Ozone-induced lipid peroxidation and membrane leakage in isolated rat alveolar macrophages: protective effects of taurine

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Preincubation of alveolar macrophages in the presence of taurine resulted in a significant elevation of the intracellular content of this nutrient. Ozone exposure was associated with further increases in free intracellular taurine content. This mobilization of taurine seems to be a defense response to oxidants, since taurine supplementation decreased oxidant injury resulting from exposure of these cells to 0.45 ppm ozone for 30 min. Results indicate that taurine enrichment (100, 250, or 500 μM extracellular taurine) enhanced the ability of ozone-exposed cells to exclude trypan blue dye, decreased lipid peroxidation, lessened the ozone-induced decline in total ATPase, and decreased the leakage of glutathione. Taurine supplementation also decreased protein leakage and lessened the ozone-induced decline in Na^+/K^+ ATPase but only with 100 μM extracellular taurine (i.e., the plasma level of this nutrient). These data suggest that taurine is mobilized from the bound to free state in response to ozone exposure and that it acts to protect alveolar macrophages from ozone-induced damage. The data are consistent with the theory that taurine acts as a membrane stabilizer and/or an antioxidant.

Keywords: taurine; pneumocytes; antioxidant; ion flux; ozone exposure

Introduction

The precise role of taurine, 2-amino ethanesulfonic acid, in human and animal nutrition has remained elusive despite intensive investigation in recent years. Evidence has accumulated supporting the hypothesis that taurine protects cellular membranes against toxic compounds including bile acids, xenobiotics, and oxidants. Taurine has been demonstrated in different biological systems to act as a direct (primary) antioxidant that scavenges oxygen-free radicals and as an indirect (secondary) antioxidant that prevents increases in membrane permeability that result from oxidant damage.[1-3] In the lung, dietary taurine supplementation has been reported to protect against bronchiolar damage induced by oxidant gas, NO_2.[4]

Lung oxidant injury results in metabolic changes such as lipid peroxidation[5,6] and the mobilization of intracellular antioxidants such as glutathione,[7,8] ascorbic acid,[9,10] and vitamin E.[11-13] Although exposures to high oxygen tensions, NO_2, or ozone result in similar morphological and metabolic changes in the lung, the distribution of the injury varies: NO_2 and ozone toxicities primarily involve the epithelium of conducting airways and alveoli near the terminal bronchioles (proximal alveoli), whereas high oxygen tensions induce damage at the levels of the trachea through the distal alveoli.[14]

Pneumocytes found on the alveolar surface exhibit a wide range of sensitivities to oxidant injury.[15-20] When considering alveolar macrophages, type I and type II epithelial cells, type II cells are the most resistant while type I cells are the most susceptible to oxi-

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dant damage. This sequence of susceptibility to oxidants correlates with intracellular levels of taurine in these alveolar cells suggesting that taurine might exert a protective effect against oxidant injury.[21,22] Recently, we have demonstrated that high taurine levels in alveolar macrophages and type II cells were due to the existence of a specialized transport system to accumulate taurine.[21] Further support for a possible antioxidant function of taurine in the alveolar region of the lung was provided by our study of the time sequence of metabolic changes occurring in rat alveolar macrophages during ozone exposure.[23] One of the responses of these cells to oxidant injury was an increase in cytoplasmic taurine. This was associated with a delay of more extensive types of cell damage, such as membrane leakage and decreased cell viability. We concluded that in response to oxidant injury, taurine was mobilized from a bound to a free state in these cells.

The purpose of this study was to investigate further the role of taurine as a possible pulmonary antioxidant. This study evaluated whether elevated cellular taurine levels would modify or prevent ozone-induced oxidative damage in isolated rat alveolar macrophages. To accomplish this, cells were preloaded with various levels of taurine and the effects of in vitro exposure to ozone on various cellular parameters were monitored.

Materials and methods

Animals, housing, and diet

Cesarean-derived, specific pathogen-free, male Sprague-Dawley rats were obtained from Charles River Laboratory (Wilmington, MA, USA). The animals were shipped in filtered cartons and quarantined for one week after arrival and before use in the experiments. One rat in 50 was sacrificed and examined for abnormal pathology and/or infection and the results were judged as negative. The rats were housed in polycarbonate cages (3 rats/cage) and placed in laminar flow hoods within the NIOSH animal facility (AAALAC-approved). The lighting cycle was maintained at 16 hr light, 8 hr dark. Relative humidity averaged 60 ± 4%, and the environmental temperature was controlled at 70–72° F.

The rats were fed a standard laboratory chow diet (Wayne Lab Blox, Wayne Feeds, Peoria, IL, USA), which was sterilized prior to use. Both food and filtered tap water were supplied ad libitum.

Isolation of lung cells

Alveolar macrophages were isolated as previously described.[21] Male Sprague-Dawley rats (200–300 g) were anesthetized with pentobarbital sodium (220 mg/kg body wt). Alveolar macrophages were obtained by pulmonary lavage using ten 8-ml aliquots of ice-cold calcium- and magnesium-free Hank's Balanced Salt Solution (pH 7.4), concentrated by centrifugation at 550*g* for 10 min at 4° C (Sorvall RC-3 Centrifuge, Sorvall Instruments, Newtown, CT, USA), and resuspended in HEPES-buffered medium (10 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$, 5 mM KCl, and 145 mM NaCl; pH 7.4).

The number of cells and the purity of the cell suspension were determined with a Coulter electronic cell counter equipped with a cell-sizing attachment (Model ZB, Coulter Instruments, Hialeah, FL, USA). The cellular preparations contained 84.4 ± 1.2% alveolar macrophages.

Pre-incubation of lung cells with taurine

Isolated alveolar macrophages were pelleted by centrifugation at 550*g* for 10 min at 4° C and resuspended in HEPES-buffered medium (pH 7.4) containing 0, 100, 250, or 500 μM taurine. The cell suspensions were incubated for 30 min at 37° C in a shaking water bath to allow uptake and accumulation of taurine as demonstrated in our previous study.[21]

Exposure of lung cells to ozone

Prior to ozone exposure, the taurine-loaded alveolar macrophages (36–51 × 10^6 cells) were resuspended in 10 ml HEPES-buffered medium containing 0–500 μM taurine and allowed to adhere to the bottom surface of a 75 cm^2 tissue culture flask for 30 min at 25° C.

The ozone exposure system has been described previously by our laboratory.[23] Briefly, ozone was delivered via teflon tubing from the generator (Model #1000, McMillan Electronics Corp., Houston, TX, USA) to the inlet port of the flask, passed over the cells, and withdrawn from the outlet port to an ozone monitor (Model #1003-AH, Dasibi Environmental Corp., Glendale, CA, USA). The flask was mounted on a rocking mixer. The cells were exposed to 0.45 ± 0.05 ppm ozone for 30 min at 25° C while the flask was rocked slowly from side to side (22 cycles/min) to allow direct contact between the cells and the oxidant gas.

Recovery of lung cells

After ozone exposure, the medium was decanted and centrifuged at 500*g* for 10 min to remove non-adherent cells. The supernatant was frozen at −4° C overnight and assayed the next day.

The flasks were incubated with 10 ml ice-cold calcium- and magnesium-free Hank's buffer and placed on ice in a cold room (4° C) for 10 min before the surface was scraped to remove adherent cells. This procedure was repeated for a total of three washes. The adherent cells were concentrated by centrifugation at 550*g* for 10 min. The non-adherent and adherent cell fractions were suspended in HEPES-buffered medium, counted, and then combined prior to conducting cellular assays.

Cell assays

The exclusion of trypan blue dye was determined microscopically as previously described.[24] Briefly, cell

suspensions were incubated with 0.04% (wt/vol) trypan blue dye for 4 min at 25° C. The cells were then treated with 1% buffered formalin and the percent viability determined as (the number of cells excluding dye/total number of cells) × 100.

Chemiluminescence was measured as previously described.[23,25] Alveolar macrophages (5×10^5) were suspended in 5 ml of HEPES-buffered medium containing 100 μl of 10 mg % luminol (5-amino-2, 3-dihydrol-1,4-phthalzinedione). Zymosan-stimulated samples contained 2 mg/ml zymosan. The samples (5 ml total volume) in plastic vials were incubated at 37° C in a shaking water bath and counted for 0.5 min at 3–5 min intervals for 30 min in a Packard Tricarb Scintillation Counter (Model #3255) operated in the out-of-coincidence mode. From plots of cpm versus time, total chemiluminescence was estimated gravimetrically and expressed as relative chemiluminescence per 10^6 cells.

Membrane ATPase activities were measured as inorganic phosphate liberated from ATP. The assay buffer (HEPES) contained 2.5 mM ATP and 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). To estimate the activity of the Na^+-K^+ ATPase by difference, 1 mM ouabain (Sigma) was added to half the samples. After incubation of approximately 5×10^5 cells in medium at 37° C for 1–2 hours, the reaction was stopped by boiling the samples for 10 min. Cell debris was removed by centrifugation at 8700*g* for 5 min (Microfuge, Beckman Instruments Co., Palo Alto, CA). Inorganic phosphate was measured by the method of Ames.[26] To 1 volume of supernatant (350 μl), 2 volumes of the phosphate reagent [0.26 M ferrous sulfate in acid molybdate solution (17% ammonium molybdate · 4 H_2O in 0.6 M sulfuric acid)] were added and the color was allowed to develop for 30 min. The samples were centrifuged again at 8700*g* for 5 min to remove precipitated material, and the absorbances of the supernatants were read at 700 nm and compared to a standard curve from known phosphate concentrations. Activity was expressed as nmol Pi/hr/10^6 cells.

Intracellular taurine concentrations were measured by an HPLC technique which we developed.[27]

Assays of the Medium

Lipid peroxides released into the medium were assayed by the method of Ottolenghi,[28] as modified by Hunter.[29] The absorbance of thiobarbituric acid-reactive substances in the sample was read at 532 nm on a Gilford Model 300-N spectrophotometer (Gilford Instrument Co., Oberlin, OH, USA), and values were expressed as nmol malonaldehyde/10^6 cells using a molar extinction coefficient of $E = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.[30]

Protein leakage into the medium was estimated with the Bio-Rad technique (Bio-Rad Laboratories, Richmond, CA, USA) based on the method of Bradford[31] using bovine serum albumin (Sigma) as the standard. Values were expressed as mg protein/10^6 cells.

Leakage of reduced (GSH) and oxidized (GSSG) glutathione into the medium was determined using the method of Hissin and Hilf[32] as modified by Van Der Zee et al.[33] From standard curves of known GSH and GSSG (Sigma) concentrations, values were calculated and expressed as μg glutathione/10^6 cells.

Potassium ion leakage was measured using atomic absorption spectroscopy (Model #5000, Perkin Elmer Instruments). Samples were diluted in 0.5% $LaCl_3$ (Fisher Scientific, Pittsburgh, PA, USA). Values were calculated from a standard curve of known potassium concentrations (Fisher Scientific) and expressed as ppm/10^6 cells.

Statistical analysis

Data were expressed as means ± SE of between 3–8 separate experiments. Statistical differences between data points were estimated using one-way ANOVA.[34] Significance was set at $P < 0.05$.

Results

The actual intracellular taurine concentrations of alveolar macrophages preincubated in 0–500 μM taurine prior to ozone exposure are given in *Figure 1*. The taurine content of the cells increased with increasing extracellular taurine. This result was expected since rat alveolar macrophrages have been shown to actively transport and accumulate this nutrient via a specialized sodium-taurine co-transport mechanism.[21]

The free intracellular taurine concentrations of alveolar macrophages after exposure to ozone are given in *Figure 2*. Comparing data from *Figures 1* and *2*, cytoplasmic taurine rose by approximately 1.6, 1.3, 5, and 7-fold in ozone-exposed cells incubated at 0, 100, 250, and 500 μM extracellular taurine, respectively. This ozone-induced taurine mobilization was significant in macrophages supplemented with 250 and 500 μM extracellular taurine.

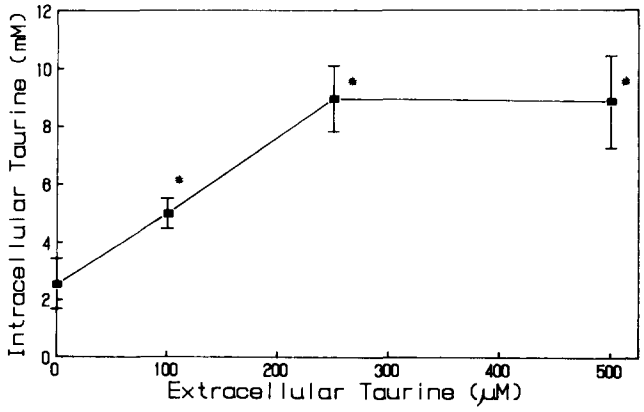

Figure 1 Effect of preincubation of rat alveolar macrophages with variable extracellular taurine concentrations on intracellular taurine content prior to ozone exposure. Accumulation of taurine is both time and concentration dependent. The asterisk (*) indicates a significant increase above the value for cells incubated in 0 μM taurine at the $P < 0.05$ level

Table 1 Effect of taurine supplementation on ozone-induced injury to alveolar macrophages

Parameter	Extracellular Taurine Concentration (μM)[a]			
	0	100	250	500
Cell viability (%)	60.6 ± 3.0	84.1 ± 1.7*	86.0 ± 1.3*	78.3 ± 1.3*
Lipid peroxidation (nmol MDA/10^6 cells)	4.6 ± 0.3	1.4 ± 0.4*	2.1 ± 0.9*	2.3 ± 0.7*
Total ATPase (nmol Pi/hr/10^6 cells)	30.8 ± 7.0	65.9 ± 4.0*	64.0 ± 2.0*	91.3 ± 4.2*
GSH leak (μg/10^6 cells)	0.42 ± 0.14	0.03 ± 0.01*	0.04 ± 0.02*	0.05 ± 0.02*
Protein leak (μg/10^6 cells)	15.9 ± 4.2	5.2 ± 1.4*	10.0 ± 1.0	11.4 ± 2.6
Na^+/K^+ ATPase (nmol/Pi/hr/10^6 cells)	4.4 ± 6.4	29.5 ± 3.4*	2.3 ± 3.0	4.3 ± 8.4

Note: Alveolar macrophages were preincubated in media of different taurine concentrations at 37° C for 30 min prior to being cultured at various taurine levels and exposed to ozone (0.45 ppm for 30 min at 25° C).
[a] Values are mean ± SE from 3–8 separate experiments.
* Significantly different from the value at 0 μM taurine ($P < 0.05$).

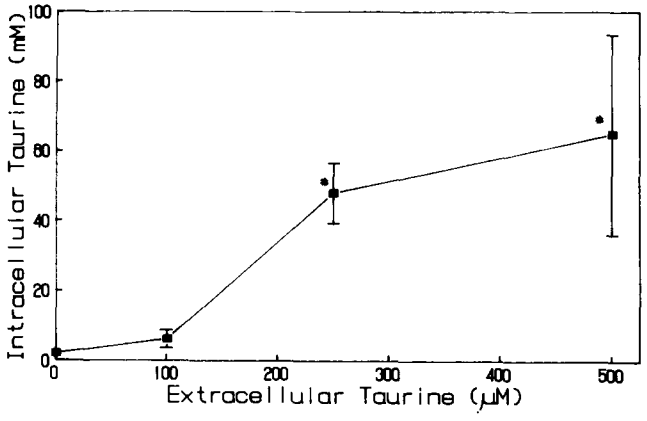

Figure 2 Intracellular taurine content of alveolar macrophages exposed to 0.45 ± 0.05 ppm ozone for 30 min. Cells were preincubated in media of different taurine concentrations at 37° C for 30 min prior to being cultured at various taurine levels and exposed to ozone. At all extracellular taurine levels, free intracellular taurine rises in response to ozone exposure. The asterisk (*) indicates a significant increase from the value for unexposed cells supplemented at the respective extracellular taurine level ($P < 0.05$)

Ozone exposure of alveolar macrophages has been shown to decrease cellular viability, induce lipid peroxidation, decrease total ATPase and Na^+/K^+ ATPase levels, and cause leakage of glutathione and protein.[23] Data in *Table 1* indicate that taurine enrichment decreased ozone-induced damage as measured by these cellular parameters. At 100 μM extracellular taurine (i.e., the approximate plasma level of this nutrient),[27] viability was increased by 38%, lipid peroxidation decreased by 70%, total ATPase increased by 113%, Na^+/K^+ ATPase increased by 625%, GSH leak decreased by 93%, and protein leak decreased by 67% compared to levels measured for ozone-exposed cells incubated in the absence of extracellular taurine. In the case of viability, lipid peroxidation, total ATPase, and GSH leak, this protection from oxidant injury was also significant at 250 and 500 μM extracellular taurine. However, supra-plasma levels of taurine did not significantly elevate Na^+/K^+ ATPase or prevent protein leak.

As shown previously, ozone exposure of alveolar macrophages increased chemiluminescence, decreased cell recovery, and increased leakage of GSSG and potassium ions.[23] In the present study, taurine supplementation at any level failed to significantly alter these ozone-induced changes (data not shown).

Discussion

At 0 μM exogenous taurine, the values for the parameters monitored in the present study are in relatively close agreement with those from our previous study of the time sequence of metabolic changes occurring in isolated rat alveolar macrophages after in vitro ozone exposure.[23] Briefly, in vitro exposure of alveolar macrophages to 0.45 ppm ozone for 30 min resulted in a generalized decline in cellular viability. At 0 μM extracellular taurine, ozone exposure decreased trypan blue exclusion by 9%, increased zymosan-stimulated chemiluminescence by 16-fold, increased lipid peroxidation by 2.3-fold, increased protein leakage by 20%, increased K^+ leakage by 94%, decreased total ATPase activity by 40% and Na^+/K^+ ATPase by 90%, and increased GSH and GSSG leak by 1.8-fold and 48%, respectively. The mechanism for these responses to ozone exposure may be as follows. First, ozone reacts with membrane lipids causing lipid peroxidation.[35,36] Lipid peroxidation could result in a breakdown of the permeability barriers of plasma membranes.[37] Therefore, the second sequence of events would be a generalized decrease in membrane integrity resulting in leakage of protein and K^+ from the cells. K^+ leakage may reflect both the decline in Na^+-K^+ ATPase activity[23] and influx of Ca^{2+} followed by a calcium-induced K^+ leak.[38] In addition to increased membrane leakage, lipid peroxidation may also alter membrane conforma-

tion so that macrophages generate more radicals (measured as chemiluminescence) at rest and in response to zymosan particles.

The results of this study indicate that taurine may function as an antioxidant in rat alveolar macrophages at its physiological concentration of 100 μM (i.e., the plasma concentration of taurine in the rat).[27] At this level of supplementation, taurine significantly protected alveolar macrophages from ozone-induced damage. That is, recovered cells demonstrated an increase in viability as judged by trypan blue exclusion, a decrease in lipid peroxidation, a decrease in the ozone-induced decline in total and Na^+-K^+ ATPase activity, and a lessening of the leak of reduced glutathione and protein. Protection against ozone-induced cell damage was less obvious at supra-physiological concentrations of external taurine, since the decline in Na^+/K^+ ATPase and the leak of protein were not significantly prevented at 250 and 500 μM extracellular taurine. The suggestion that taurine may protect alveolar macrophages from oxidant injury agrees with its ability to protect bronchioles from oxidant injury due to NO_2 exposure.[4]

Taurine has been proposed as both a direct and indirect antioxidant.[1,3] As a direct antioxidant, taurine would act to quench radicals derived from the interaction of ozone with membrane lipids.[35,36] As an indirect antioxidant, taurine would act to stabilize the plasma membrane and thus prevent oxidant-induced increases in membrane permeability. In support of taurine's role as a membrane stabilizer, taurine has been shown to prevent Ca^{2+} influx in cat cerebral cortex resulting from treatment with ouabain[39] and to prevent K^+ leakage in dog heart.[40] In contrast, support for taurine as a direct antioxidant was given by Nakashima et al.[41] who reported that taurine was able to mitigate CCl_4-induced lipid peroxidation in rat liver. Data from the present study are consistent with both views, since taurine significantly reduced lipid peroxidation (direct effect) and significantly increased membrane integrity (indirect effect).

The data on intracellular taurine concentrations after exposure to ozone deserve attention. In our previous study of the time course of metabolic changes occurring in alveolar macrophages during ozone exposure, free cytoplasmic taurine increased with ozone exposure. Therefore, we concluded that taurine was mobilized from cellular bound stores to the free state in response to oxidant injury.[23] In the present study, extremely high intracellular taurine concentrations resulted in alveolar macrophages incubated with 250 μM or 500 μM taurine prior to ozone exposure. There are two possible explanations for this effect: either (1) cells incubated in high taurine levels were stimulated to increase their rate of taurine uptake from the medium in response to ozone exposure; or (2) the cells were mobilizing taurine from bound stores to the free state in response to ozone exposure. Taurine uptake is Na^+ and energy dependent.[21] Since Na^+-K^+ ATPase activity in ozone-exposed cells at 250 or 500 μM taurine was very low, the size of the inwardly directed concentration gradient for Na^+ should have decreased. Thus, it is unlikely that taurine uptake had increased. Therefore, the second explanation seems more likely.

In conclusion, in vitro exposure of alveolar macrophages to ozone results in lipid peroxidation and a decline in membrane integrity and cellular viability.[23] Alveolar macrophages exhibit a Na^+-taurine co-transport system which is able to accumulate taurine within the cell to levels far exceeding those of the external medium.[21] At plasma levels of exogenous taurine, alveolar macrophages are protected partially from ozone-induced damage, i.e., oxidant-induced lipid peroxidation, the fall in membrane integrity and decreases in ATPase activity are partially prevented (*Table 1*). Therefore, taurine seems to act as an antioxidant in these pneumocytes.

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