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Research Article

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Lamellar Body Depletion in Dogs Undergoing Pulmonary Artery Occlusion

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ABSTRACT We have investigated the relationship between pulmonary artery occlusion (PAO) and the surfactant system of the lung by studying the ultrastructural responses of type II alveolar pneumocytes to PAO of 4–12 h duration in 16 mongrel dogs. In six of these animals, the occluded lung was allowed to reperfuse for 6 h before killing and in four animals subjected to PAO of 4 h duration, the occluded lung was ventilated with 5% CO_2 balance air. PAO by itself resulted in a dramatic 80% reduction in the volumetric density of lamellar bodies (LB) in the type II cells. This resulted predominantly from a decrease in volume of the individual LB. Although reperfusion was associated with an increase in LB volume density toward normal, 6 h of reperfusion was insufficient to re-establish normal type II cellular morphology. Ventilation of the occluded lung with 5% CO_2 prevented LB depletion indicating that alveolar CO_2 tension may affect the release and/or synthesis of LB in type II pneumocytes.

INTRODUCTION

Unilateral pulmonary artery occlusion initiates a sequence of physiological and biochemical events that result in the development of focal hemorrhage, edema, and atelectasis in the occluded lung (1, 2). Although these morphological changes are generally reversible over a period of several months, during which time a marked increase in the bronchial circulation occurs (3), the precise mechanisms responsible for producing the injury remain to be clarified. It is well established that acute pulmonary artery occlusion produces a severe reduction in the alveolar carbon dioxide tension in the occluded lung, which directly initiates the contraction of airway smooth muscle and leads to a reduction in lung volume and a shift in ventilation away from the occluded lung (4–7). These changes in airway mechanics are initially reversible with the administration of supplemental CO_2 to the inspired gas (5, 7). As the duration of occlusion increases, the addition of CO_2 to the inspired gas is no longer capable of reversing the decrease in compliance and ventilation to the occluded lung (8). This irreversibility coincides with the development of atelectasis, hemorrhage, and edema in the occluded lung, which in turn has been attributed to loss of the alveolar surface lining material, surfactant (8–10). Although the ability of pulmonary surfactant to reduce surface tension is decreased after pulmonary artery occlusion, it remains unclear whether this alteration in function contributes to the development or results as a consequence of the alveolar edema and hemorrhage that occur (8, 11–13). If pulmonary surfactant is actually quantitatively depleted after pulmonary artery occlusion, as suggested by Morgan and Edmunds (14), then type II alveolar pneumocytes might show depletion of their lamellar bodies (LB)[1], which are recognized as the intracellular storage sites of the surface active phospholipids. To test this hypothesis we have used electron microscopy to evaluate ultrastructural changes in type II pneumocytes during pulmonary artery occlusion and reperfusion in dogs. Because previous research has indicated that synthesis of phospholipids and type II cellular morphology may be altered by changes in pH, and/or CO_2 tensions (15–20), the role that alveolar hypocapnia might play in mediating changes in type II cellular ultrastructure after pulmonary artery occlusion was also investigated.

METHODS

16 mongrel dogs weighing 16–28 kg were anesthetized intravenously with sodium pentobarbital (28 mg/kg body wt) and intubated with a modified Carlen's bronchospirometric catheter to permit independent ventilation of the right and

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[1] *Abbreviations used in this paper*: LB, lamellar body; PAO, pulmonary artery occlusion.

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left lungs. A dual cylinder Harvard animal ventilator (Harvard Apparatus Co., Inc., S. Natick, Mass.) was used to deliver a tidal volume of 15 ml/kg body wt with 45 and 55% of this volume being delivered to the left and right lungs, respectively. Respiratory rate was adjusted to maintain a $PaCO_2$ of 35±5 mm Hg and the lungs were hyperinflated to 45 ml/kg body wt every 15 min. All animals were subjected to left pulmonary artery occlusion by fluoroscopically positioning a double lumen Dotter-Lukas catheter (C. R. Bard, Inc., Billerica, Mass.) in the left pulmonary artery and inflating the balloon with 4–9 ml of 0.9% saline. Occlusion was considered complete when end-tidal CO_2 concentration on the occluded side fell to <0.5% as monitored with a Godart model 146 CO_2 analyzer (Godart-Statham, Bilthoven, Holland). The adequacy of balloon occlusion in the group whose occluded lungs were ventilated with 5% CO_2 was assessed by intravenously injecting 1 mCi of ^{99m}Tc-labeled human albumin macroaggregates (Union Carbide Corp., Medical Products Div., Tuxedo, N. Y.) before sacrifice and imaging the excised occluded lung with a Searle Pho-Gamma II (Anger) scintillation camera (Searle Radiographics Inc., Des Plaines, Ill.). The radioactivity of the left lung was no greater than background in all four animals and, therefore, balloon occlusion was considered complete. The adequacy of bronchospirometric division was determined by ventilating one lung with 100% oxygen, and monitoring expired nitrogen tension from the contralateral lung with an Ohio model 700 nitrogen analyzer (Ohio Scientific, Inc., Aurora, Ohio). If no decrease in expired nitrogen concentration occurred on the contralateral side, division was considered complete.

Three groups of animals were studied: occlusion, reperfusion, and occlusion plus 5% CO_2. The six animals in the occlusion group were studied upon completion of pulmonary artery occlusion (PAO) of 4, 8, and 12 h duration in pairs. The six animals in the reperfusion group were similarly subjected to occlusive intervals of 4, 8, and 12 h in pairs. However, at the end of the occlusive interval, the balloon was deflated allowing the left lung to reperfuse for 6 h in each animal. The occlusion plus 5% CO_2 group consisted of four animals all subjected to 4 h of PAO, during which time the occluded lung was ventilated selectively with 5% CO_2 balance air.

Protocol for tissue preparation. All animals were given sodium heparin 2,000 U i.v. just before killing with sodium pentobarbital. The chest was opened rapidly, the lungs removed and the right and left lower lobes perfused via the airway with 2% glutaraldehyde in 0.1 M sodium phosphate buffer at 25 cm water pressure. The tissue was fixed for 24 h at room temperature after which time, five 1-mm^3 blocks were cut from each of four randomly selected areas from each lower lobe. The blocks were then postfixed in 1% osmium tetroxide for 2 h at 4°C, dehydrated in acetone and embedded in Luft's Epon (Shell Chemical Co., Houston, Tex.). One block from each of the four areas in each lower lobe was then thin-sectioned on a Porter-Bloom ultramicrotome with a diamond knife. These sections were mounted on copper grids, stained with uranyl acetate, lead citrate and 30–48 type II pneumocytes photographed from each lower lobe with a Philips 200 electron microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.).

Morphometric analysis. All type II pneumocytes were printed at a final magnification of 15,000–20,000 and a 9864A digitizer (Hewlett Packard Co. Palo Alto Calif.) linked to a model 9810 calculator was used to measure planimetrically the cross-sectional surface areas of the cells, their nuclei, and their individual LB. We derived the cytoplasmic volumetric density of the LB fraction, Eq. 1, as the ratio of the sum of the cross-sectional areas of the individual LB divided by the cross-sectional cytoplasmic area (A) where $A_{cyto} = A_{cell} - A_{nucl}$:

$$V_v(\text{LB/cyto}) = \frac{\sum_{n} A_{LB_n}}{A_{cyto}}. \quad (1)$$

Weibel has shown that the volume of a given structure is related to its mean cross-sectional area, A, by $V = \beta \cdot A^{3/2}$, where β is a coefficient determined by the structure's shape (21). Based on the assumption that PAO does not change the shape of type II cells or their LB, we have computed and expressed the volume of the type II cells and their LB from the occluded lung (occl) as a percentage of their volumes (V) in the nonoccluded lung (nonoccl) as follows:

$$\%\left(\frac{\text{V occl}}{\text{V nonoccl}}\right) = \left(\frac{\text{A occl}}{\text{A nonoccl}}\right)^{3/2}(100). \quad (2)$$

To ascertain whether or not LB shape was significantly altered by occlusion we measured the minor to major axis ratios of 120 randomly selected LB from each experimental group. The shape of type II pneumocytes was evaluated qualitatively by observing over 100 cells from each group with a JEOL JSM-35 scanning electron microscope (JEOL, Tokyo, Japan).

Statistical analysis. To determine if duration of occlusion affected the cell measurements 2 × 3 × 2 analyses of variance for a mixed design were conducted. All of the analyses were conducted on a computer with the BMDP2V statistical program (22). In each analysis, the between subject variables were type of occlusion group (occlusion vs. reperfusion group) and duration of occlusion (4 vs. 8 vs. 12 h). The within subject variable was the lung side (occluded vs. nonoccluded side). There were no effects that included the duration of occlusion variable, therefore, the data have been averaged over time within each group and the nonpaired *t* test employed to examine group differences.

RESULTS

Occlusion group. Table I presents the morphometric data for the type II cells from the occluded and nonoccluded control lungs of the six animals studied. Although no differences in Type II cellular or nuclear areas were detected, occlusion resulted in highly significant reductions in mean LB area (−65%), LB volume density (−80%), and the number of LB transected per cell (−53%).

Frequency histograms of the minor to major axis ratios for LB from the occluded and control lungs are shown in Fig. 1. The arithmetic mean axis ratio ($\bar{R}$) ±SD for the occluded lung $\bar{R} = 0.77 \pm 0.14$ was not different from the value obtained for the control lung; $\bar{R} = 0.74 \pm 0.15$. Qualitatively, no differences in the shape or surface morphology of type II pneumocytes from the occluded vs. the control lungs were detected by scanning electron microscopy. Although no significant change in cell volume occurred, a dramatic 78% reduction in LB volume, $P < 0.001$, was observed, which would predominantly account for the 80% reduction in LB volume density (Fig. 2).

Reperfusion group. Although the mean nuclear cross-sectional area was slightly greater in the cells

TABLE I
Occlusion Group Data

Dog	Duration of occlusion	Mean cell area		Mean nuclear area		Mean LB area		Volumetric fraction percent		Number of LB on cross section	
		Occlusion	Control	Occlusion	Control	Occlusion	Control	Occlusion	Control	Occlusion	Control
	h	μm^2		μm^2		μm^2		*LB/Cyto*			
1	4	37.8	39.8	12.0	13.1	0.22	0.43	3.0	12.1	4.6	7.3
2	4	33.8	29.2	13.0	7.8	0.10	0.42	1.8	17.7	3.4	9.0
3	8	33.7	36.4	8.7	9.1	0.17	0.52	4.7	18.7	4.5	10.2
4	8	30.9	34.0	8.8	10.2	0.13	0.43	1.8	13.1	3.0	7.7
5	12	38.1	33.6	13.1	10.7	0.20	0.59	4.2	20.2	5.8	8.7
6	12	28.5	28.8	9.2	8.6	0.20	0.51	5.3	20.5	5.5	8.2
	Mean	33.8	33.6	10.8	9.9	0.17*	0.48	3.5*	17.1	4.5*	8.5
	SD	3.8	4.2	2.1	1.9	0.05	0.07	1.5	3.6	1.1	1.0
	CV	0.11	0.13	0.19	0.19	0.29	0.15	0.43	0.21	0.24	0.12

CV, cell volume.
* $P < 0.001$.

from the occluded-reperfused lungs, no significant differences in cellular or cytoplasmic areas were found (Table II). Highly significant reductions in mean LB area (−58%) and LB volume density (−62%) were again observed. However, the average number of LB observed per transected cell was not significantly decreased.

That occlusion followed by reperfusion had no major impact on the shape of LB can again be deduced from the similarities in the frequency histograms for the experimental and control lungs (Fig. 1), where shape is indexed by the minor to major LB axis ratio. The arithmetic mean axis ratio for the occluded-reperfused lungs ($\bar{R} = 0.81 \pm 0.11$) did not differ from the value of $\bar{R} = 0.82 \pm 0.10$ for the control lungs. As for the occlusion group, scanning electron microscopy revealed no quantitative differences in the shape or surface morphology of type II pneumocytes between the occluded-reperfused and control lungs.

Although no significant change in cell volume was observed, a severe 70% reduction in individual LB volume was observed; again predominantly accounting for the decrease in LB volume density, Fig. 2.

Comparing the morphological changes in the type II cells from the occluded-reperfused lungs with those subjected to occlusion alone (Fig. 2), we found that reperfusion significantly increased the LB volumetric fraction of the type II cells, whereas cell volume and individual LB volume were not changed. No differences in type II cellular morphometry were observed between the cells from the control lungs of the occlusion and reperfusion groups.

Occlusion plus CO_2 group. No differences in type II cellular morphometry were found between the occluded lungs ventilated with 5% CO_2 and the non-occluded control lungs (Table III, Figs. 1 and 2).

DISCUSSION

Since Charles Macklin's original hypothesis in 1946, considerable evidence has accumulated indicating that type II alveolar pneumocytes are responsible for the synthesis, storage, and excretion of surface active phospholipids in the mammalian lung (23–26). Whereas immaturity of the phospholipid biosynthetic activity of these cells has been linked with the pathogenesis of the respiratory distress syndrome of the newborn (27–29), no pathophysiological link has been definitively established between type II cellular dysfunction and diseases affecting mature lungs. However, PAO is one disease process in which damage to the surfactant system has been purported to play a significant pathophysiological role.

In 1960, Finley et al. reported that extracts of canine lungs subjected to PAO had a decreased ability to reduce surface tension (8). Although this observation was confirmed by other investigators (9, 10, 30), it subsequently has been shown that blood or plasma also impairs the surface tension lowering capabilities of normal lung extracts (11, 12). Subsequently, deflation pressure volume curves, alveolar bubble stability, and phospholipid contents were shown to be normal in morphologically normal regions of dog lungs with PAO (13, 14). However, in regions of the occluded lung manifesting typical focal morphological abnormalities of alveolar hemorrhage, edema, and atelectasis, the phospholipid content as well as bubble stability were decreased (14). As a result, it remains unsettled

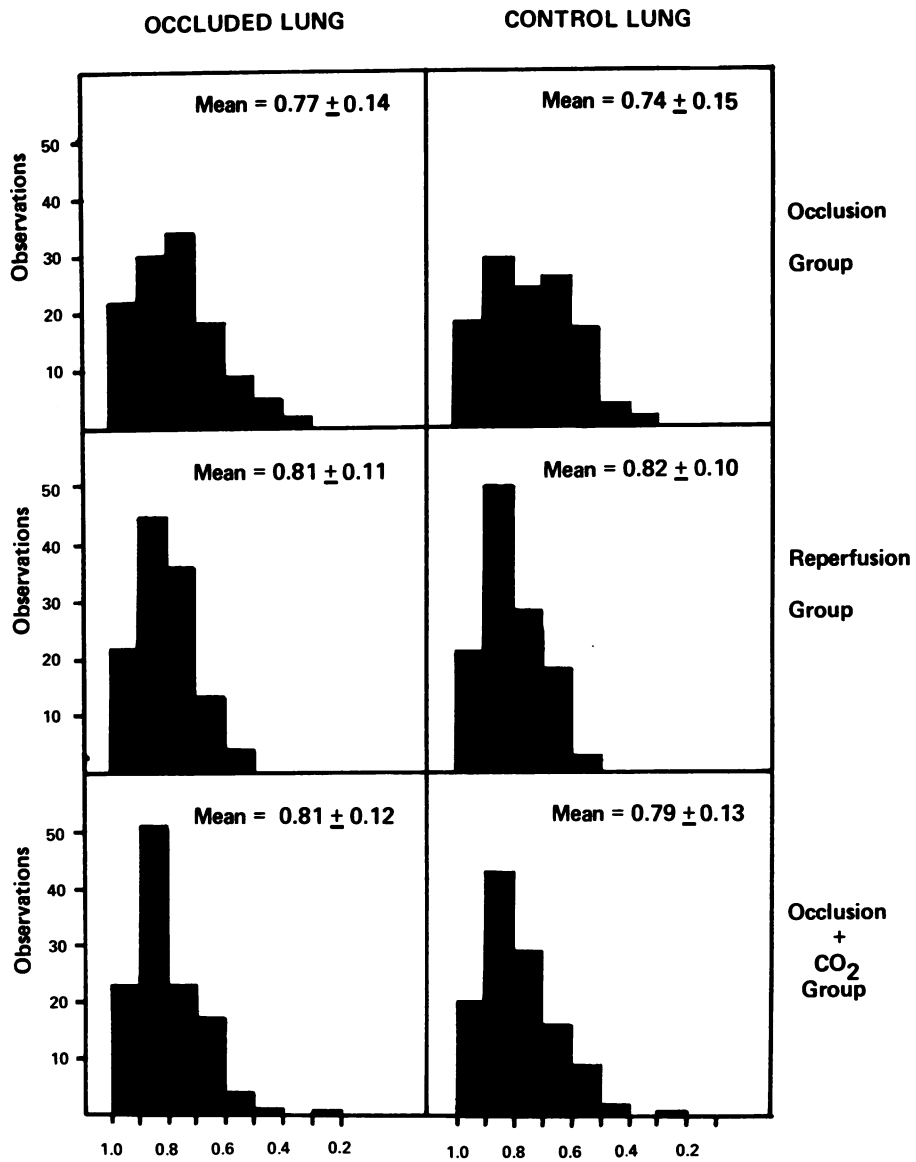

FIGURE 1 Frequency histograms for the minor to major LB axis ratios for the experimentally occluded and nonoccluded control lungs for the occlusion, reperfusion, and occlusion plus CO_2 groups studied.

whether PAO primarily damages the surfactant system resulting in atelectasis and edema or whether the observed dysfunction in surface tension lowering ability results from the contamination of the alveolar space with blood constituents.

Under our experimental conditions, PAO resulted in a dramatic decrease in LB volume density before the development of alveolar edema or hemorrhage. We believe that this decrease represents a true depletion in pulmonary LB content rather than a spurious decrease attributable to an increase in cell number, size, or change in shape for the following reasons: (*a*) The short duration of these experiments allowed insufficient time for cellular division to alter significantly total cell number. (*b*) The lack of differences in mean cell area coupled with similar coefficients of variation support an equality in cell volume and size distribution. (*c*) The scanning electron microscopic observations revealed no obvious alterations in cellular shape.

The decrease in LB volume density that we observed resulted predominantly from a decrease in the size of the individual LB as LB shape, indexed by the minor to major axis ratios, showed no appreciable change after PAO. The quantitatively similar reductions in LB

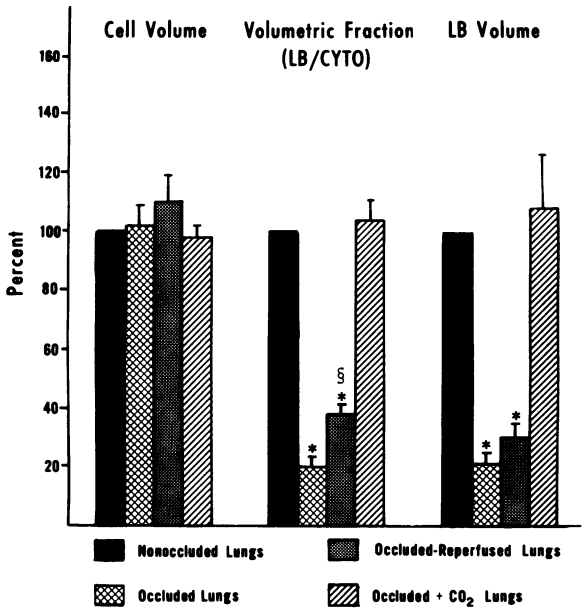

FIGURE 2 Changes in type II cell volume, LB volumetric fraction, and LB volume in the experimentally occluded lungs expressed as a percentage of the value for the nonoccluded control lungs indicated by the solid bars, which have been normalized to 100%. $^*P < 0.001$ vs. values for the nonoccluded lungs; $\S P < 0.05$ occluded-reperfused lung data vs. occluded lung data.

volume density (−80%) and individual LB volume (−79%) suggest that little change in LB numerical density occurred.

Reperfusion of 6 h duration was associated with a small but significant return in the ultrastructural composition of the type II pneumocytes toward control as assessed by the increase in LB volume density compared to occlusion. However, reperfusion of this duration was insufficient to return the morphological appearance of these cells to normal.

After making the initial observation of LB depletion, our investigation into the mechanisms responsible for regulating intracellular LB depletion was focused around two major physiological consequences of PAO: (*a*)cessation of blood flow with potential cellular deprivation of metabolic substrates for biosynthesis and (*b*) the possibility that alveolar hypocapnia would produce a sufficiently severe degree of extracellular alkalosis and/or bicarbonate depletion to induce dysfunction of cellular metabolism. Although substrate deprivation has been proposed as a potential etiological factor responsible for lung injury after PAO, direct evidence to support this hypothesis has been lacking.

In contrast, alveolar hypercapnia is known to play a significant role in mediating alterations in lung mechanics subsequent to PAO (4, 5, 7) and a reduction in CO_2 concentration has been shown to result in a lower rate of glucose incorporation into palmitate, phosphatidylglycerol, and phosphatidylcholine fractions of lung phospholipids (18, 19). Also, it is known that fatty acid synthesis is critically dependent on the reaction of bicarbonate with acetyl-CoA to produce malonyl-CoA which is the rate limiting step in *de novo* fatty acid synthesis (31). More recently, Merritt and Farrell (20) have shown that alkalosis as well as acidosis adversely affect the synthesis of [^{14}C]lecithin from [^{14}C]choline in fetal rat lung slices. They further documented an effect of pH on the specific activities of choline kinase, cytidyl transferase, and choline phosphotransferase, suggesting that pH induced alterations

TABLE II
Reperfusion Group Data

Dog	Duration of occlusion	Mean cell area		Mean nuclear area		Mean LB area		Volumetric fraction percent		Number of LB on cross section	
		Occlusion	Control	Occlusion	Control	Occlusion	Control	Occlusion	Control	Occlusion	Control
	h	μm^2		μm^2		μm^2		*LB/Cyto*			
7	4	39.5	34.5	10.1	9.0	0.22	0.70	6.1	19.5	8.7	8.0
8	4	35.3	39.1	11.5	8.9	0.21	0.47	5.7	12.8	6.4	9.0
9	8	35.9	35.9	7.4	7.3	0.24	0.67	7.6	21.7	9.8	10.2
10	8	36.8	39.9	10.2	8.6	0.20	0.63	5.3	18.3	7.1	9.7
11	12	40.2	31.3	10.8	7.7	0.30	0.50	6.3	13.7	6.4	6.7
12	12	37.2	34.0	10.4	8.2	0.27	0.44	7.7	16.8	7.7	9.8
	Mean	37.5	35.8	10.1*	8.3	0.24‡	0.57	6.5‡	17.1	7.7	8.9
	SD	2.0	3.3	1.4	0.7	0.04	0.11	1.0	3.4	1.4	1.3
	CV	0.05	0.09	0.14	0.08	0.17	0.19	0.15	0.20	0.18	0.15

CV, cell volume.
* $P < 0.05$.
‡ $P < 0.001$.

TABLE III
Occlusion Plus CO_2 Group Data

Dog	Duration of occlusion	Mean cell area		Mean nuclear area		Mean LB area		Volumetric fraction percent		Number of LB on cross section	
		Occlusion	Control	Occlusion	Control	Occlusion	Control	Occlusion	Control	Occlusion	Control
	h	μm^2		μm^2		μm^2		*LB/Cyto*			
13	4	36.8	38.0	9.3	9.2	0.58	0.43	19.9	16.9	10.1	11.6
14	4	29.7	31.7	9.4	9.4	0.48	0.50	19.5	21.3	8.4	9.7
15	4	37.0	35.5	9.6	8.6	0.48	0.61	12.2	13.0	7.2	6.0
16	4	35.7	36.5	6.4	8.3	0.59	0.55	22.9	20.1	11.9	10.1
	Mean	34.8	35.4	8.7	8.9	0.53	0.52	18.6	17.8	9.4	9.4
	SD	3.4	2.7	1.5	0.5	0.06	0.08	4.5	3.7	2.0	2.4
	CV	0.10	0.08	0.17	0.06	0.11	0.15	0.24	0.21	0.21	0.26

CV, cell volume.

in enzymatic function could alter the rates of phospholipid biosynthesis.

With these considerations in mind we prevented the development of alveolar hypocapnia by selectively ventilating the occluded lung with 5% CO_2 balance air. Our finding that the type II cells in the occluded, CO_2-supplemented lungs showed no evidence of LB depletion strongly suggests that alveolar CO_2 tensions play a major role in LB depletion. Whether this effect is mediated directly by the low alveolar CO_2 tension or is secondary to changes in hydrogen ion or bicarbonate concentrations remains undetermined.

Although ultrastructural observations within the initial 24 h after PAO have not been published, Balis et al. (32) have reported that dog lungs occluded from 1 to 5 d showed increased intra-alveolar myelin figures as an early change and later revealed "degenerative changes" in the type II cells located in areas of alveolar hemorrhage. 5 d after PAO in dogs, Huber and Edmunds (2) have reported qualitative changes in LB consisting of increased numbers of thinner and concentric lamellations. Of related interest are the reports of two groups studying the effects of total cardiopulmonary bypass on pulmonary ultrastructure in dogs. Balis et al. (33) reported that qualitatively, the LB appeared abnormally small but unchanged in number after 2 h of cardiopulmonary bypass. Whereas Sobonya et al. (34) found a quantitative reduction in the number of LB per cell in one dog bypassed for 2 h compared to a single control animal. Although cardiopulmonary bypass cannot be directly equated with PAO, both conditions act similarly in producing cessation of pulmonary arterial blood flow.

In summary, our data show that PAO has a definite impact on the LB content of type II pneumocytes. Because LB are considered the intracellular storage and secretory organelle for surface active phospholipids, PAO must be considered to have a definite effect on the surfactant system of the lung. Although we interpret LB depletion as reflecting intracellular depletion of phospholipids, these morphological observations alone do not establish any quantitative or qualitative dysfunction of the intralveolar surface lining layer, where the important physiological function of surface tension reduction is exerted. The changes in cellular LB content after 4 h of occlusion were clearly dependent on the alveolar CO_2 tension, suggesting the possibility that secondary alterations in hydrogen and/or bicarbonate ion concentrations may play important roles in regulating the LB content of type II cells and in mediating lung injury after PAO.

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