Calorie-related rapid onset of alveolar loss, regeneration, and changes in mouse lung gene expression

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Massaro, Donald, Gloria DeCarlo Massaro, Alex Baras, Eric P. Hoffman, and Linda Biadasz Clerch. Calorie-related rapid onset of alveolar loss, regeneration, and changes in mouse lung gene expression. Am J Physiol Lung Cell Mol Physiol 286: L896–L906, 2004.—Calorie restriction, followed by ad libitum refeeding, results, respectively, in loss and regeneration of pulmonary alveoli. We now show 35% of alveoli are lost within 72 h of onset of calorie restriction (5% decreased daily chow intake), and an additional 12% of alveoli are lost over a subsequent 12 days of calorie restriction. Tissue necrosis was not seen. Within 72 h of refeeding, after 15 days of calorie restriction, the number of alveoli returns to precollapse restriction values. Microarray lung gene profiling, in conjunction with Western and RNase protection assay, demonstrate an increase of granzyme and caspase gene expression 2–3 h after onset of calorie restriction. By 12 h, granzyme and caspase expression is no longer increased, but tumor necrosis factor death receptor expression is elevated. At 336 h, Fas death receptor expression is increased. Because granzymes are found only in cytotoxic lymphocytes (CTLs) and natural killer (NK) cells, we suggest calorie restriction activates these cells, initiating a series of molecular events that results in alveolar destruction. The evidence of involvement of CTLs and NK cells and the absence of necrosis are similar to alveolar destruction in chronic obstructive pulmonary disease.

apoptosis; caspases; cytotoxic lymphocytes; granzymes; microarray; natural killer T cells

THE REGULATION, molecular basis, and time required for the turnover (loss and formation) of pulmonary alveoli are poorly understood. Pulmonary emphysema, an important component of chronic obstructive pulmonary disease (COPD), is characterized by apoptosis of alveolar wall cells (2), little or no necrosis (6), and, surprisingly, an all-or-nothing (68, 71) progressive destruction of individual alveoli (26, 56, 67). These changes are thought to be due to insufficient intra-alveolar antiprotease activity, inflammation, and oxidant stress (3, 6, 16, 26, 55). Experiments on animals and humans with emphysema suggest proteases and oxygen radicals released by neutrophils, lymphocytes, and macrophages play a key role in alveolar destruction (6, 11, 24, 27, 50, 63). However, therapies of COPD based on these notions of pathogenesis have been notably unsuccessful in slowing the rate of loss of diffusing capacity, as has cessation of cigarette smoking (56, 67). Furthermore, a means of inducing alveolar regeneration in COPD has not been established.

Calorie restriction of adult rodents, followed by ad libitum access to food, activates, respectively, endogenous programs of alveolar destruction (23, 30, 32, 52) and of alveolar regeneration (33, 39, 52). Starvation in adult humans leads to emphysema-like changes in the lung (9, 12, 73), suggesting the endogenous program(s) of alveolar destruction is conserved in humans. We now show in adult male mice that calorie restriction results, within 2 h, in molecular changes in lung consistent with activation of pathways of apoptosis; by 72 h of calorie restriction, alveolar loss has occurred. Ad libitum refeeding after 15 days of calorie restriction results in alveolar regeneration within 72 h. Some of the changes of gene expression during calorie restriction-induced alveolar destruction are similar to those found in COPD (3, 6, 11).

MATERIALS AND METHODS

Animals and experimental manipulations. Adult male C57BL/6J mice were purchased from Jackson Laboratory. Upon arrival at Georgetown, they were housed three to four per cage in the Department of Comparative Medicine on a 12:12-h light-dark schedule and were allowed ad libitum Ralston Purina Laboratory Chow 5001. After at least 1 wk at Georgetown, the mice were housed one per cage, and the amount of chow eaten each day was measured. On the basis of the average daily weight of chow eaten over 4–5 days by each mouse, we diminished the daily allotment of chow provided to some mice by 2/3 (33). After being calorie restricted, some mice were allowed ad libitum access to food; other mice were always allowed ad libitum access to food. All mice were always provided tap water ad libitum.

The average daily food consumption in mice fed ad libitum was ~4.2 g/day. Diminishing daily Ralston Purina Laboratory Chow 5001 intake by 2/3 provides 30 IU of retinol each day. The minimum daily requirement of retinol for mice is 1–2 IU (61). Therefore, this study is not influenced by retinol deficiency, which requires months of a retinol-free diet to produce (61), but can alter alveolar structure (4). We killed animals by cutting large vessels in the abdomen after establishing a surgical level of anesthesia with xylazine (~10 mg/kg) plus ketamine (~75 mg/kg). All procedures were approved by the Georgetown University Animal Care and Use Committee and comply with the United States Department of Agriculture and National Institutes of Health guidelines.

Morphological and morphometric studies. Mice were anesthetized with xylazine plus ketamine. The trachea was intubated, the diaphragm punctured, and cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, was infused into the trachea at a transpulmonary pressure of 20 cmH2O. The trachea was ligated, and the lungs were removed from the thorax. Fixation was continued at 0–4°C for 2 h. Lung volume was measured by volume displacement (54). Lungs

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were cut into blocks. Blocks were selected for further processing using a systematic sampling technique (14), washed in cacodylate buffer, postfixed for 1 h at 4°C in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated, and embedded in epoxy resin (38).

We cut serial sections at ~0.8-μm thickness and sectioned three blocks per animal. Each group of serial sections was cut to a depth of 150–250 μm. To identify alveolar air spaces, gas-exchange structures were followed through a complete set of prints of serially sectioned lung (38, 39). An alveolus was defined as a structure with a mouth that communicated with a common air space; the latter was designated an alveolar duct.

The selector method (13) was used to choose alveoli for analysis (38, 39). This method allows alveoli to be selected for measurement based on number, uninfluenced by size, shape, or orientation. Thirty alveoli were analyzed per mouse. The volume of an alveolus was estimated by the point-sampled intercepts method (22), and the number of alveoli was calculated as previously described (38).

To determine alveolar surface area, sections ~0.8 μm thick were cut from each of 10 tissue blocks, which provided 10 sections per mouse, and stained with toluidine blue. Lung sections were photographed using a Reichert Microstar IV microscope and Polaroid film 667; final prints were at a magnification of ×160. Point and intersection counting was used to determine alveolar surface area (70). Volume density was estimated by counting the number of points that fell on the object under analysis (alveolar tissue, gas-exchange air space, or conducting structures) divided by the total number of test points (70). All measurements were made without knowledge of the manipulation to which the mice had been subjected.

Expression profiling. The right and left lung from each mouse were placed in a separate piece of aluminum foil with lungs from four other mice on the same diet, i.e., food ad libitum or calorie restricted (Fig. 1). Each package of aluminum foil had right and left lungs from two (2- to 96-h time points) or four (336-h time point) different mice. All the lungs in each foil package were disrupted to form one homogenate representing each package. Total RNA was separately extracted from each homogenate using TRIzol reagent (GIBCO BRL) and purified further using RNeasy (Qiagen). The RNA was converted into double-stranded cDNA using SuperScript Choice System (GIBCO BRL) with an oligo(dT) primer containing T7 RNA polymerase promoter (Genset). Double-stranded cDNA was purified by phenol/chloroform extraction and then used for in vitro transcription with an ENZO BioArray RNA transcript-labeled kit. Biotin-labeled cRNA was purified by RNeasy (Qiagen) and randomly fragmented before hybridization to an Affymetrix Murine MG-U74Av2 GeneChip using an Affymetrix Fluidics Station 400 and a Hewlett Packard G2500A Gene Array scanner. Expression profiling data can be found at NCBI GEO (http://www.ncbi.nlm.nih.gov/geo), accession series GDS241 (Alveoli destruction time course). Supplementary Table S3 is available online at the AJP–Lung Cellular and Molecular Physiology web site.1

Analysis of expression profiling. We used an experimental design that attempts to account for potential diurnal variation in gene expression (Fig. 1). GeneSpring 5.1 (Silicon Genetics) software was used to analyze the data. Because all RNA analyses were performed on

1 The Supplementary material for this article (Supplementary Table S3) is available online at http://ajplung.physiology.org/cgi/content/full/00333.2003/DC1.
duplicate Affymetrix gene chips, the GeneSpring global error model (http://www.silicongenetics.com) was used to estimate variations between chips and between treatments except for the results from the 336-h experiments. On the latter, an unpaired, two-tailed t-test analysis was used to determine the statistical significance of differences of gene expression between ad libitum-fed and calorie-restricted mice (59).

RESULTS

Body mass changed rapidly with altered food intake. Body mass fell 10% within 12 h of onset of calorie restriction (Table 1). Two-thirds of the fall that took place during 15 days of calorie restriction occurred within 72 h of initiating calorie restriction. By 12 h of refeeding, after 15 days of calorie restriction, body mass increased 28%. Body mass was not different among groups of mice always allowed chow ad libitum (Table 1).

Morphology of gas-exchange tissue during altered food intake. We did not detect evidence of tissue necrosis (Fig. 2). Alveolar walls appeared thinner and alveoli larger in calorie-restricted mice compared with ad libitum and with calorie-restricted ad libitum-refed mice.

Calorie restriction caused a fall in the volume of gas-exchange tissue; ad libitum refeeding raised it. Neither total lung volume (Fig. 3A) nor gas-exchange air volume (Fig. 3B) differed between ad libitum-fed and calorie-restricted mice. However, the volume of gas-exchange tissue was 30% lower after 15 days of calorie restriction than in ad libitum-fed mice; this difference was not present by 72 h of ad libitum refeeding (Fig. 3C).

Alveolar loss was detected within 72 h of onset of calorie restriction and regeneration within 72 h of ad libitum refeeding. The volume of individual alveoli increased 44% within 72 h of calorie restriction (Fig. 4A). This represented ~75% of the increase of the volume of individual alveoli that occurred over the entire 15 days of calorie restriction. The number of alveoli exhibited a correspondingly rapid decline (Fig. 4B). Alveolar surface area also fell within 72 h of calorie restriction (Fig. 4C). By 72 h of ad libitum refeeding, after 15 days of calorie restriction, the volume of individual alveoli and the number of alveoli had returned to values present in mice never calorie restricted. We did not detect a statistically significant increase of alveolar surface area after ad libitum refeeding. This failure may reflect the fact that area changes to the power 2, volume to the power 3, and hence area changes are only 2/3 as large as those of volume.

Comparison of gene expression from the same lungs on duplicate chips. A scatter plot analysis of mRNA from mice allowed food ad libitum or that were calorie restricted for 15 days, respectively, revealed 93.4 ± 0.2 and 93.4 ± 0.3 of the intensity of points fell within two standard deviations of the line of identity (Fig. 5). As expected, there was greater variation between duplicate chips at low levels of expression than at high levels (Fig. 5). The greater variance impairs detection of intergroup differences among genes expressed at low levels.

Analysis of lung gene expression during calorie restriction. The expression of all genes and expression sequence tags (ESTs) queried in our experiments may be obtained from the

![Fig. 2. Morphological response to CR and calorie restriction-refeeding (CR-RF). Mice were fed ad libitum for 3 (A) or 15 (C) days; other mice were calorie restricted for 3 (B) or 15 (D) days; some of the latter were refed ad libitum for 3 days (E). Bar scale = 50 μm.](http://ajplung.physiology.org/)

Alveolar turnover and lung gene expression
database “Public Expression Profile Resource” (http://microarray.cnmcresearch.org/pgadatable.asp). Because of the massive amount of data generated by microarray gene profiling, we have focused on only two categories of genes as identified by GeneSpring ontology software. We selected genes involved in cell death or proteolysis (Supplementary Table S3, Figs. 6 and 7) because these biological processes are considered important in experimental cigarette smoke-produced emphysema, emphysema in human COPD, and in lung calorie-related apoptosis, increased proteolysis, and alveolar loss (2, 3, 6, 11, 24, 27, 39, 64). However, many other cellular changes, e.g., related to glucose and lipid metabolism (5, 21, 64), take place in response to calorie restriction and may be reflected in the gene expression results shown in Supplementary Table S3 and Figs. 6 and 7. Furthermore, we have shown only genes whose expression in lungs of calorie-restricted mice compared with lungs of ad libitum-fed mice had a P value ≤0.05 by the GeneSpring global error model or by an unpaired, two-tailed t-test. With these restrictions, 120 genes were higher in lungs of calorie-restricted mice, and 137 were lower compared with lungs of ad libitum-fed mice.

Within 2 h of onset of calorie restriction, the mRNA of a TNF ligand (TWEEN) and of granzyme A, an IL-1β converting enzyme (24), was elevated in lungs of calorie-restricted mice (Supplementary Table S3). By 3 h, granzyme B protein concentration, which was determined by Western analysis, was increased in lungs of calorie-restricted mice (vide infra). The 2- and 4-h mRNA values for caspase 3, considered individually, did not achieve statistically significant differences among treatment groups. However, when the 2- and 4-h calorie restriction results were combined (n = 4), and the 2- and 4-h ad libitum-fed results were combined (n = 4), caspase 3 mRNA was 1.3-fold higher in lungs of calorie-restricted mice (P = 0.038). Four hours after institution of calorie restriction, the mRNA of kallikrein, caspase 14, TNF receptor superfamily member 17, and an aryl hydrocarbon receptor, which activates proteosomes, were higher in lungs of calorie-restricted mice than in lungs of ad libitum-fed mice (Supplementary Table S3). These findings are consistent with increased serine protease activity (kallikrein), diminished cell-extracellular matrix interaction (caspase 14) (46), and increased signaling via TNF ligand (TWEEN) and TNF receptors (TNF receptor superfamily member 17). By contrast, at the same time, a RING finger mRNA, which targets proteins for proteasomal destruction (17) and programmed cell death 8, an apoptosis-inducing factor (62), were diminished in lungs of calorie-restricted mice (Supplementary Table S3).

These findings suggest the early onset of four routes to the previously demonstrated (39) alveolar wall cell death: 1) TNF mediated, 2) destruction of extracellular matrix (caspase 14), 3) caspase-independent cell death (granzymes A and B) (36), and 4) caspase-dependent cell death (caspase 3 and granzyme B) (36). Granzymes A and B are serine protease activators of cell death produced only by CTLs and NK cells and are stored in secretory granules (36). CTLs require 20–24 h to become activated and form granules (36). NK cells in the steady state bear granules containing granzymes and other cytotoxicity effectors that can be released instantaneously (36). Therefore, it is likely NK cells are the initial source of cell-destroying granzymes after the onset of calorie restriction.

By 12 h of calorie restriction, evidence of DNA damage was indicated by growth arrest and DNA damage-inducible 45γ (Gadd 45γ) mRNA being higher in calorie-restricted mice than ad libitum-fed mice (Supplementary Table S3).
The main role of Gadd genes is to block proliferation at G1 and G2 check points in response to DNA damage (33, 74). The mRNAs of casinolytic protease X and of ubiquitin-specific protease 2, which are involved in destruction of cellular proteins (20, 33), were also elevated in lungs of calorie-restricted mice (20, 33). In contrast to the molecular effects of calorie restriction that favor matrix destruction and apoptosis within the first 12 h, the higher concentration of Bcl-2, which interrupts an apoptosis cascade (49), the lower concentrations of the mRNA of three ADAM (a disintegrin and metalloprotease) family proteins (ADAM 7, 17, and 28), and of programmed cell death 8 mRNA do not favor apoptosis (10, 41, 58).

The simultaneous presence of molecular changes favoring and opposing apoptosis may reflect the lung’s cellular heterogeneity, some cells being expendable, others not. In this regard, endophilin, which is involved in endocytic removal of plasma membrane receptors (43), was elevated in calorie-restricted mice and thereby could determine which alveolar wall cells respond to the nonspecific presentation of extracellular signals to all alveolar wall cells. It is also important to acknowledge these changes of gene expression are at the level of mRNA, whereas the functions of the genes are effected by proteins.

After the first 4 h, the mRNAs of granzyme A and of granzyme B either were not different between lungs of calorie-restricted and ad libitum-fed mice or were lower in the former than the latter (Supplementary Table S3). However, evidence consistent with cell death mediated by TNF receptors (increased mRNAs of Tnfrsf 1a and of TRAF 2) persisted through 336 h (Supplementary Table S3). The mRNA of an ADAM family member (ADAM 23) was higher in lungs of calorie-restricted mice than in lungs of ad libitum-fed mice (Supplementary Table S3). This is relevant to the increase of TNF receptors because ADAM family proteases cause release of soluble TNF (41), which can induce apoptosis (48). The mRNA of angiotensin-converting enzyme, which is formed in endothelial cells and whose protein is required for TNF-α-induced apoptosis of alveolar epithelial cells (69), was elevated at 24, 48, and 96 h. Furthermore, the transcription factor CCAAT/enhancer-binding protein, which is induced by stress (19) and whose activation by IL-12 may induce expression of TNF-α (29), was increased at 72, 96, and 336 h. The mRNA of tissue plasminogen activator, whose protein converts plasminogen, an inactive serine protease, to plasmin, an active serine protease, was elevated at 24 and 96 h. Granzyme C mRNA was elevated 2.4-fold at 96 h in lungs of calorie-restricted mice (Supplementary Table S3). Granzyme C, as granzyme A, can produce caspase-independent cell death but is also a powerful cause of cell death by pathways different from those used by granzymes A and B (36). By 336 h, Fas-associated factor mRNA was elevated in lungs of calorie-restricted mice, suggesting the Fas ligand death pathway had been activated. However, and consistent with the morphometric results (Figs. 3 and 4), the gene expression results at 336 h indicate apoptosis had diminished but that extracellular matrix remodeling persisted. These findings are consistent with the substantial apoptosis (39), alveolar wall thinning (Fig. 3C), and alveolar loss (Fig. 4B) that occur over the first 72 h of calorie restriction and continues, albeit at a slower pace, over the full 336 h of calorie restriction. There were also changes of gene expression consistent with, and explanatory of, the previously described twofold increase of proteolysis in the lung during calorie restriction (64). For example, the mRNA of calpain 2, which is a Ca2+-activated intracellular cysteine protease (45), was elevated at 24, 48, and 336 h (Supplementary Table S3).

To verify some of the intergroup differences in gene expression identified by use of microarray, Western blot and RPA analysis were performed. The concentration of granzyme B (densitometry units/milligram lung protein) in lungs of calorie-restricted mice, 3 h after the onset of calorie restriction, was (means ± SE) 8.5 ± 0.2 (n = 4), and in lungs of mice fed ad libitum and killed at the same time, it was 7.7 ± 0.1 (n = 3, P = <0.02). We also compared the microarray assessment of
time in the 24-h cycle limited the potentially confounding factor of diurnal variation in gene expression. Duplicate processing, i.e., separate isolation of RNA and hybridization of RNA from an equal mix of right and left lungs on duplicate chips, provided two sets of data and is expected to control for differences in RNA isolation, biotinylation of RNA, and variability in hybridization to the GeneChip. The complete agreement, for the genes tested, of the array analysis with Western blot and RPA supports the validity of the methods and increases confidence in the expression results we did not try to confirm. Considering only genes that exhibited statistically significant intergroup differences further increases confidence in the results of the microarray experiments.

Calorie restriction. Periods of food scarcity in air-breathing organisms must have occurred since the Devonian when lung fish estivated during periods of drought. To survive such periods of not eating, organisms produce substrate, by destruction of comparatively nonessential structures, for gluconeogenesis to provide glucose for the brain and amino acids to maintain muscle. For example, among marine iguanas, those that shrink long bones to the greatest extent during food scarcity survive the longest (72). With respect to the lung, its only essential function is to provide sufficient gas-exchange surface to meet the organism’s need for oxygen. Therefore, some lung tissue would be expendable as total organismal (42) and lung oxygen consumption (21) fall during calorie restriction. The lung’s respiratory quotient decreases during calorie restriction (21), indicating use of lipid rather than glucose, the lungs preferred substrate in ad libitum-fed animals (26). Studies with 2-deoxy-D-glucose, which is not metabolized beyond being phosphorylated, reveal the diminished utilization of glucose during calorie restriction is at a step(s) beyond phosphorylation of glucose (5). In addition to decreased utilization of glucose, calorie restriction doubles the rate of proteolysis in the lung (64). Therefore, in the lung, calorie restriction results in metabolism of lipid in preference to glucose and in substantial proteolysis; the former would increase glucose available for use by the brain, and the latter would provide amino acids for gluconeogenesis and to maintain muscle. Refeeding elevates O2 consumption (42), thereby increasing the need for alveolar surface area, and lung regeneration rapidly takes place.

Alveolar destruction. At each time queried, some intergroup differences of gene expression favored cell death and tissue destruction, others cell survival. This suggests that, in a cellularly heterogeneous organ, certain cell types are selected for destruction, others for survival. As a whole, the results of our gene expression studies are consistent with the following during calorie restriction: 1) NK cells, activated by as yet unknown stimuli, initiate the molecular changes responsible for alveolar destruction; 2) degradation of extracellular matrix is a key initiating event in alveolar wall cell apoptosis and alveolar destruction; and 3) apoptosis of alveolar wall cells initially occurs by granzyme-induced, TNF receptor, and ADAM family pathways, later by Fas pathways.

The cytotoxicity of CTL and NK cells is effected by their secretion of proteases, e.g., granzymes, present in secretory granules (48). Granzymes are serine proteases produced only

Fig. 5. Scatter plot of replicate expression values. Each point represents duplicate measurements of the same gene. If replicate expression values were identical, all points would fall on the diagonal line of identity. In the ad libitum-fed mice, 93.4 ± 0.2% of the replicates fell within 2 SD of the line of identity (A); for calorie-restricted mice, 93.4 ± 0.5% of the replicates fell within 2 SD of the line of identity (B).
Fig. 6. Temporal expression of genes related to apoptosis. Hierarchical clustering was used to order the genes. Blue indicates downregulation of genes in CR mice compared with the same genes in ad libitum-fed mice at the same time. Red indicates upregulation of genes from CR mice compared with the same gene from ad libitum-fed mice at the same time. The intensity of the color directly reflects the magnitude of increase or decrease of gene expression.
Fig. 7. Temporal expression of genes related to proteolysis. The experimental manipulations, clustering, and color code are as described in Fig. 6.
These considerations suggest alveolar loss associated with loss of elastic tissue recoil is not, or cannot be, endogenously regulated; alveolar loss without loss of elastic tissue recoil is endogenously regulated. Because apoptosis of alveolar wall cells occurs in all these forms of alveolar wall destruction (2, 6, 39), the apoptosis does not seem to drive the process unless, and this remains to be seen, apoptosis of specific lung cells, e.g., alveolar capillary endothelial cells (31), is determinative.

**Potential clinical relevance.** Although alveolar destruction occurs in a general population as part of biological aging (60, 65), the most widely recognized and medically important cause of alveolar destruction is COPD (3, 6, 26), for which cigarette smoking is the major risk factor (25, 57). The main current theories of the pathogenesis of COPD are intra-alveolar protease-antiprotease imbalance, chronic inflammation, oxidant damage, and an interaction among these (3, 6, 16, 26, 53). Smoking cessation slows the loss of conducting airway function (1, 44). However, therapies aimed at the currently considered etiological basis of alveolar destruction (3, 16), including remarkably, even smoking cessation (53, 56, 67), fail to slow the loss of gas-exchange function in individuals with COPD.

In addition to polymorphonuclear leukocytes (26) and macrophages (24, 27, 63), CTL and NK cells, via their secretory products, which include granzymes and TNF, are thought to play a role in alveolar destruction in emphysema (3, 11, 50). Our findings demonstrating the remarkably early onset of increased expression of granzymes, caspases, and TNF ligands and receptors strongly suggest these molecules are important to alveolar destruction in calorie-related alveolar loss, as they are thought to be in COPD (3, 11, 50).

The evidence of involvement of granzymes, which are serine proteases, in calorie restriction-related alveolar loss, the likelihood calorie restriction-related alveolar loss is highly conserved from “mouse to man” (9, 10, 12, 23, 30, 39, 52, 73), and the failure of endogenous serine antiproteases to control granzyme activity in the inflamed lung (66) suggest these properties of lung have been conserved because they allow lung destruction during food scarcity, thereby providing a survival advantage. The evidence that CTL, NK cells, granzymes (40), and TNF play an important role in alveolar destruction in COPD (11, 40, 50) and in calorie restriction-related alveolar loss raises the possibility that COPD and calorie restriction share the same molecular signals for alveolar loss. The fact that alveolar loss slows as the duration of calorie restriction increases and is reversed with ad libitum refeeding indicates there are endogenous regulators of the process of alveolar destruction and regeneration. We raise the possibility that in COPD, even in the absence of calorie restriction, the pathways of calorie restriction-related alveolar destruction are inappropriately activated, and endogenous controls fail, or the inappropriate stimulus for alveolar destruction persists, accounting for the, so far, irremediable, progressive loss of gas-exchange function (56, 67).

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### Table 2. **Comparison of lung gene expression assessed by microarray and RPA analysis**

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<th>Caspase</th>
<th>Fold Change of mRNA Calorie Restriction/Fed Ad Libitum</th>
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Mice were calorie restricted for 48 h or never calorie restricted. Lungs were excised, RNA was isolated, and the mRNA of 5 caspases was measured by microarray analysis and by RNase protection assay (RPA). NC, no difference between calorie-restricted and ad libitum-fed mice.

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by NK cells and by activated CTLs (48). However, naive CTLs do not contain preformed granzymes; their formation, following CTL activation, takes 1–3 days (48). By contrast, NK cells have preformed granules that contain granzymes that can be released within minutes after the NK cell receives an appropriate signal (48). Therefore, NK cells are probably responsible for molecular evidence of cell death and alveolar destruction that is present very shortly after initiation of calorie restriction. The rapid onset, in a nocturnal mammal, of changes in gene expression after institution of calorie restriction during day/night, is consistent with the frequent episodes of food consumption in mice even during daytime (34).

Granzyme A, whose mRNA is elevated 2 h after onset of calorie restriction, does not play a primary role in apoptosis but does destroy extracellular matrix proteins (36, 48). This supports the notion that an early key event in apoptosis of alveolar wall cells may be disruption of the extracellular microenvironment, which initiates cell death by apoptosis. Granzyme B can also cause apoptosis by altering the extracellular environment but can more directly initiate an intracellular cascade that leads to apoptosis (36). The ADAM family of serine proteases regulates cell behavior by modifying the extracellular matrix (50); it also causes the release of soluble TNF, which can initiate apoptosis (34, 40). The increase of ADAM mRNA and of TNF receptor mRNA after 12 h of calorie restriction, in view of the ability of ADAM proteins to release soluble TNF, suggests a common upstream regulator of ADAM and TNF receptor genes.

**Alveolar loss with, or without, a change of lung tissue elastic recoil.** It is becoming apparent that alveolar destruction can occur with, or without, loss of lung tissue elastic recoil. For example, alveolar destruction in humans with COPD (8) and alveolar destruction produced by the instillation of elastase (37) or active caspase 3 (2) are associated with a decrease of lung elastic tissue recoil. By contrast, calorie restriction and corticosteroids both cause alveolar loss (7, 39), but without a loss of lung recoil (15, 18, 51). Alveolar loss in COPD, and that produced by exogenous elastase, continues even after the inciting agent has been removed, e.g., cessation of cigarette smoking in COPD (56, 67) and absence of elevated elastase activity in experimental exogenous elastase-induced emphysema (37). The number of alveoli lost between 3 days and 15 days of calorie restriction is not different (Fig. 4). These considerations suggest alveolar loss associated...