Altered Alveolar Macrophage Function in Calorie-restricted Rats

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Alveolar macrophage functions associated with clearance of bacteria from the lung were assessed in male Fischer 344 rats maintained on a 25% calorie-restricted diet. Calorie-restricted and *ad libitum*-fed (control) rats were exposed to concentrations of ozone known to compromise phagocytic function of alveolar macrophages. Ozone suppressed alveolar macrophage phagocytosis of latex beads *in vitro* in *ad libitum*-fed rats, but not in calorie-restricted rats. In fact, caloric restriction enhanced phagocytic function in both control and ozone-exposed animals. *Ad libitum*-fed rats exposed to ozone and challenged with *Streptococcus zooepidemicus* experienced a prolonged infection and influx of polymorphonuclear leukocytes (PMN), whereas calorie-restricted rats exposed to ozone cleared the bacteria in 24 h without an inflammatory response. Bacterial endotoxin-stimulated *in vitro* production of nitric oxide and tumor necrosis factor (TNF)-α as well as expression of TNF-α and interleukin-6 messenger RNAs were all lower in alveolar macrophages isolated from calorie-restricted rats. Together, the data suggest that caloric restriction enhances resistance to gram-positive bacteria, while lowering the production of proinflammatory mediators elicited by endotoxin, a component of gram-negative bacteria. Although increased bacterial resistance is considered beneficial, reduction in the lung's ability to induce inflammatory mediators can have both positive and pathophysiologic consequences. **Dong, W., M. K. Selgrade, M. I. Gilmour, R. W. Lange, P. Park, M. I. Luster, and F. W. Kari. 1998. Altered alveolar macrophage function in calorie-restricted rats. Am. J. Respir. Cell Mol. Biol. 19:462–469.**

Ample evidence exists that caloric restriction modulates homeostasis and impacts the sensitivity of host responses to various natural and environmental insults. For instance, caloric restriction retards age-associated pathophysiologic changes (1–4) as well as various types of degenerative diseases, including cancer in rodents (5–7). Although the subject has not been extensively investigated, several studies have shown that feed restriction or fasting enhances host defenses against infection in animals and humans. Feed restriction reduced the age-associated decline in antibody production following challenge with influenza virus (8) and dramatically decreased mortality caused by the cerebral malaria parasite in mice (9). Similarly, acute fasting has been shown to increase host resistance against *Listeria monocytogenes* challenge in mice (10, 11), enhance delayed cutaneous hypersensitivity to *Candida albicans*, increase serum monocyte bactericidal activity in obese patients (11), and increase the response to influenza vaccine in anorexia nervosa patients (12).

Feed restriction and/or fasting also affects nonspecific phagocytic responses and inflammation. Prolonged fasting decreases serum neutrophil chemotaxis (13) and reduces the intensity of inflammation and levels of proinflammatory cytokines such as tumor necrosis factor (TNF) α and interleukin (IL)-6 in the salivary glands of $NZBxNZWF_1$ mice for an autoimmune disease model (14). Phagocytosis of opsonized sheep red-blood cells by alveolar macrophages (AM) in Fischer rats was studied on fasted or 20–95% restricted regimens (15). Phagocytosis increased shortly

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Abbreviations: alveolar macrophages, AM; bronchoalveolar lavage, BAL; glyceraldehyde-3-phosphate dehydrogenase, G3PDH; interleukin, IL; lactate dehydrogenase, LDH; lipopolysaccharide, LPS; polymorphonuclear leukocytes, PMN; reverse transcriptase-polymerase chain reaction, RT-PCR; tumor necrosis factor, TNF.

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after fasting (2 d), but decreased after prolonged fasting (3–6 d). Increased phagocytosis also occurred in rats fed a 40% restricted diet.

Alveolar macrophages constitute the first line of defense against respiratory infections and are primarily responsible for clearance of gram-positive bacteria from the lung via phagocytosis and intracellular killing (16). Alveolar macrophages also elaborate proinflammatory mediators including reactive oxygen species, nitric oxide, and cytokines which regulate inflammatory responses (16). Previous studies have demonstrated that ozone exposure suppresses alveolar macrophage function in animals (17–19) and humans (20). Ozone-suppression of alveolar macrophage phagocytosis results in increased mortality in mice challenged with a relatively avirulent Group C *Streptococcus*. Delayed clearance of the bacteria in the lungs following ozone exposure has been demonstrated in both mice and rats (19, 21, 22). Recently, we have shown that dietary restriction mitigates ozone-induced lung inflammation in rats, in part, via increasing pulmonary storage of ascorbate (23). Thus, we hypothesized that caloric restriction might also mitigate the negative effects of ozone on alveolar macrophage function, enhance bacterial clearance, and prevent increased mortality from streptococcal challenge. In this study, we explored the effects of caloric restriction on alveolar macrophage functions thought to be important in the control of bacterial infections.

Materials and Methods

Animals and Dietary Treatment

Male Fischer 344 rats (Charles River, Raleigh, NC) were received at 8 wk of age. Animals were individually housed in polycarbonate cages and maintained under AAALAC-approved conditions in a pathogen-controlled environment. After a 1-wk acclimation period, the dietary treatments were imposed as described below. Distilled water was freely available.

Ad libitum-fed (control) animals were allowed unrestricted access to feed, the consumption of which was estimated by daily weighing of the feed containers. The daily mean consumption of the *ad libitum*-fed group was multiplied by 0.75 and the resultant mass of food was provided to the calorie-restricted animals at 7:00 A.M. daily for 21 d. The semipurified diets were formulated such that the *ad libitum*-fed and calorie-restricted groups are isonutrient with respect to vitamins, minerals, fat, and protein; the caloric restriction came exclusively from carbohydrates. The composition of these diets and the specific-nutrient consumption by the two dietary groups for a typical experiment are described in Table 1. Typically, the body weight at the beginning of the experimental feeding period averaged 195 ± 2 g ($n = 20$). Those rats fed *ad libitum* for 3 wk gained 57.3 ± 3 g ($n = 10$), while the calorie-restricted animals gained 14.9 ± 2 g ($P < 0.01$). All dietary regimens exceeded the nutrient requirements of rats as recommended by the National Research Council (NAS, 1995).

Ozone Exposure

Rats were housed in individual wire compartments and exposed for 3 h in the morning to either filtered air or 0.8 ppm ozone in Rochester-type chambers as described previously (24). Ozone was generated from oxygen using a silent arc discharge generator (OREC, Phoenix, AZ), and its entry into the chambers was controlled by a mass flow controller. The chamber concentration of ozone was monitored continuously using chemiluminescent ozone analyzers (Bendix, Lewisburg, WV), which were calibrated biweekly using a Dasibi transfer standard. Ozone levels were within 2% of the target concentration throughout the study. Temperature and relative humidity ranged from 69 to 73°F and 40 to 60%, respectively, for all exposures. In experiments which involved aerosol infection, the caged animals were subsequently placed in a similar chamber under negative pressure with respect to room air (25) and exposed to aerosolized bacteria as described below.

Bacteria and Infection

Batch slants of *Streptococcus zooepidemicus*, isolated from a pneumonic guinea pig lung and originally described as *Streptococcus pyogenes* or Group C *Streptococcus*, were obtained from lyophilized aliquots of the organism and stored at 4°C. Two days prior to infection, the bacteria were inoculated onto 5% blood agar plates and grown overnight at 37°C. Fresh colonies were then used to inoculate tubes containing 5 ml of Todd-Hewitt broth (THB). Following overnight incubation at 37°C, the bacteria were washed in phosphate-buffered saline (PBS; pH 7.2) and

TABLE 1

Diet composition and nutrient consumption for typical calorie restriction experiment

Ad Libitum			Calorie-restricted		
Diet Composition* (*g/100 g diet*)		Nutrient Consumption (*g/rat/d*)	Diet Composition† (*g/100 g diet*)		Nutrient Consumption (*g/rat/d*)
Protein	20.3	3.77 ± 0.27	Protein	26.5	3.67 ± 0.0
Carbohydrate	66	12.27 ± 0.27	Carbohydrate	55	7.62 ± 0.0‡
Fat	5	0.93 ± 0.27	Fat	6.6	0.92 ± 0.0
Mineral mix	3.5	0.65 ± 0.27	Mineral mix	4.6	0.64 ± 0.0
Vitamin mix	1.2	0.22 ± 0.27	Vitamin mix	1.6	0.22 ± 0.0
Energy (kcal/g diet)	3.9	72.5 ± 2.7 (kcal/rat/d)	Energy (kcal/g diet)	3.9	53.7 ± 0.0‡ (kcal/rat/d)

* Product No. D11520, Research Diets, Inc., New Brunswick, NJ. *Ad libitum* consumption averaged 18.59 ± 0.27 g/rat/d.
† Product No. D11521, Research Diets, Inc. Restricted consumption averaged 13.87 ± 0.0‡ g/rat/d.
‡ Significantly different from corresponding *ad libitum* value ($P < 0.05$).

resuspended in THB at a concentration of 1–2 $\times$ 10^9 bacteria/ml. Aerosol infection of rats was carried out as previously described (19). Briefly, both groups of rats were placed together in an exposure chamber where 4 ml of the bacterial suspension were aerosolized in a nebulizer (No. 40; DeVilbiss, Somerset, PA) operating at 15 lb/sq in for 15–20 min followed by a 5–min purge. Deposition was determined by taking lungs at time 0 from both groups. As Figure 1 indicates, titers of bacteria in the lung at time 0 were the same for both groups. Flow rate through the chamber was 160 L/min (10 air changes/h). Animals were killed according to the schedule described below.

Bacterial Inactivation *In Vivo*

The intrapulmonary inactivation of the bacteria (*S. zooepidemicus*) was assessed in ozone-exposed control or calorie-restricted rats over a 48-h period after infection. Immediately, and 6, 24, and 48 h after infection, five animals from each treatment group were anesthetized with sodium pentobarbital (150 mg/kg, i.p.), the tracheas cannulated, and the lungs lavaged 3 times with warm saline (37°C, 35 ml/kg body weight). Lavage fluid was pooled for each rat, diluted 5-fold, and plated out (0.1 ml) in duplicate on blood agar. Following a 24-h incubation at 37°C, the resultant β-hemolytic colonies were counted and the $\log_{10}$ colony-forming units (CFU)/ml of lavage fluid was calculated. The minimal detectable level for this procedure is 10 CFU/ml.

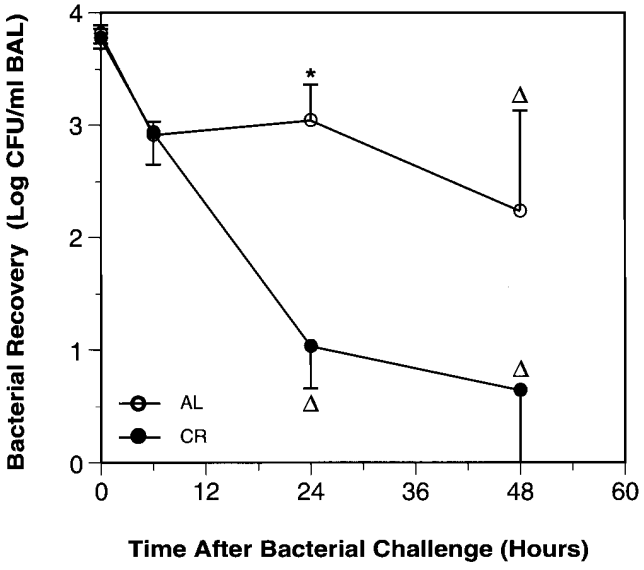

Figure 1. The effect of caloric restriction on *in vivo* bacterial clearance following ozone challenge. The data were expressed as CFU remaining in the lungs of rats at 0, 6, 24, or 48 h after a 3-h exposure to air or 0.8 ppm ozone and aerosol infection with *S. zooepidemicus.* Values represent means ± SEM $\log_{10}$ bacteria/ml BAL fluid. Sample numbers equal 10 for 0-, 6-, and 24-h groups and four for 48-h group. *Asterisk* indicates significant difference between *ad libitum* (AL)-fed and calorie-restricted (CR) groups ($P < 0.05$). *Open triangle* indicates significant difference from the control of AL or CR group ($P < 0.05$).

Pulmonary Cell Populations

Cells obtained by lavage were enumerated on a hemocytometer and viability was assessed by trypan blue exclusion. Following dilution, 5 $\times$ 10^4 cells from each sample were prepared by cytospin for Diff-Quik staining (American Scientific, Sewickley, PA). Differential counts were performed on 200 cells for identification of AM, polymorphonuclear leukocytes (PMN), and lymphocytes.

Alveolar Macrophage Isolation

Animals were euthanized with CO_2, the tracheas cannulated, the lungs resected, and bronchoalveolar lavage (BAL) performed by infusing the lung six times with a total of 40 ml of Ca^{2+}/Mg^{2+}-free PBS. BAL fluid was centrifuged (450 $\times$ *g* for 10 min) and erythrocytes were lysed with ammonium chloride lysing buffer. The pelleted cells were diluted to a concentration of 1 $\times$ 10^6 cells/ml in RPMI 1640 culture medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 2 mM L-glutamine (GIBCO BRL). Cell viability assessed by trypan blue exclusion was always greater than 98%. The cells were initially incubated for 30 min (5% CO_2, 37°C) to allow adherence of AM to the surface of microscope slides or culture plates. Non-adhered cells were then removed by rinsing three times with warm media. In pathogen-free rats, greater than 98% of the adherent cells obtained by this procedure are AM (26).

In Vitro **Phagocytosis**

To assess the effect of caloric restriction on alveolar macrophage phagocytosis following ozone exposure, animals were exposed to 0.8 ppm ozone or chamber air (control) for 3 h and BAL cells were collected. Following centrifugation (450 $\times$ *g*, 10 min), the AM were resuspended to a final concentration of 5 $\times$ 10^5 cells/ml in serum-free RPMI 1640 medium containing 5 mM glutamine, and 0.5-ml aliquots were plated in quadruplicate in microscope slide tissue-culture chambers (Nunc, Naperville, IL). The cells were initially incubated for 30 min (5% CO_2, 37°C) to allow adherence of AM to the surface of microscope slides. Non-adhered cells were then removed by rinsing and inverting the chambers 3 times with warm medium. Fluorescent latex beads (1.46 μm in diameter; Coulter, Hialeah, FL) were then added at a cell-to-bead ratio of 1:50 in 0.3 ml of RPMI and the cultures were incubated on a revolving platform for 3 h (37°C, 5% CO_2). Following the incubation period, the culture chambers were inverted and the cells fixed and stained with Diff-Quik. Prior to examination, the slides were dipped 3 times in methylene chloride for a total of 15 s (5 s each time) to dissolve extracellular polystyrene beads. A phagocytic index was determined by examining the number of beads in each of 200 cells.

In Vitro **Alveolar Macrophage Culture**

Alveolar macrophages (5 $\times$ 10^5 cells/ml) were seeded in 1-ml volumes into 24-well culture dishes for supernatant collection, or in 3-ml volumes into 12-well culture dishes for RNA extraction. Following incubation at 37°C and 5% CO_2 for 1 h and the removal of nonadhered cells, the cultures were treated with lipopolysaccharide (LPS; Sigma,

St. Louis, MO) and incubated for 2 h for RNA isolation or 18 h for supernatant collection. These time points were previously shown to be optimal for cytokine messenger RNA (mRNA) expression and secretion, respectively, in AM (26, 27). Each experiment was conducted with cells harvested from the BAL fluid pooled from five animals on each of the two dietary treatments.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells in each well were homogenized in 1 ml of Ultraspec RNA (Biotecx Laboratories, Houston, TX) and total cellular RNA was extracted according to the manufacturer's procedure. To synthesize complementary DNA, 1.0 μg of RNA was resuspended in a 20-μl final volume of the reaction buffer (25 mM Tris-HCl, 37.5 mM KCl, 10 mM dithiothreitol, 1.5 mM $MgCl_2$, 10 mM of each deoxynucleotide triphosphate, pH 8.3 [Perkin-Elmer Cetus, Foster City, CA]) containing 0.5 μg oligo d(T) 12–18 primer (GIBCO BRL). After the reaction mixture reached 42°C, 200 U SuperScript RT (GIBCO BRL) was added into each tube, incubated for 30 min at 42°C, and stopped by denaturing the enzyme at 99°C for 5 min. The reaction mixture was diluted with distilled water to 100 μl. PCR primers for rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH), TNF-α, and IL-6 were purchased commercially from Clontech (Palo Alto, CA). The sequences of the primers were as follows: (*1*) G3PDH (sense: 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′, antisense: 5′-CATGTAGGCCATGAGGTCCACCAC-3′); (*2*) TNF-α (sense: 5′-TACTGAACTTCGGGGTGATTGGTCC-3′, antisense: 5′-CAGCCT-TGTCCCTTGAAGAGAACC-3′); and (*3*) IL-6 (sense: 5′-CAAGAGACTTCCAGCCAGTTGC-3′, antisense: 5′-TTGCCGAGTAGACCTCATAGTGACC-3′).

Amplified PCR products along with the molecular weight marker, ΦX174 DNA HaeIII digest (Sigma), were separated electrophoretically on 1% agarose gel (UltraPure; Sigma) at 75 V for 60 min and visualized by ultraviolet illumination after staining with 0.5 μg/ml ethidium bromide. Gels were photographed with Type 55 positive/negative film (Polaroid, Cambridge, MA). The relative changes in mRNA transcripts were determined using the Eagle Eye II Still Video System (Stratagene, La Jolla, CA). Densitometric analysis of the captured image was performed using NIH Image 1.54 image analysis software. The area under the curve was normalized against G3PDH content (28).

TNF-α Bioassay

TNF-α activity was measured in culture supernatants using the L929 mouse fibroblast (ATCC, Rockville, MD) lysis bioassay in the presence of 6 μg/ml actinomycin D (29) as performed in this laboratory (26). The detection limit of the assay is 0.02 ng/ml.

Nitric Oxide Determination

Nitric oxide production by cultured AM was estimated by determination of nitrite, a stable derivative of nitric oxide, in the conditioned culture media. Briefly, 50 μl of supernatant was combined with 50 μl of Griess reagent (0.5% sulphanilamide and 0.05% napthylethylenediamine), incubated for 10 min at room temperature, and read at 570 nm in a microtiter plate reader. The concentration was determined against a standard curve employing varying concentrations of sodium nitrite (30).

Lactate Dehydrogenase (LDH) Measurement

To assess cytoplasmic leakage, LDH in culture supernatants was measured immediately after supernatants were harvested using a single reagent system (LD-L 20; Sigma) and measured at 340 nm (27).

Statistical Analysis

Data were analyzed using analysis of variance. The explanatory variables were LPS concentrations, hours after bacterial challenge, feed regimen (*ad libitum* or calorie-restricted), and inhalation exposure (air or ozone). The response variables were bioassay endpoints, bacterial CFU, differential cell counts, and phagocytic index. When appropriate, pairwise comparisons were performed as subtests of the overall analysis. Statistically significant differences were reported when the P value was less than 0.05. The significance levels of multiple comparisons were adjusted from raw numbers using a modified Bonferroni correction.

Results

The effect of caloric restriction on phagocytic activity was assessed in AM isolated from control and ozone-treated rats using latex beads (Figure 2). The phagocytic indices were significantly elevated in AM from calorie-restricted rats relative to *ad libitum*-fed controls in both air- and ozone-exposed groups. Ozone significantly decreased the phagocytic index in *ad libitum*-fed but not calorie-restricted rats.

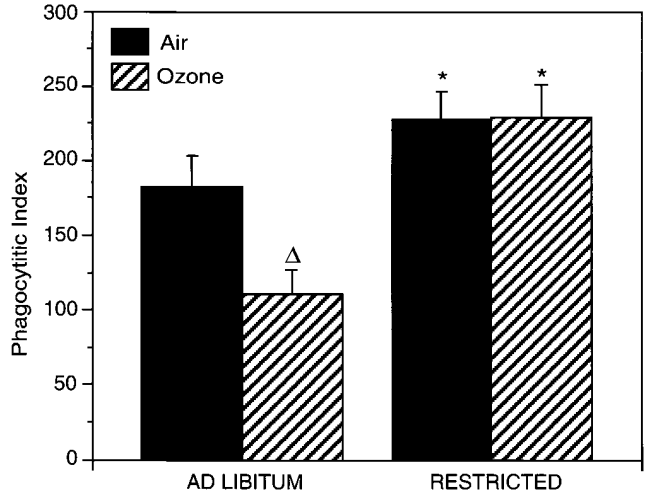

Figure 2. The effect of caloric restriction on *in vitro* phagocytosis of latex beads by AM following ozone challenge. The number of beads ingested per 200 AM from rats exposed to air or 0.8 ppm ozone for 3 h was enumerated and expressed as a phagocytic index ($n = 10$). *Asterisk* indicates significant difference between *ad libitum*-fed and calorie-restricted groups of air or ozone treatment ($P < 0.05$). *Open triangle* indicates significant difference from the air control ($P < 0.05$).

TABLE 2

Effect of caloric restriction on total and differential cell counts in BAL fluid from ozone-exposed rats at various times after bacterial infection

	Ad Libitum				Calorie-restricted			
Time after Infection (*h*)	Total Cell No. ($\times 10^5$)	PMN (%)	LYM (%)	MAC (%)	Total Cell No. ($\times 10^5$)	PMN (%)	LYM (%)	MAC (%)
0	1.10 ± 0.14	0.56 ± 0.24	0.56 ± 0.24	98.89 ± 0.39	1.31 ± 0.21	0.89 ± 0.35	0.33 ± 0.17	98.78 ± 0.40
6	1.13 ± 0.11	15.50 ± 2.18*	0.38 ± 0.26	84.00 ± 2.33*	1.31 ± 0.11	1.88 ± 0.35†	0.50 ± 0.27	97.89 ± 0.63†
24	1.13 ± 0.08	32.63 ± 4.09*	1.38 ± 0.46	66.00 ± 4.31*	1.35 ± 0.12	5.63 ± 1.99†	0.88 ± 0.13	93.50 ± 2.04†
48	1.70 ± 0.32	11.50 ± 4.87*	0	88.50 ± 4.87*	0.97 ± 0.68	4.63 ± 1.78	0	96.00 ± 1.78

Definition of abbreviations: LYM = lymphocyte ; MAC = macrophage.
* Significantly different ($P < 0.05$) from 0-h time point.
† Significantly different ($P < 0.05$) from corresponding *ad libitum* value.
Means ± SEM; $n = 10$ or 15 animals/group.

Because suppression of alveolar macrophage phagocytic function by ozone can be associated with impaired clearance of *Streptococcus* from the lungs, the effects of caloric restriction on *in vivo* clearance of *S. zooepidemicus* from the lungs of animals exposed to ozone was assessed (Figure 1). Ozone impairment of bacterial clearance was evident within 24 h after exposure in the *ad libitum*-fed group, while no such impairment was found in the calorie-restricted group after exposure. Differences in bacterial clearance between *ad libitum*-fed and calorie-restricted groups were still evident at 48 h after infection. In addition to bacterial clearance, the influx of PMN into the lungs occurring in rats exposed to bacterial challenge has been used as an indicator of host resistance. Hence, the effect of caloric restriction on total and differential cell counts in BAL fluid from ozone-exposed rats was examined at 0, 6, 24, and 48 h after bacterial challenge (Table 2). There was a significant increase in pulmonary infiltration of PMN following infection in ozone-treated *ad libitum*-fed rats. In contrast, no such increase occurred in calorie-restricted rats, suggesting that enhanced susceptibility to *Streptococcus* usually associated with ozone exposure was mitigated in calorie-restricted rats via augmented alveolar macrophage phagocytic function. The effects of caloric restriction on bacterial clearance and inflammation in rats infected in the absence of ozone were not assessed because the bacteria are cleared rapidly (within 24 h) and there is no inflammatory response without ozone (19); hence, there is no opportunity for caloric restriction to improve the situation.

The effects of caloric restriction on the induction of inflammatory mediators in the lung was then examined. Alveolar macrophages from *ad libitum*-fed and calorie-restricted rats were cultured with LPS and the release of nitric oxide was determined. For both groups, nitric oxide production increased at LPS concentrations above 0.5 ng/ml compared with untreated controls (Figure 3). Nitric oxide production was significantly lower in the calorie-restricted group compared with that of the *ad libitum*-fed group for both the constitutive baseline (8.8 μM) and LPS-stimulated level. It should be noted that at the concentrations of LPS tested there was no cytotoxicity, as evidenced by lack of LDH release (Figure 3, *inset*).

Bacterial endotoxin was also used to stimulate the expression and production of proinflammatory cytokines in AM. Alveolar macrophages from both dietary groups were incubated for 18 h with increasing concentrations of LPS and the supernatants collected for LDH and TNF-α quantitation (Figure 4). TNF-α secretion in culture supernatant was significantly increased by LPS at concentrations above 0.25 ng/ml in both *ad libitum*-fed and calorie-restricted groups compared with their respective controls. Again, no treatment-related change in LDH membrane leakage was found at LPS concentrations tested (Figure 4, *inset*). Both the basal (constitutive) and LPS-stimulated levels of TNF-α were significantly lower in the calorie-restricted group than

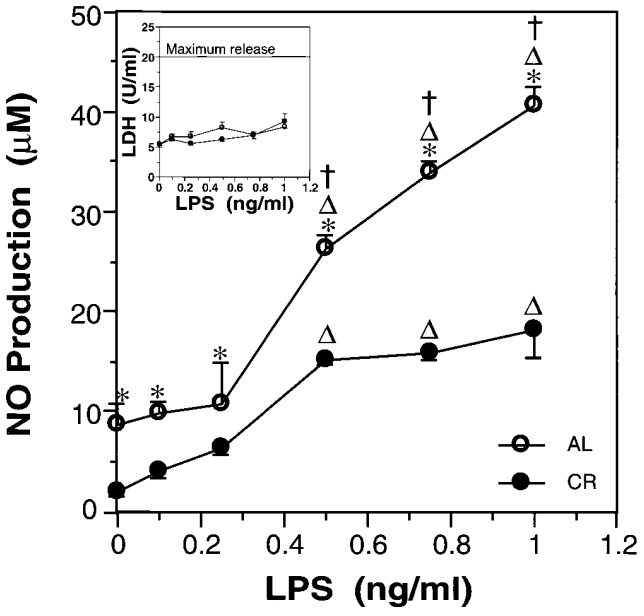

Figure 3. Effect of caloric restriction on LPS-induced nitric oxide production. Rat AM (1×10^6 cells per well) were treated with 0, 0.1, 0.25, 0.5, 0.75, or 1 ng/ml LPS for 18 h. Nitric oxide and LDH production were measured as described in MATERIALS AND METHODS. Values represent means ± SEM of four individual wells ($n = 4$). *Asterisk* indicates significant difference between *ad libitum* (AL)-fed and calorie-restricted (CR) groups ($P < 0.05$). *Open triangle* indicates significant difference from the control of AL or CR group ($P < 0.05$). *Dagger* indicates significant difference between AL and CR groups after adjustment for the existing difference without LPS treatment ($P < 0.05$). Results are representative of replicate experiments.

in the *ad libitum*-fed group, and this difference persisted with increasing concentrations of LPS up to 0.75 ng/ml.

To determine the effect of caloric restriction and LPS on inflammatory cytokine gene expression, AM from *ad libitum*-fed and calorie-restricted groups were treated with increasing concentrations of LPS for 2 h and RT-PCR was conducted on isolated RNA. As previously shown (28), cytokine TNF-α and IL-6 mRNA transcripts were present at low basal levels. Both basal and LPS-induced TNF-α and IL-6 mRNA levels were reduced in AM from calorie-restricted animals compared with those isolated from *ad libitum*-fed rats (Figure 5).

Discussion

Alveolar macrophages constitute the first line of defense against microbial infection in the lung (16). Previous studies have demonstrated that alveolar macrophage phagocytic activity is impaired by ozone exposure and that this is closely associated with enhanced disease following challenge with a relatively avirulent organism, *S. zooepidemicus* (19, 21). In rats, ozone-enhanced infection is characterized by a delayed clearance of bacteria from the lung and an influx of PMN. Compared with *ad libitum*-fed rats, ozone-induced suppression of bacterial clearance and the subsequent infiltration of PMN were largely prevented in calorie-restricted rats *in vivo*. Furthermore, phagocytic activity was higher *in vitro* in AM isolated from calorie-restricted rats. Based on our observations of differential responses *in vitro*, it is reasonable to assume that proinflammatory cytokine expression was lower in AM in calorie-restricted rats than in *ad libitum*-fed rats, thereby causing less infiltration of PMN into the lungs. Considered together, this suggests that caloric restriction enhanced phagocytic function of AM *in vivo*. It is possible, of course, that in addition to being more phagocytic, AM from calorie-restricted rats are inherently more bactericidal than those from *ad libitum*-fed rats. However, this study did not assess endpoints specifically related to bacterial killing such as nicotinamide adenine dinucleotide phosphate-reduced oxidase activity and iNOS-driven nitric oxide production. Because alveolar macrophage function was enhanced by caloric restriction even in air-exposed rats, caloric restriction may increase resistance against more virulent, gram-positive infections independent of ozone exposure. Differences in basal phagocytic function (i.e., those exhibited in air controls) are evident in strains of mice exhibiting differential susceptibilities to ozone-enhanced diseases (21). The effect of dietary restriction on ozone dosimetry in the BAL environment measured by the binding of ^{18}O to protein and cells of BAL fluid was previously studied and discussed extensively (23). The reduced ozone deposition in diet-restricted rats may be attributed partially to the increased concentrations of ascorbate in BAL fluid. From our present study, we believe that protection of ozone-induced impairment of macrophage phagocytic function in calorie-restricted rats may also be attributed, in part, to the increased levels of antioxidants in the fluid bathing the lung surface, which minimizes the ability of ozone to reach significant biologic targets.

When phagocytosis of AM is sufficient, an inflammatory response to *S. zooepidimicus* apparently is not needed to clear this gram-positive organism from the lungs and does not occur as evidently in the calorie-restricted rats. However, the ozone-impaired phagocytosis in *ad libitum*-fed rats prompted a robust inflammatory response by recruiting PMN to the site of infection. Activated PMN facilitate bacterial clearance, but they can cause tissue damage as well. In contrast, an inflammatory response is usually an important component of the lung's defense against gram-negative bacteria as well as a source of lung injury. Endotoxin elicits many of the inflammatory events seen following gram-negative bacterial infection. A typical response to such an infection in the lung is characterized by three events: an increase in local blood supply; an increase in capillary permeability caused by retraction of the endothelial cells; and an increase in migration of inflammatory cells, especially neutrophils, from capillaries to the sites of infection (31). Chemotactic events are regulated largely by inflammatory mediators such as nitric oxide and cytokines including TNF-α and IL-6. Systemic or pulmonary LPS challenge elevates plasma and lung TNF-α and IL-6 levels. Similarly, LPS treatment of AM *in vitro* increases gene expression of proinflammatory cytokines and chemokines including TNF-α and IL-6 (28). Macrophages are also able to generate large amounts of nitric oxide shortly after exposure to cytokines or LPS (32, 33). If overproduced dur-

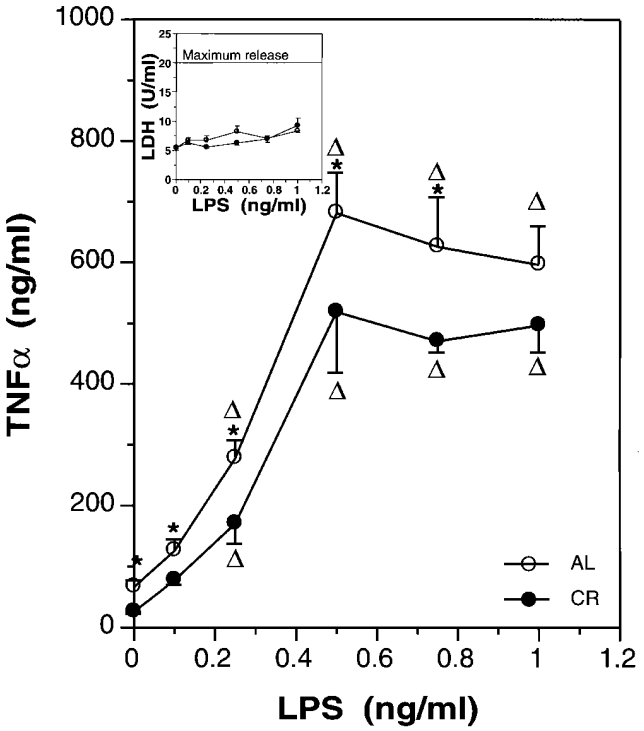

Figure 4. Effect of caloric restriction on LPS-induced TNF-α secretion. Rat AM (1×10^6 cells per well) were treated with 0, 0.1, 0.25, 0.5, 0.75, or 1 ng/ml LPS for 18 h. TNF-α and LDH secretion was measured as described in MATERIALS AND METHODS. Values represent means ± SEM of four individual samples ($n = 4$). *Asterisk* indicates significant difference between *ad libitum* (AL)-fed and calorie-restricted (CR) groups ($P < 0.05$). *Open triangle* indicates significant difference from the control of AL or CR group. Results are representative of replicate experiments.

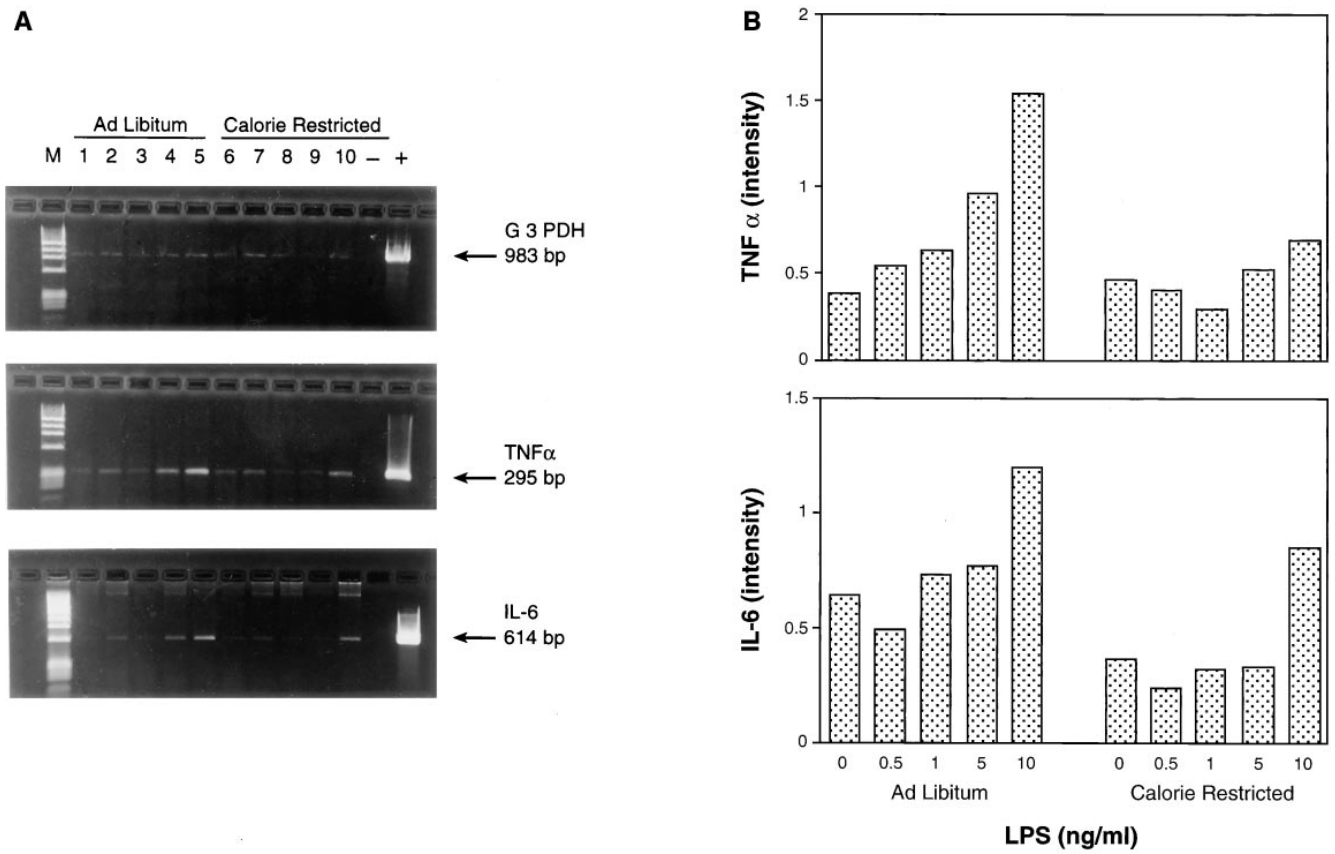

Figure 5. (*A*) Effect of caloric restriction on LPS-induced increase in TNF-α and IL-6 mRNA. Rat AM (3×10^6 cells per well) were treated for 2 h with 0, 0.5, 1, 5, or 10 ng/ml LPS. RT-PCR was used to determine relative changes in TNF-α and IL-6 mRNA expression. M = molecular weight marker (ΦX174 DNA HaeIII digest); *lanes 1–5* = 0, 0.5, 1, 5, and 10 ng/ml LPS, respectively, in *ad libitum* (AL) group; *lanes 6–10* = 0, 0.5, 1, 5, and 10 ng/ml LPS, respectively, in calorie-restricted (CR) group; *lanes* − and + represent negative (water) and positive (commercial) controls, respectively, for each of the primers used. Loading equivalence was verified by equal intensities of G3PDH mRNA. The expected sizes of the PCR products are: 983 base pairs (bp) for G3PDH, 295 bp for TNF-α, and 614 bp for IL-6. (*B*) Relative changes in TNFα and IL-6 mRNA. The video image intensities of TNF-α and IL-6 mRNA transcripts in (*A*) were determined using the Eagle Eye II Still Video System and are expressed in arbitrary units. Densitometric analysis of the captured image was performed using the NIH Image 1.54 image analysis software. The area under the curve for TNF-α or IL-6 was normalized against corresponding G3PDH contents.

ing infection, nitric oxide precipitously causes local tissue damage and vasodilation, which increases blood flow to the site of infection. Since significantly lower levels of nitric oxide were found in calorie-restricted rats, it is reasonable to assume that nitric oxide-induced inflammation and tissue damage associated with a gram-negative infection would be greatly reduced in calorie-restricted rats; however, clearance of the bacteria might be impaired. The reduced production of nitric oxide in calorie-restricted animals is not due to lack of dietary arginine, the precursor of nitric oxide, because both dietary groups consumed similar levels of protein. Nor can these effects be attributed to reduction in any other nutrient becasue the consumption of all nutrients except carbohydrates were identical in both dietary groups (Table 1). We have previously shown that caloric restriction results in increased concentrations of ascorbate in the lung lavage fluid (23). In view of special energy requirements needed for oxidative burst, it is tempting to speculate that there may be interactions between extracellular ascorbate concentrations, glucose availability, and macrophage function. Indeed, interactions between transport of ascorbate/dihydroascorbate and glucose/hexose as a regulatory feature have been suggested in work with rabbit ciliary epithelium (34), human neutrophils (35), and oocytes expressing mammalian transport proteins (36). Clearly, more work is required to elaborate on these possibilities.

TNF-α, a primary proinflammatory cytokine, exhibits both paracrine and autocrine effects to activate macrophages, eosinophils, and neutrophils. Thus, TNF-α stimulates its own expression as well as other cytokines and chemokines (28). TNF-α also elevates the expression of endothelial and leukocyte adhesion molecules such as intercellular adhesion molecule-1, thereby facilitating the migration of inflammatory cells from capillaries to the sites of infection. Since both TNF-α and IL-6 levels in calorie-restricted rats were lower than those in *ad libitum*-fed rats, we speculate that the concentrations of other proinflammatory cytokines, chemokines, and mediators may also be lower in calorie-restricted rats, thus further dampening inflammatory responses.

In summary, this study demonstrated that caloric restriction enhanced alveolar macrophage phagocytic activity and improved resistance to challenge with a gram-positive bacteria while also suppressing the production of inflammatory mediators such as nitric oxide and TNF-α. While the latter observation may result in less tissue damage due to infection, it may also impair clearance of bacteria, particularly gram-negatives, from the lung as a result of depressed neutrophil recruitment or inability to develop a normal immune response. Interestingly, the effects of caloric restriction on alveolar macrophage phagocytosis and production of inflammatory mediators were observed *in vitro*. Hence, the reported changes represent a direct and memorable effect on the cellular physiology per se in addition to possible changes in the *in vivo* milieu. If this proves to be a general phenomenon, it suggests that energy-related set-points may be transduced via the immune system to amplify or attenuate host response to environmental stress.

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