Dietary Restriction Mitigates Ozone-induced Lung Inflammation in Rats: A Role for Endogenous Antioxidants

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Studies were undertaken to determine whether dietary restriction protects against acute pulmonary oxidant challenge. Male F344 rats were fed NIH-31 diet either *ad libitum* or at restricted levels equal to 75% that of *ad libitum* intake. After 3 wk of dietary adaptation, animals were exposed by inhalation to 2.0 ppm ozone (O_3) for 2 h or chamber air and evaluated for cellular and biochemical indices of pulmonary toxicity. Compared to air controls, bronchoalveolar lavage fluid (BALF) from O_3 exposed *ad libitum* fed rats contained increased protein (145 versus 380 μg/ml), PMN infiltration (0 versus 11%) and fibronectin (45 versus 607 U/ml). Diet restriction abrogated these indicators of pulmonary inflammation induced by ozone. Binding of $^{18}O_3$ to BALF protein and cells was significantly decreased in diet restricted rats while BALF ascorbate and glutathione levels, but not α-tocopherol or urate, were elevated compared to *ad libitum* fed rats. Taken together, these results indicate that dietary restriction affords protection against O_3-induced oxidant toxicity. Protection is mediated partially by increases in ascorbate in the fluid bathing the lung surface, thereby providing an antioxidant sink which minimizes the ability of O_3 to reach biological targets.
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It is generally recognized that the nutritional, physiological, and pharmacological status of the host markedly impacts its sensitivity to environmental stressors (1–3). Excessive body weight is a significant risk factor in a number of diseases contributing to enhanced morbidity and mortality in humans including cardiovascular and neoplastic diseases. Dietary restriction, with concomitant body weight reduction, increases longevity and ameliorates a variety of spontaneous and chemical-induced pathologies in experimental models including spontaneous cancers and cardiovascular lesions (4, 5). Dietary modulation of these age-related pathophysiologies may be closely related to the fact that diet restriction exerts an antioxidant action against lipoperoxidation, free radical mediated glycation and DNA damage (6–8). Since pro-oxidant activity is one of the major contributors to inflammation, we hypothesized that dietary restriction would influence the response to inflammatory stimuli via modulation of the antioxidant status.

Acute ozone (O_3) inhalation results in transient airway inflammation and diminished pulmonary function in experimental animals and humans (9, 10). This response is characterized by quantifiable indicators in bronchoalveolar lavage fluid (BALF), including neutrophil infiltration, increased protein, generation of inflammatory cytokines, and the release of arachidonic acid metabolites (10). The increased BALF protein appears to result from plasma leakage through the alveolar-capillary barrier of the lung (11, 12). The primary target cells in the lung are thought to be type I and II epithelial cells where oxidative damage may initiate an inflammatory response (13). While O_3 itself is not a radical, it initiates radical-mediated lipid peroxidation which is minimized by antioxidants such as vitamins C and E (14, 15).

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Abbreviations: bronchoalveolar lavage fluid, BALF; glutathione, GSH; polymorphonuclear cells, PMNs.

The work reported here describes our efforts to study the effect of dietary restriction on pathophysiological and biochemical processes associated with O_3-induced lung inflammation. Thus, we have employed inhalation exposure to O_3 as an oxidative challenge to the lung, and measured inflammatory responses and endogenous antioxidant status. Further, by exposing animals to ^{18}O-labeled O_3, subsequent quantitation of ^{18}O binding in various lung compartments provided dosimetric indices of the toxic insult to relevant targets. Our findings indicate that dietary restriction affords protection against ozone-induced toxicity via, at least in part, increases in ascorbate and glutathione concentrations in BALF.

Materials and Methods

Animals and Treatment

Male Fischer 344 rats were procured at 8 wk of age ($\sim$ 195 g) from a commercial vendor (Charles River, Raleigh, NC). Animals were individually housed in polycarbonate cages and maintained under AAALAC-approved conditions in a pathogen-free environment. Deionized water was available *ad libitum*. After acclimation for 3–4 days, the rats were prededicated to specific diets and ozone treatments and the dietary treatments were imposed. Unless noted, NIH-31 diet (Ziegler Bros Inc., Gardners, PA), an open-formula, cereal based diet was used for the feed studies. *Ad libitum* animals were allowed constant access to feed, the consumption of which was determined by daily weighing. The daily mean consumption in the *ad libitum* group was multiplied by 0.75 and the resultant mass of food was offered to the diet restricted animals at 7:00 A.M. In one experiment, semipurified diets were formulated such that the *ad libitum* and restricted groups would be isonutrient with respect to vitamins, minerals, and protein. The composition of these diets are described in Table 1. All dietary regimens exceeded the nutrient requirements of rats as recommended by the National Research Council (16).

After 20 consecutive days of *ad libitum* or feed restriction, rats were placed in stainless steel exposure cages and exposed to 2.0 ppm O_3 or $^{18}O_3$ for 2 h in 0.3 m^3-Rochester-type exposure chambers. Rats exposed to filtered room air were used as controls. Generation of $^{18}O_3$ was accomplished by substituting isotopically pure (98.11 atom%) $^{18}O_2$ (Isotech Inc., Miamisburg, OH) for normal O_2 (primarily $^{16}O_2$) in a silent arc generator (model 03V-0; Orec Inc., Tucson, AZ), according to previously described procedures. The concentration of O_3 was measured continuously using a chemiluminescent analyzer (Bendix model L8002, Louisburg, WV). These analyzers were calibrated every two weeks with a Dasibi transfer standard, which was referenced quarterly to a primary ultraviolet O_3 standard, as previously described (17).

Bronchoalveolar Lavage Fluid (BALF) Collection

BALF was performed as described previously (9). Briefly, rats were anesthetized with 5% halothane, the abdominal vasculature was severed to bleed the animal, a tracheal cannula was inserted to about 0.5 cm above the carina, and the whole lung was lavaged five times with separate aliquots of saline warmed to 37°C at a volume equal to 30 ml/kg of body weight. Since this volume is based on allometric equations relating body weight to the lung volume and is approximately equal to the total lung capacity of the rat, the entire lung surface is lavaged and comparative measurements between animals can be expressed per ml of BALF (17). Cells were pelleted by centrifugation at 1,000 $\times$ *g* for 15 min. A high speed pellet (which is known to contain most of the surface-active lipid material) was separated from the cell-free supernatant of the lavage fluid by centrifugation at 27,000 $\times$ *g* for 30 min. The cell pellet and the high speed pellet were stored at -80°C, then lyophilized. The analysis of ^{18}O was made on about 0.5 mg of lyophilized pellets.

Analysis of Dried Tissues ^{18}O

The assay of lyophilized tissues for excess ^{18}O employed a modification of the method of Santrock and Hayes (18). This system uses an elemental analyzer to convert O_2 in dried tissues to CO, a column filled with I_2O_5 to convert CO to CO_2 and an isotope ratio mass spectrometer for analysis of the resulting CO_2. The CO_2 mass ratios (46/44 daltons) were related to the $^{18}O/^{16}O$ ratios by use of standards included in each sample run. The $^{18}O/^{16}O$ ratios of unexposed tissues were compared with those of exposed tissues to determine whether there was a detectable (statistically significant) increase in the $^{18}O/^{16}O$ ratio in exposed tissues. The mean $^{18}O/^{16}O$ ratio of each tissue was

TABLE 1

Diet composition and nutrient consumption in carbohydrate-calorie restriction experiment

*Ad Libitum**			Diet Restricted†		
Diet Composition* (*g per 100 g diet*)		Actual Nutrient Consumption g/rat/day	Diet Composition† (*g per 100 g diet*)		Actual Nutrient Consumption g/rat/day
Protein	20.3	2.7	Protein	26.5	2.7
Carbohydrate	66	8.8‡	Carbohydrate	55	5.6
Fat	5.0	0.7	Fat	6.6	0.7
Mineral mix	3.5	0.5	Mineral mix	4.6	0.5
Vitamin mix	1.2	0.16	Vitamin mix	1.6	0.16
Energy (Kcal/100 g)	39.1	5.2 Kcal‡	Energy (Kcal/100 g)	38.7	3.9 Kcal

* Diet #D11520 (Research Diets Inc., New Brunswick, NJ). Each rat consumed 13.4 $\pm$ 0.3 g/day (n = 10).
† Diet #D11521 (Research Diets Inc). Each rat consumed 10.3 $\pm$ 0.2 g/day (n = 10).
‡ Consumption significantly different from restricted amount ($P < 0.01$; Student's t-test).

subtracted from each sample and converted to "μg excess ^{18}O/gm dry weight." Values from the unexposed tissues were used to determine background levels of ^{18}O.

Cellular and Biochemical Determinations

Processing of BALF, lung tissue and plasma for protein, ascorbic acid, uric acid, glutathione (GSH) and α-tocopherol concentrations were conducted as previously described (19). Briefly, BALF supernatants were acidified using perchloric acid (PCA; 3% final concentration) and lungs were homogenized in 3% PCA. After proteins were sedimented by centrifugation (20,000 × *g*) supernatants were analyzed by HPLC/electrochemical detection for ascorbate, and an enzymatic recycling assay for GSH. Protein was assayed by coomassie blue using bovine serum albumin as a standard. Total cells from BALF were enumerated using a Coulter Counter (Coulter, Hialeah, FL). For differential cells counts, BALF cell pellets were suspended in saline, and pelleted onto a microscope slide by cytocentrifugation (Shandon Inc., Pittsburgh, PA) and stained with Diff-Quick (Dade Diagnostics, Aguada, PR).

Cytokine Assays

TNFα activity from tissue culture supernatants was measured by quantitating cytolytic activity against the L929 target cell line in the presence of actinomycin D (20). Cytolysis was determined from the reduction in mean absorbance at 570 nm relative to control wells (culture medium only) using a microplate reader. Reference wells containing known amounts of recombinant murine TNFα (Genzyme, Cambridge, MA) were used to generate a standard curve. Rat IL-6 was determined using IL-6-responsive 7TD1 cells, a generous gift of Dr. K. Connolly, Glaxo, Research Triangle Park, NC, via the hexoaminidase method (21). Briefly, 7TD1 cells were washed twice in Iscove MEM medium, adjusted to 2×10^4 cells/ml and 0.1-ml aliquots were added to wells of a 96-well microtiter plate. Equal aliquots of test sample or murine IL-6 standard (R&D Systems, Minneapolis, MN) were added in triplicate to the plates. After incubation for 4 days, the plates were centrifuged, the medium was removed by inverting and gently blotting, and the plates were washed with phosphate buffered saline (PBS). Sixty μl of substrate [1 vol. 7.5 mM p-nitrophenyl-N-acetyl-D-glucosaminide (Sigma, St. Louis, MO) in 0.1 M sodium citrate and 1 vol. 0.5% Triton X-100 in distilled water] was added to each well and the plates were incubated for an additional 4 h at 37°C. Color reagent (0.1 M glycine/NaOH buffer, pH 10.4) was added to each well (90 μl) and absorbance was measured at 405 nm (reference at 650 nm) on a microplate reader. Values were obtained from the standard curve with unknowns being determined from linear regression.

Fibronectin

Soluble fibronectin was quantitated by an indirect ELISA procedure as previously described (22). Briefly, test samples were serially diluted in PBS containing 0.075% Tween 20 and 0.1% bovine serum albumin (BSA) (0.1 ml) and incubated overnight at 4°C with 0.1 ml of a 1:25,000 dilution of rabbit anti-mouse fibronectin (Chemicon, Temecula, CA). The mixture was transferred to microtiter plates (Immulon 2; Dynatech, Chantilly, VA) which had been coated overnight with 2 μg/ml of mouse fibronectin (Chemicon) in 0.2 ml of buffer (10 mM Tris-HCl, 140 mM NaCl, pH 9.6). After incubation at room temperature (RT) for 30 min, the wells were washed with PBS containing 0.05% Tween 20 followed by addition of 0.2 ml of a 1:1,000 dilution of goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Chemicon). The plates were incubated at RT for an additional 90 min, washed and peroxidase substrate was added. The reaction was stopped with 2% oxalic acid in water after 30 min at RT and the absorbency determined at 415 nm. The concentration of fibronectin was determined from a standard curve prepared with purified mouse fibronectin (Sigma). The data are expressed as units of fibronectin/ml of BALF fluid.

Statistics

The animal experiments were designed for 2 × 2 factorial analysis to evaluate effects of *ad libitum* versus restricted feeding, exposure to ozone versus control chamber air, and the interactions of these factors. Individual experiments were conducted using $n = 5$ for each of the four treatments. Data for replicate experiments were pooled for presentation purposes.

Results

To determine whether dietary restriction protects against acute oxidant challenge in the lung, rats were fed either *ad libitum* or at restricted levels equal to 75% that of *ad libitum* intake. After 3 wk of dietary adaptation, animals were exposed by inhalation to ozone or chamber air and evaluated 24 h later for cellular and biochemical indicators of pulmonary toxicity and inflammation. Prior to commencement of the feeding studies, the initial body weight of the rats averaged 195 g (Table 2). The *ad libitum* fed animals gained approximately 58 g during the 3-wk dietary treatments while the weight gain of the restricted animals during the same period averaged 13 g. Overall average body weights of animals destined for the air versus O_3 treatments did not differ. The input volume of lavage fluid was indexed to the sacrifice body weight. Therefore, the initial lavage volume was approximately 20% less in the restricted groups ($P < 0.01$). The volume recovery of the BALF from rats exposed to ozone did not differ statistically from air-exposed rats. There was no effect of diet or O_3 treatment on the volume recovery of the BALF (Table 2). Total cellularity in the BALF fluid was not affected by ozone at the 24 h time point, but was slightly reduced in feed restricted animals (Figure 1). For animals fed *ad libitum*, ozone resulted in marked increases in total polymorphonuclear cell (PMNs) which was prevented by feed restriction. Feed restriction also attenuated protein, fibronectin, and IL-6 levels in BALF induced by O_3 when compared with levels observed in the *ad libitum* group (Figure 2). There was no treatment-related effects on lactate dehydrogenase (LDH) concentrations in the BALF indicating that the effects were not related to frank toxicity nor were there any changes in lung TNF levels, with all groups having low concentrations (data not shown).

TABLE 2

*Effects of diet restriction and acute ozone exposure on selected parameters**

Parameter	*Ad Libitum*		Diet Restricted		Significance		
	Control	Ozone	Control	Ozone	Diet	Ozone	Interaction
Initial body weight (g)	195 ± 2 (10)†	195 ± 4 (10)	193 ± 3 (9)	197 ± 2 (10)	NS	NS	NS
Pre-exposure BW (g)	253 ± 2 (10)	254 ± 2 (10)	208 ± 2 (9)	207 ± 2 (10)	$P < 0.01$	NS	NS
Weight change (g)	57.3 ± 3 (10)	58.1 ± 2 (10)	14.9 ± 2 (9)	10 ± 2 (10)	$P < 0.01$	NS	NS
BALF fluid determinations							
Lavage volume in (ml)	7.5 ± 0.1 (10)	7.4 ± 0.1 (10)	6.0 ± 0.1 (9)	6.0 ± 0.1 (10)	$P < 0.01$	NS	NS
Lavage recovery (%)	72 ± 3 (10)	72 ± 1 (10)	72 ± 0 (9)	72 ± 0 (10)	NS	NS	NS

* Animals were fed *ad libitum* or at restricted levels (75%) for 20 days and then exposed to air (control) or O_3 (2.0 ppm for 2 h). After a 24 hour recovery period, rats were anesthetized and BALF fluid was collected and analyzed as described.
† Mean ± SEM (*n*)

To help establish the mechanism by which feed restriction protects against pulmonary inflammation by ozone, the effect of dietary restriction on O_3 dosimetry in the bronchoalveolar environment was determined. Groups of rats were subjected to the dietary treatments previously described, exposed to 2.0 ppm of ^{18}O-enriched O_3 (or control air) for 2 h and killed for quantitation of ^{18}O binding to dry matter in the low-speed (cellular) and high-speed (surfactant) pellets of the recovered bronchoalveolar fluid. As shown in Figure 3, the dietary treatments had no influence on the background determinations in the air control samples, while ozone exposure resulted in substantial ^{18}O binding in both pellets ($P < 0.01$). There was decreased binding in both the cellular (Figure 3a) and surfactant (Figure 3b) fractions in ozone-exposed animals that were fed restricted compared with *ad libitum* diets. However, the decrease was only significant in the surfactant fraction.

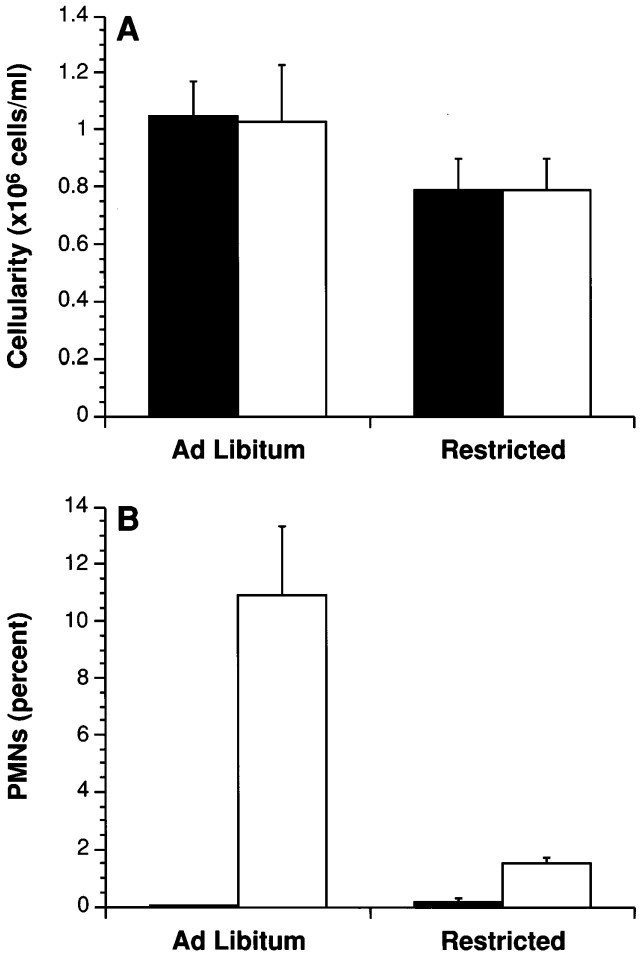

Figure 1. Lung cellularity and PMNs in diet restricted rats exposed to O_3. Rats were fed *ad libitum* or at restricted (75%) levels for 20 days and exposed by inhalation to air (*closed bars*) or O_3 (2.0 ppm for 2 h) (*open bars*). BALF fluid was collected and analyzed as described in MATERIALS AND METHODS. The height of each bar represents the mean ± SEM from 5 rats for determinations of cellularity (*A*) or PMNs (*B*).

Changes in the oxidant tone of the bronchoalveolar environment as a result of the O_3 challenge was evaluated by quantitating ascorbate, total glutathione (GSH), urate, and α-tocopherol. In *ad libitum* fed animals, the concentration of ascorbate in the BALF was markedly reduced following O_3 exposure (Figure 4a). This was in contrast to diet restricted rats where BALF ascorbate was increased in air-exposed rats and remained high after O_3 treatment. O_3 exposure increased ascorbate in the whole lung homogenates in the restricted, but not the *ad libitum* group (Figure 4b). This may be due to the mobilization of internal stores as plasma levels of ascorbate were elevated in the diet restricted animals given air, compared to *ad libitum* rats, and declined after ozone exposure (Figure 4c). Control levels of GSH in the BALF fluid were higher in the diet restricted group and decreased following O_3 treatment to values that were comparable to those in the *ad libitum* fed rats (Figure 4d). Uric acid in the lung homogenates was not influenced by either the dietary treatment or exposure to O_3 (Figure 4e). Similarly, there were no treatment related effects on α-tocopherol levels in the BALF, which averaged about 9 μg/g tissue over all treatments (Figure 4f).

In the forementioned experiments, we intentionally imposed a general dietary restriction, and the consumption of all dietary components was decreased by ~ 25% in the restricted groups. Thus, it was not possible to attribute the above results to the restriction of a particular nutrient or non-nutrient component. Diets were, therefore, formulated wherein the concentrations of vitamins, minerals, fat and protein were supplemented by 25% in the restricted diet allowing feed-restricted animals to consume 25% less

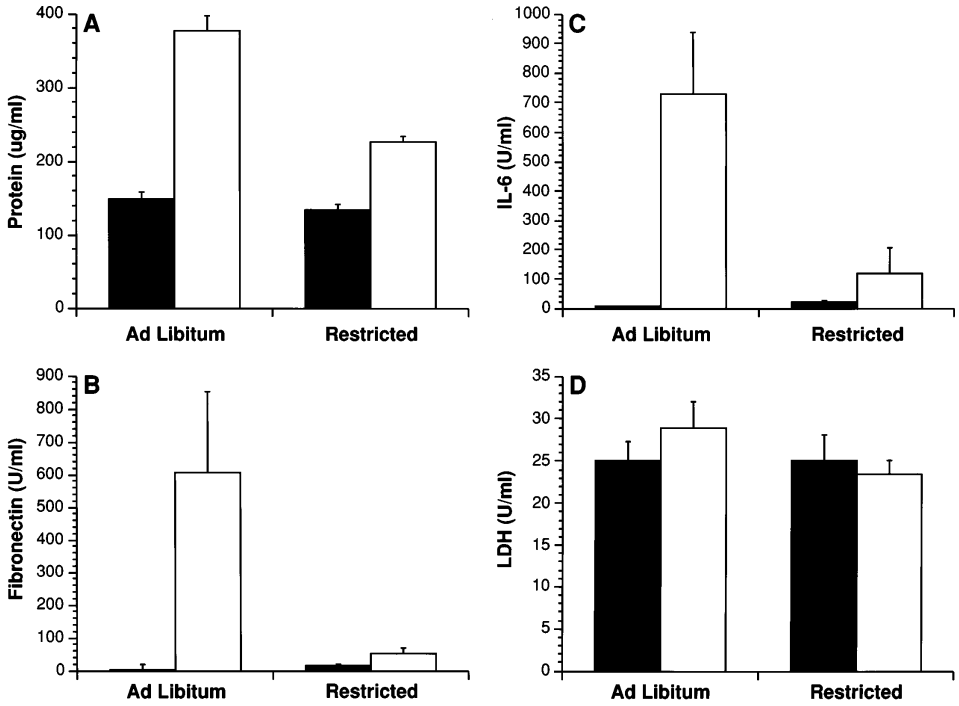

Figure 2. Effect of dietary restriction on selected indicators of toxicity and inflammation following O_3 exposure. Animals were fed *ad libitum* or at restricted (75%) levels for 20 days and exposed by inhalation to air (*closed bars*) or O_3 (2.0 ppm for 2 h) (*open bars*). BALF fluid was collected and analyzed as described in MATERIALS AND METHODS. Values represent mean ± SEM from 5 rats for determinations of protein (*A*), fibronectin (*B*), IL-6 (*C*) and LDH (*D*).

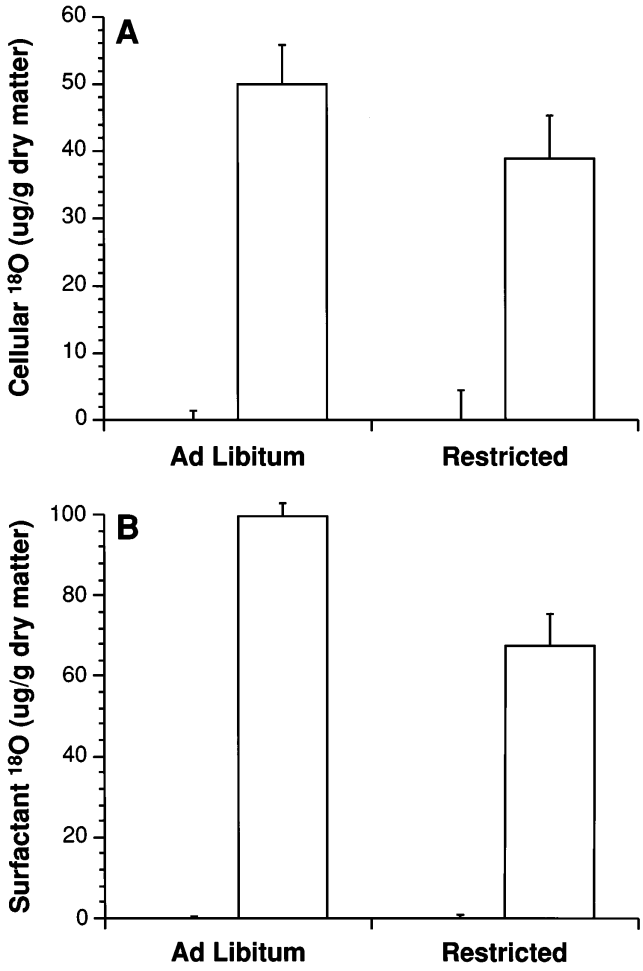

Figure 3. Effect of dietary restriction on ^{18}O-incorporation. Rats were fed *ad libitum* or at restricted levels (75%) for 20 days and exposed by inhalation to chamber air (*closed bars*; not seen) or ^{18}O enriched O_3 (2.0 ppm for 2 h) (*open bars*). BALF fluid was collected as described and subjected to either low speed (1,000 × *g*) or high speed (27,000 × *g*) centrifugation to harvest cells (*A*) or surfactant (*B*), respectively, for ^{18}O quantitation. Values represent mean ± SEM from 10 rats per group (average from 2 experiments) for the low speed determinations or 15 rats (average from 3 experiments) for high speed. Statistical analysis (2 × 2 factorial design) of data from *panel A* reveal a highly significant ($P < 0.001$) treatment effect (air versus O_3) but an insignificant effect from dietary treatment ($P = 0.26$). Similar analysis of data in *panel B* indicate highly significant effects of dietary treatment ($P < 0.001$), ozone treatment ($P < 0.001$), and their interaction ($P < 0.008$).

carbohydrate calories while keeping the intake of all other nutrients and calorie sources comparable between the two groups (Table 1). After the 3-wk feeding period, the rats were exposed to $^{18}O_3$ as described. As seen in Table 3, the dietary treatments caused a marked difference ($P < 0.01$) in $^{18}O_3$ binding in both the high and low speed pellets of BALF. Pellets isolated from the exposed animals in the restricted groups bound about 30% less $^{18}O_3$ than those from the *ad libitum* group.

Discussion

Previous studies have demonstrated that pulmonary toxicity associated with O_3 inhalation in humans and experimental animals is characterized by an inflammatory response initiated by oxidative damage (10, 17). As such, this response can be influenced *in vitro* as well as *in vivo* by exogenous antioxidants such as vitamin C and α-tocopherol (14, 23, 24). Since dietary modulation can influence the ox-

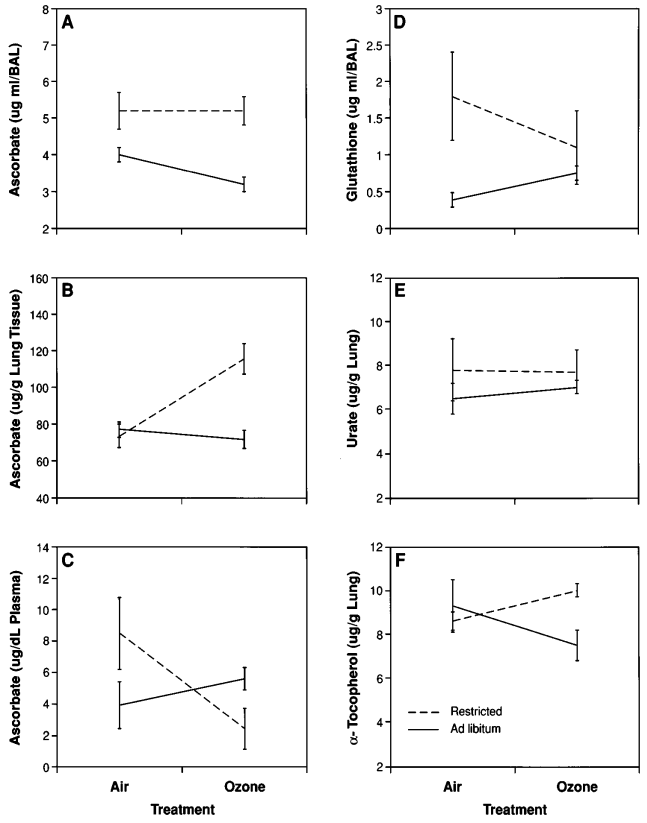

Figure 4. Antioxidant concentration in *ad libitum* (*solid line*) or diet restricted (*dashed line*) rats following O_3 exposure. Rats were treated as described in previous figure legends and BALF fluid, lung tissue and plasma collected for quantitation of antioxidants as described in MATERIALS AND METHODS. Values represent the mean ± SEM of 8 rats for determination of ascorbate in BALF fluid (*A*), ascorbate in lung homogenate (*B*), ascorbate in plasma (*C*), BALF-associated glutathione (*D*), urate (*E*) and α-tocopherol (*F*). Statistical analysis (2 × 2 factorial design) of data from *panel A* indicates a significant treatment effect (air versus O_3; $P < 0.05$), dietary effect (*ad libitum* versus dietary restricted; $P < 0.01$) and interaction between these factors ($P < 0.05$). Analysis of data in *panel B* revealed that both factors and their interaction were significant ($P < 0.01$). Analysis of data in *panel C* revealed that neither diet nor ozone treatment caused significant effects but their interaction was significant ($P < 0.05$). Analysis of data in *panel D* revealed that the effect of diet was significant, while the effect of ozone treatment and the interaction of diet and treatment was not significant.

idant status of the host (7, 8), we hypothesized that any dietary restriction would alter pulmonary toxicity induced by O_3. The results of these studies indicate this is true as restriction protected against acute O_3-induced inflammation and toxicity as assessed by standard indicators of pulmonary toxicity. Compared to *ad libitum* fed animals, diet-restricted rats exposed to equivalent concentrations of O_3 responded with decreased total protein, IL-6 and fibronectin accumulation in the BALF and decreased PMN infiltration when evaluated 24 h after O_3 challenge. Thus, mediators of both fibrotic and inflammatory responses were affected by feed restriction. It is interesting to note that in rats, fibronectin levels are also modulated in the liver following feed restriction (25).

The importance of exogenous antioxidants, especially ascorbate, in lung defense mechanisms is well known. For example, vitamin C has been reported to protect against cellular infiltration from cigarette smoke (26), as well as chronic obstructive pulmonary diseases (23). The antioxidant status of several organs, such as the liver, is increased by diet restriction (6–8) although to our knowledge this has not been evaluated in the lung. The present studies suggest both diet-specific and chemical-specific changes in oxidant status may occur in the lung. Increases in BALF ascorbate and glutathione correlated with diet but only glutathione levels decreased from O_3 exposure. Neither treatment affected urate or α-tocopherol levels. These observations are consistent with previous studies demonstrating that changes in ascorbate are associated with diet, while glutathione levels are frequently modulated by oxidative stress caused by toxic chemicals (7, 24, 27, 28).

The observation that dietary restriction was accompanied by a decrease in $^{18}O_3$ binding in the lung suggests that this treatment may influence the dosimetry of the inhaled O_3 to biological targets, thereby partly mitigating inflammatory effects. Our results showing increased antioxidants in BALF occur in diet restricted, O_3-treated animals are consistent with the interpretation that dietary restriction stimulates endogenous antioxidant status in the BALF fluid thereby enhancing sequestration and detoxification of reactive species. In this respect, ozone-induced pulmonary toxicity is more severe in vitamin C-deficient guinea pigs (27). Binding of $^{18}O_3$ to surrogate surfactant protein *in vitro* is also inhibited by addition of physiological concentrations of ascorbate and to a lesser extent GSH (Hatch, unpublished observations). From these studies it was shown that a 26% increase in ascorbate concentrations would cause a 14% decrease in $^{18}O_3$ incorporation. If the basal ascorbate concentrations in the BALF were higher, the difference in $^{18}O_3$ incorporation would decrease. This would suggest that a substantial portion of the difference between ^{18}O incorporation in food restricted and *ad libitum* rats results from ascorbate in the BALF. Thus ascorbate, unlike tocopherol, reacts so rapidly with ozone (29), that it offers sacrificial protection to the lungs. This is evidenced by the consumption of ascorbate over the 2-h period when animals are exposed to O_3 (Figure 3). Indeed, ascorbate has been shown to convert O_3 to water and dioxygen (30). As these two molecules are not retained by the tissue, it would account for the apparent loss of ^{18}O binding to the biological targets (Table 3). Since feed restriction can lower basal metabolic rates, thereby decreasing the animal's respiration/ventilation (2, 28), it is also possible that the amount of inhaled O_3 is less. While further work will be required to assess the relative contribution of this mechanism in attenuating inflammation, the observation that O_3 binding to lung surfactant and cells was mitigated by carbohydrate-calorie restriction, as well as by restriction of the complete diet, indicates that the anti-inflammatory activity is not due to malnutrition and is attributable to factors modulated, at least in part, by energy consumption.

Factors responsible for concentrating ascorbate in the alveolar extracellular fluid remain largely unknown. Mammalian facilitative hexose transporters are a significant pathway for the cellular uptake and accumulation of vita-

TABLE 3
Effects of caloric restriction on ^{18}O binding in BALF fluid

	Ad Libitum		Diet Restricted		Significance			Single Comparison
Parameter	Control	Ozone	Control	Ozone	Diet	Ozone	Interaction	*Ad Lib* versus Restricted Ozone
^{18}O incorporation into low speed pellet (μg ^{18}O/g dry matter)	0.0 ± 0.15 (5)	43.3 ± 5.9 (5)	0.0 ± 0.5 (5)	20.6 ± 2.6 (5)	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.05$
^{18}O incorporation into high speed pellet (μg ^{18}O/g dry matter)	0.0 ± 0.28 (5)	152.2 ± 11.3 (4)	0.0 ± 0.6 (5)	8.0 ± 17.3 (5)	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.05$

* Animals were fed *ad libitum* or at restricted levels as described in MATERIALS AND METHODS, and exposed to air (control) or $^{18}O_3$ (2.0 ppm for 2 h). Rats were anesthetized immediately after termination of exposure and BALF was analyzed as described.
† Mean ± SEM (*n*).

min C. In fact, interactions between transport of ascorbate/dihydroascorbate and glucose/hexose as a regulatory feature have been suggested in work with rabbit ciliary epithelium (31), human neutrophils (32), and oocytes expressing mammalian transport proteins (33). Blood ascorbate levels have even been implicated in the control of glucose-directed insulin secretion from pancreatic islet cells (34). Although limited, the present data on ascorbate concentrations in the lavage fluid, lung homogenates and blood are suggestive of a transport mechanism in which plasma ascorbate is mobilized via lung capillaries to replenish the ascorbate being consumed in the bronchoalveolar lining fluid. The systemic remobilization of ascorbate and vitamin E have been observed under conditions of dietary restriction (35), or oxidant challenges to the lung (36, 37).

In conclusion, we demonstrate that feed restriction, via enhancing pulmonary antioxidant status, protects against lung toxicity. We cannot preclude the possibility that alterations in oxidant status or other biochemical alterations due to feed restriction may also influence cellular processes which influence O_3 toxicity, such as corticosteroid production (3), and further studies will be required to elaborate these possibilities.

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