Bishai JM, Mitzner W. Effect of severe calorie restriction on the lung in two strains of mice. Am J Physiol Lung Cell Mol Physiol 295: L356–L362, 2008. First published May 30, 2008; doi:10.1152/ajplung.00514.2007.—There is a body of literature in animal models that has suggested the development of emphysema following severe calorie restriction. This has led to the notion of “nutritional emphysema” that might have relevance in COPD patients. There have been few studies, however, that have looked closely at both the mechanics and lung structure in the same animals. In the present work, we examined lung mechanics and histological changes in two strains of mice that have substantial differences in alveolar size, the C57BL/6 and C3H/HeJ strains. We quantified the dynamic elasticity and resistance at 2.5 Hz, the quasistatic pressure volume curve, and the alveolar chord lengths in lungs inflated to a lung capacity at 25–30 cmH2O. We found that after 2 or 3 wk of calorie restriction to 1/3 their normal diet, the lungs became stiffer with increased resistance. In addition, the lung capacity was also decreased. These mechanical changes were reversed after 2 wk on a normal ad libitum diet. Histology of the postmortem fixed lungs showed no changes in the mean alveolar chord lengths with calorie restriction. Although the baseline mechanics and alveolar size were quantitatively different in the two strains, both strains showed similar qualitative changes during the starvation and refeeding periods. Thus, in two strains of mice with genetically determined differences in alveolar size, neither the mechanics nor the histology show any evidence of emphysema-like changes with this severe caloric insult.

METHODS

Animal models. Two inbred mouse strains were used in this study, the C57BL/6 (B6) and the C3H/HeJ (C3) strains (Jackson Laboratories, Bar Harbor, ME). All animal work was approved by the Johns Hopkins Animal Care and Use Committee. Twenty-three 10- to 12-wk-old male mice from each strain were individually housed, and daily food consumption in each mouse was monitored for 2–3 wk. Once determined, one-third of this daily intake was calculated and administered to each mouse at the same time each day. Mice were subjected to calorie restriction (CR) for a period of 2 (n = 15) or 3 wk (n = 8). After this time, one-half of each group was used for pulmonary function testing and histological analysis, whereas the remaining mice were refed ad libitum chow for a duration of 2 wk (B6 n = 8, C3 n = 4). Control mice were fed ad libitum chow for the duration of the protocols with the CR and refed mice. All mice were allowed tap water ad libitum and were housed on a 12:12-h light-dark cycle at an ambient temperature of 21–22°C. To test for possible growth changes in control mice during the brief 2–3 wk of the experiment, we made measurements in four additional B6 and C3 mice at 11 wk. There were no significant differences in any variable between these mice and those in the control groups at the experimental time points. We also found no differences in pressure-volume (PV) curves or lung volumes with 2 or 3 wk of CR, and therefore, data from the two periods were combined for the CR group.

Dynamic resistance and elastance. To measure dynamic lung mechanics, animals were anesthetized with intraperitoneal injections of mixture of ketamine (65 mg/kg) and xylazine (13 mg/kg). Once sedated, a tracheostomy was performed, and a cannula (18G) was inserted. Each animal was ventilated (Flexivent; Scireq, Montreal, PQ, Canada) in the supine position with a tidal volume of 0.2 ml of 100% oxygen at a rate of 150 breaths/min, with a positive end-expiratory pressure of 2 cmH2O. A deep inspiration (to 30 cmH2O for 5 s) was given, and then the animal was returned to normal ventilation. Five minutes later, a sinusoidal oscillation at 2.5 Hz was applied to determine dynamic resistance (Rts) and elastance (Ers). Following the perturbation, the mouse was returned to normal ventilation for 1 min. This cycle was repeated twice, and the average values are reported.

Quasistatic PV curves. After determination of Rts and Ers, ventilation was stopped, and the tracheal cannula was occluded for 5 min, which led to complete degassing of the lungs by absorption atelectasis. Quasistatic PV curves were performed as previously reported (32). Briefly, air was infused with a syringe pump, and airway pressures were recorded. The data were corrected for the compliance of the circuit and analyzed using custom-written software.

Effect of severe calorie restriction on the lung in two strains of mice

John M. Bishai and Wayne Mitzner

Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland

Submitted 12 December 2007; accepted in final form 27 May 2008

There is a body of literature that has described emphysema-like changes in the lung after severe caloric restriction, a condition that has been referred to as nutritional emphysema (23). This concept has arisen from observations in both humans and animal models. There is anecdotal evidence suggesting that such changes in the lung may have occurred in residents of the Warsaw Ghetto during World War 2 (35), and there are also more recent studies in anorexic women showing a decreased lung computed tomography (CT) density (2, 19). Less severe levels of malnutrition have also been correlated with the severity and prognosis of COPD (20, 31). In addition to these human studies, there are studies in small mammals supporting the notion that even short periods of starvation can lead to an emphysematous condition. Nutrition; airway resistance; lung elastance; alveolar size

Address for reprint requests and other correspondence: W. Mitzner, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205 (e-mail: wmitzner@jhsp.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
pressure and volume were recorded on a PowerLab digital data acquisition system running Chart v.3.3 software (ADInstruments, Castle Hill, Australia). Once a maximal pressure of 35 cmH₂O was reached, lungs were deflated to -10 cmH₂O at a rate of 3 ml/min. Two sequential PV loops between -10 and 35 cmH₂O were then acquired. Residual volume was measured at a pressure of -10 cmH₂O during the first deflation. Since mouse lungs never reach a true maximal lung volume (32), and at pressures below 35 cmH₂O, the inflation limbs often do not show any evidence of flattening, we chose to define a lung capacity (V₃₅) as the volume from the second inflation curve at 35 cmH₂O, a pressure beyond which all mouse strains show progressive stiffening. Specific compliance of the quasistatic respiratory system was computed from the PV relationships as the slope of the deflation limb from 10 to 0 cmH₂O divided by the lung volume at 35 cmH₂O.

Fixation and morphometry. Upon completion of pulmonary function testing, lungs were inflated with warmed (50–55°C) 1% low-melt agarose at 25–30 cmH₂O (10). The inflation pressure was measured continuously until the agarose started to gel (~1 min). The stopcock was then closed, and the whole animal was placed in a refrigerator at 4°C for at least 2 h. The mice were then removed, and the cooled agarose-filled lungs were excised and weighed. This was followed by volume measurement using water displacement (30). Inflated lung volumes averaged 88–91% of the V₃₅ in both strains. We did not measure the air fraction of these inflated lungs or any further tissue processing shrinkage, but in previous work, we have found that the subsequent procedures with methacrylate embedding add an additional ~20% linear shrinkage. Lung tissues were then placed in 10% phosphate-buffered formalin for at least a week, after which they were rinsed with water and placed in 70% ethyl alcohol. Before histological sectioning, the left lung was dissected, and the inferior and superior 3 mm along the long axis of the lung was removed. The remaining tissue was cut into three 2- to 3-mm-thick sections, dehydrated in ethanol, and embedded in glycol methacrylate (Polysciences, War- rington, PA). Three-micrometer-thick sections were cut and stained with Coomassie blue. Three slices were selected from each of the apical surfaces of the upper, middle, and lower regions of the left lung. Images were acquired using a Nikon Digital Camera DXM1200 (Nikon, Tokyo, Japan) at ×40 magnification. The entire cross-sectional area of each section bounded by the visceral pleura was photographed, with approximately six images per section. Mean chord lengths were measured using ImageJ software (NIH, Bethesda, MD) with sampling grid lines 17 μm apart ensuring one to two chords per alveolus. As was previously done, lower and upper cutoffs of 8 μm and 250 μm, respectively, were applied making certain that no capillaries, arterioles, veins, or bronchioles were included (33). It should be noted, however, that including all chords had no effect on the difference between lungs in the different experimental conditions. With all chords included, the mean is slightly smaller, since there are far more tiny chords (of 1 or 2 pixels) than large ones. Overall, our technique as applied enabled the measurement of ~50,000–65,000 chords/mouse, which were averaged to obtain the air space mean chord length (Lₘ) of each animal, and is reported as corrected back to the air-filled lung. We estimated air space surface area (SA) as previously described from the Lₘ and fresh lung volume (SA = 4 V/Lₘ) (36). The volumes used for this calculation were those of the air-filled lung at full air inflation (V₃₅).

Statistical analysis. All data are presented as the means ± SE. Body weights, lung volumes, dynamic elastance and resistance, static compliance, Lₘ, and SA were all analyzed for the effect of CR and strain by two-way ANOVA (GraphPad Prism 4, www.graphpad.com). Dynamic resistance and elastance, lung volume, static lung compliance, and Lₘ were analyzed using a one-way ANOVA to compare the Lₘ of each group. Bonferroni multiple comparisons corrections were applied to all variables for the difference between control, CR, and refeed mice in each strain. A corrected P value ≤ 0.05 was accepted as significant.

RESULTS

Body weight. Average daily body weight loss is represented as a percent loss from baseline for B6 and C3 mice (Fig. 1). Body mass averaged 27.3 ± 0.23 and 26.0 ± 0.54, in B6 and C3, respectively, at the onset of the study, and were not significantly different. With calorie restriction, there was a significant weight loss in each strain, averaging, 30–40% loss after 2 wk of CR, which was not different between B6 or C3 mice ($P > 0.05$). Reported B6 weight loss is comparable to values reported by Massaro and colleagues (16). Although periods of 2 and 3 wk of CR were implemented in this study, the weight had begun to stabilize after 2 wk. This may explain why there were no differences in PV curves or lung volumes were apparent between the two time periods. Body weights in both strains had returned to the pre-CR levels within 4 days of ad libitum refeeding.

Lung volumes. Average deflation limbs from quasistatic PV curves of B6 and C3 mice are shown in Fig. 2 for control, CR, and refeed groups. In the control state, the lung volumes of C3 mice (1.55 ± 0.10 ml) are significantly larger than those in the B6 strain (1.28 ± 0.06 ml), supporting the genetic differences in lung volumes among mouse strains previously reported (35a). All mice undergoing CR show a significant reduction in lung volume (to 1.33 ± 0.07 and 1.02 ± 0.05 ml in C3 and B6 mice, respectively). With refeeding, both strains are able to recover their respective volumes lost during CR.

Dynamic resistance and elastance. The effect of severe CR on dynamic resistance of the respiratory system in vivo is shown in Fig. 3. Both strains show a significant increase in dynamic respiratory resistance. The percentage increase is significantly smaller in C3 mice (8%) undergoing CR compared with B6 (16%). The increased resistance in both strains is reversed with refeeding.

Similar to resistance, dynamic lung elasticity also increased significantly with CR (Fig. 4). Specifically, B6 mice showed a 26% increase in lung elastance compared with a 17% rise in C3.
mice. With refeeding, the B6 elastance returned to control, but the C3 lungs had not yet recovered their original elastance at the time of measurement.

From the quasistatic PV curves, we quantified the slope of the deflation limb between 0 and 10 cmH2O and calculated this deflation compliance. Results are shown in Fig. 5. This compliance shows the same trend as the elastance in Fig. 4, but when normalized to the lung volume (i.e., the specific compliance), there are no longer any effects of CR in either strain. This indicates the changes in lung elasticity result from the changes in lung volume.

Lung morphometry. Representative low power sections in each strain from the control and 2 wk of CR time points are shown in Fig. 6. There is no obvious visual effect of CR in either strain. The quantitative effect of CR on lung structure for both strains as determined by the mean alveolar chord length (Lm) is shown in Fig. 7. Figure 7 confirms that, while there are significant differences (P < 0.001) between the Lm in the normal control C3 and B6 strains, as previously reported (33), there were no changes in Lm in either strain during the CR or after refeeding. Figure 8 shows the estimated alveolar SA.

DISCUSSION

In this study, we found that 2 or 3 wk of a severe CR to 1/3 the normal diet, led to acute reversible decreases in lung size and SA, and increases in elastance, changes that are the opposite of what one would expect with emphysema. In addition to these changes in lung structure and function, there were no significant changes in the mean air space chord lengths at any time point during the CR or recovery. Thus we found no evidence of nutritional emphysema in either strain of mouse. The two strains of mice were chosen based on previous work (33) that showed substantially larger alveoli in the C3 strain. In fact, compared with the B6 strain, the normal C3 lung itself could almost be interpreted as mildly emphysematous (33). However, despite this genetic difference in alveolar size, neither strain showed any changes in alveolar size with the CR, and the changes in mechanical properties were also similar in the two strains. These results were surprising, since we tried to...
match the experimental conditions previously used in mice subject to CR (16). We used the same strain, same age, and same level and duration of food restriction and observed a similar same weight loss as was previously reported in this study. In the following discussion, we will attempt to resolve this apparent difference with several published studies.

First, we should note that our results with lung mechanics are in fact not too dissimilar from what has been published in other species. Only one early study has shown an increased lung volume after CR in rats (29), and this was done with saline inflation. Most other studies where lung volumes were measured in rats and hamsters have shown either no change in lung volumes (13) or decreased lung volume with CR (11, 12, 25–27), consistent with what was measured in the present study in the mouse. In addition, in the few studies where quasistatic or dynamic elastance was measured, the results were ambiguous, sometimes showing the increase we have observed (5, 11), and sometimes showing a decrease (12, 29), but often no change (13, 25, 27). Fewer studies have been done with CR (16) and recovery (15) in the mouse, and none have measured any mechanical variables. Overall, in considering the whole body of published literature, the evidence from measurement of lung volumes and mechanics offers very little support for CR leading to a larger, more compliant, emphysema. The strongest support in the literature for such a nutritional emphysema comes from the histological evidence.

In the first study in rats by Sahebjami and Vassallo (28), a significant increase in mean linear intercept was reported. This observation in rats was supported by several subsequent studies by this same group and others (11, 12, 29). Other studies do not report the mean linear intercept, but rather use either point counting (13) or the selector method to show that there is a decrease in the number of alveoli, suggesting that the remaining alveoli were enlarged. This method was also used to show alveolar volume enlargement (averaging 70%) after CR in the mouse (16). In the present work, we found that there was a decrease in SA (Fig. 8), and with the reasonable assumption that there was little change in shape of the alveoli with CR, this finding is consistent with there being fewer alveoli after CR. We note that both a reduction in the number of alveoli and SA was also found by Massaro et al. (16). The major difference in our study, however, is that the alveoli were not increased in size. At the present time, we cannot offer any clear explanation of this key difference we observed in our CR mice. We used the same strain (C57BL/6), same degree of CR (1/3 normal), same duration of starvation (2 wk), with a similar weight loss (~10 g). One methodological difference, however, is that in our work we sampled one or two orders of magnitude more alveoli than any of the previous studies. Although it is the objective to sample lung regions in an unbiased manner, we have observed that sections from different regions of the mouse (for instance, from the top or bottom of a lobe) often show differences in the Lm (unpublished observations). Such variability in normal lungs may make it very difficult to obtain unbiased samples, even with the best intentions, using conventional random sample selection designed for larger lungs (9, 18). In our approach, we essentially sampled all alveoli in a slice bounded by the visceral pleura. Such slices typically contain thousands of alveoli, and we averaged chords from three such slices, separated by at least 2 mm, from each left lung. Based on a recent methodological analysis of parenchymal isotropy in mouse lungs, our method should be sensitive enough to detect a difference in Lm of 4–5 μm (unpublished observations).

How this approach compares with the more detailed counting of individual alveoli using the selector method with serial stacks of sections (4) has not been done. However, since this latter method may only look at 30–50 alveoli in the entire mouse lung (16), it is likely less representative of the whole lung than the sampling of 50,000 chords in multiple two-dimensional sections. These issues could perhaps be resolved with the approach used by Fehrenbach et al. (7), but even this method will not readily be able to sample sufficient numbers to analyze the heterogeneity often found in emphysema. This point is supported by a more recent study of CR in rats that showed a substantial increase in the heterogeneity of alveoli after the CR (5). These investigators found an increase in the percent of the lung section with both smaller and larger alveoli, yet there was no change in the mean linear intercept. This anisotropy in alveolar size is apparent even in the lung parenchyma of normal mice (unpublished observations). Such a situation may lead to inadvertent bias in the measurement of individual alveoli if insufficient numbers are studied. We should also note that our sampling of only the left lung clearly could have introduced some bias if the changes in the lung with CR were restricted to specific lobes. However, we know of no
evidence that would indicate that any such systemic changes lead to such gross heterogeneities between individual lung lobes. In any event, although we presently cannot satisfactorily resolve the differences in alveolar size reported in the literature, we are confident that in the two mouse strains we studied, CR did not result in enlarged alveoli. Thus, neither from the histology nor the mechanics is there any evidence of nutritional emphysema in these mice.

Evidence for the presence of nutritional emphysema in human subjects is equally controversial. Although pulmonary function tests are not routinely performed on anorexic nervosa patients, what spirometry and plethysmography data do exist suggest that static lung volumes and compliances are not larger than predicted, and may even be smaller. In an early study by Cravetto et al. (3), it was reported that FEV₁, residual volume, and blood gases were all normal. However, lung compliance (inverse of lung elastance) was in the low to normal range. In 1992, Ryan et al. (24) reported a TLC at 75% of that predicted in anorexic patients, which was suggested to result partially from the severely weakened inspiratory muscles in these patients. Pieters et al. (21) studied a larger population of anorexic patients, 24 women between the ages of 14 and 38 years, and confirmed previous findings showing that residual volume and blood gases were within the normal range in anorexic individuals.

Recently, two studies have used CT imaging to study the radiodensity of the lungs of anorexic women (2, 19). These results showed a significant decrease in attenuation of the CT image of the lung parenchyma. The authors speculated that this was consistent with the decreased CT density seen in emphy-
ences in alveolar size leads to a slightly smaller, stiffer lung. Anorexic women have long been known to have lower heart rate and systemic blood pressure (8), factors consistent with a decreased cardiac output. If such were the case, then both the pulmonary vascular pressure and pulmonary blood volume would also be much decreased (neither has been measured in this population), and this by itself would decrease the CT density. The data reported from the studies in the Warsaw Ghetto showed that 13% of the people studied had evidence of more translucent lungs. However, there may have been many other causes of this qualitative observation under these severe conditions.

Although we did not find any evidence of emphysema, we did find a smaller, stiffer lung that recovered with refeeding. This increased stiffness was largely a result of the decreased lung volume as confirmed by the lack of any changes in the specific compliance. What might have caused the lung to be temporarily smaller during this period of severe CR? The most likely explanation is that these matrix changes led to some degree of septal folding (microatelectasis) such that the maximal lung volume was decreased. The gross heterogeneity seen in rats by Dias et al. (5) is consistent with this explanation. In our mice, however, we did not see any evidence of this gross heterogeneity, but in this regard, it should be emphasized that Dias et al. fixed their lungs at functional residual capacity, and we fixed our mouse lungs at a defined maximal pressure that where surface tension would be zero, surfactant changes could not account for our findings. There must thus be structural changes resulting from CR that are not dependent on surfactant.

In none of the previous studies of CR was respiratory or airway resistance measured. In clinical emphysema, the loss of parenchymal tethering leads to airway collapse on expiration, an effect that often results in gas trapping. In this study, we found that CR caused an increase in resistance, which recovered on refeeding in both strains. Reasons for this increase are not entirely clear, but one possibility is that if there was a loss of alveolar surface with CR, the tethering support for the airway could be decreased. A higher resistance is also consistent with the smaller lung volumes observed. Another possibility is that, since we were measuring respiratory system resistance, the increase we found may reflect an increase in the tissue viscosity. This would be consistent with the increased elastance also observed.

In summary, we have shown that CR to 1/3 of the normal diet in two strains of mice with genetically determined differences in alveolar size leads to a slightly smaller, stiffer lung. There is no evidence of any emphysema-like conditions resulting from this severe acute nutritional insult. The acute changes that were observed with 2 wk of CR were largely reversed after 2 wk of returning to a normal diet.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grants HL-10342 and HL-007534.

**REFERENCES**