compared, the 1.5-g lung will show respiratory activity 150% of that of the 1-g lung. Thus, when comparing lungs of increased weight from hyperoxic exposed animals to controls it is necessary that a weight correction factor (the relative increase in lung weight) be used if a comparison of respiratory activities, or ATP concentration, is to be made. Otherwise, if you express activity per total lung and expect a direct comparison of a 1.0- and 1.5-g lung to be equal, it would require that respiratory activity and ATP concentration per milligram mitochondrial protein or per g lung vary inversely as the weight of the lung. This does not occur. Results normalized as described above are consistent with results obtained at 1.5 ATA O_2 when edema does not occur.

The 1.0 and 1.5 ATA O_2 exposures resulted in levels of isolated mitochondria per gram of lung not significantly different from controls even when significant edema was observed on Days 3, 4, and 6 of the 1.0 ATA O_2 exposures. Thus, with edema there was a compensatory increase in mitochondrial protein in an effort to keep up with the increased energy production required to maintain homeostasis in the increased tissue mass. The decreased lung respiration rates indicate significant changes in the mitochondria in spite of the increase in total mitochondrial protein.

Respiration rates and ATP levels in pulmonary O_2 toxicity when expressed as microliters O_2/milligram mitochondrial protein per minute, and micromoles ATP/milligram mitochondrial protein, permit evaluation of hyperoxic damage to respiration and oxidative phosphorylation and changes in ATP levels. This is also more logical physiologically, since both are related to mitochondrial function.

Summary. Comparison of homogenates vs mitochondrial preparations in evaluating *in vivo* respiration and oxidative phosphorylation in lung from rats exposed to 1.5 ATA O_2 for 24, 27, and 30 hr showed that homogenates from experimentally exposed rats may contain extramitochondrial factors inhibitory to respiration which are washed out in mitochondrial isolation procedures. Thus, the use of homogenates is preferable to isolated mitochondria, since they more nearly reflect *in vivo* oxidative phosphorylation in pulmonary oxygen toxicity studies.

Intercomparison of methods of expressing lung respiration rates and ATP concentration were made in lung of rat subjected to 1.0 and 1.5 ATA O_2 exposures. Care should be exercised in selecting the means for expressing data. Our results indicate that the preferred method for expressing respiration rates is microliters O_2/milligram mitochondrial protein per minute, and for expressing ATP levels is micromoles ATP/milligram mitochondrial protein, in pulmonary O_2 toxicity studies. Evaluation of metabolic activity requires that accurate determinations of the total lung (or individual lung) weight, and some unit of the lung (preferably the organelle responsible for the activity being studied) be determined, e.g., milligram mitochondrial protein per gram lung.

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