

Ascorbate deficiency and oxidative stress in the alveolar type II cell

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Brown, Lou Ann S., Frank L. Harris, and Dean P. Jones. Ascorbate deficiency and oxidative stress in the alveolar type II cell. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 17: L782–L788, 1997.—The objective of this study was to determine the impact of limited ascorbate (Asc) availability on type II cell sensitivity to oxidant stress. Guinea pigs were fed diets with or without Asc for 18 days, and type II cells were isolated. Although lung Asc was decreased by 90% in deficient animals (scurbutic), type II cell Asc was decreased by 50%. Upon treatment with 250 μM H_2O_2 , the necrotic injury was twofold greater in scorbutic cells compared with control cells. With 100 μM H_2O_2 treatment, apoptotic injury was twofold greater in scorbutic cells compared with control cells. Although there was less necrotic injury in cells exposed to 95% O_2 , the scorbutic cells were more sensitive than control cells. Asc pretreatment protected against necrosis and apoptosis. The Asc analog isoascorbate provided partial protection and suggested that part of the protection was not chemical detoxification but was Asc specific. We conclude that limited Asc availability resulted in a functional type II cell but a cell more sensitive to oxidant-induced injury.

ascorbic acid; vitamin C; necrosis; apoptosis; isoascorbate

ASCORBATE (Asc) is a central redox-active molecule in intracellular compartments (11) as well as the epithelial lining fluid (20). Upon oxidant exposure, Asc decreases free radical formation as well as propagation of free radicals (for review see Ref. 1). In its role as an antioxidant, Asc cannot be viewed individually because it functions in concert with glutathione, α -tocopherol, and other antioxidants to prevent oxidative damage. Asc also functions in hydroxylation of collagen (19), an extracellular matrix protein that is required for normal differentiation of the alveolar type II cell, and recovery of alveoli denuded by oxidant injury (7, 8).

Like humans and primates, guinea pigs do not synthesize Asc but depend on dietary sources for maintenance of the high Asc levels in pulmonary intracellular and extracellular pools. In studies of guinea pigs fed an Asc-deficient diet for 21 days (scurbutic), the Asc content of the lung and the epithelial lining fluid dropped to 1 and 5%, respectively, of that present in Asc-fed animals (15). Despite the precipitous drop in Asc and the purported role for Asc in free radical reduction, the role of Asc in protecting against pulmonary oxidative injury in the scorbutic animal is unclear. Using ozone as the oxidant source, pulmonary injury as measured by lavage protein was induced by acute ozone exposure (4 h), and this injury was markedly enhanced by Asc deficiency in the scorbutic guinea pig model (15). This suggested that Asc served a protective role in the lung against oxidative stress. However, Asc deficiency

had minimal impact on lung injury and inflammation in animals exposed to ozone for 1 wk (15), suggesting that Asc played a minor role in protecting against oxidant-induced necrotic cell death. Thus the role of Asc in protection from oxidative injury and repair from that injury in the whole lung or any specific cell type is unclear.

Although there are over 40 different cell types, the alveolar type II epithelial cell plays several key roles in maintenance of pulmonary integrity. These cells synthesize and store surfactant phospholipids (9) and apoproteins (12), transport transepithelial ion and water to prevent edema (18), metabolize xenobiotics (6), and repopulate the denuded alveolar epithelium (5). The capacity to proliferate and repopulate the alveolar surface denuded during oxidant stress indicates that type II cells are relatively resistant to oxidant damage. In type II cells isolated from adult rats, the cells are highly enriched in Asc (3.2 mM) and transport Asc against a concentration gradient via a Na^+ -dependent uptake system (4). These studies demonstrate that the Asc concentration is high in this epithelial cell, but the ability of Asc to protect this cell type from oxidative injury or the consequence of decreased Asc availability has not been addressed.

In the current study, the effect of limited Asc availability on the Asc concentration in the type II cell was studied using the scorbutic guinea pig model. The resultant effects of decreased Asc availability on oxidant-induced necrosis and apoptosis were assessed. By comparing the capacity of Asc and isoascorbate (Iso), a nonphysiological analog, to protect type II cells from oxidant injury, the results suggested that some of the protection was specific for Asc and not due to a nonenzymatic chemical detoxification.

METHODS

Materials. Asc, Iso, and guinea pig diets (with or without Asc supplements) were purchased from ICN Biochemicals (Cleveland, OH). Elastase was obtained from Elastin Products (Owensville, MO). Dulbecco's modified Eagle's medium (DMEM)-F-12 (1:1) was obtained from Mediatech (Washington, DC). Antibiotics, calf sera, guinea pig immunoglobulin G, deoxyribonuclease I, insulin, transferrin, selenite, and H_2O_2 were purchased from Sigma Chemical (St. Louis, MO). L-[1- ^{14}C]Asc was purchased from NEN (Wilmington, DE). The terminal transferase dUTP nick end labeling (TUNEL) assay was purchased from Boehringer Mannheim (Indianapolis, IN). The fluorescent DNA dye 2,6-diamino-4-phenylindole (DAPI) was purchased from Molecular Probes (Eugene, OR).

Animals. Pathogen-free adult male Hartley guinea pigs (165–200 g) were purchased from Sasco (St. Louis, MO). Animal studies were conducted according to the *Guide for the Care and Use of Laboratory Animals* by the Department of

Health and Human Services. Guinea pigs from the same shipment were brought into the Emory Veterinarian Facilities and were maintained on their respective diets for 18 days. The Asc-supplemented diet contained 1 g Asc/kg diet (ICN Pharmaceuticals, Costa Mesa, CA), and the animals consumed 15 ± 3 g/300 g body wt. There was no detectable Asc in the deficient diet (ICN Pharmaceuticals), and the animals consumed 12 ± 5 g/300 g body wt. A single diet lot manufactured on the same milling date was used throughout the study.

Culture of type II cells. Procedures for isolation of pulmonary type II epithelial cells from guinea pigs were similar to that described by Dobbs et al. (7). Elastase was instilled into the trachea to dissociate the cells from the lung tissue. Newborn calf serum and deoxyribonuclease I were added, the tissue was minced, and the suspension was sequentially filtered through 100- and 20- μ m nylon mesh. After suspension in DMEM-F-12 that contained 2.5 mM glutamine, 0.04 mg/ml gentamicin, 20 U/ml penicillin, and 0.02 mg/ml streptomycin (denoted as DMEM-F-12), the cells were then plated onto bacteriological plastic dishes coated with 500 μ g/ml guinea pig immunoglobulin G. After 1 h, the nonadherent type II cells were removed, centrifuged, and resuspended in DMEM-F-12 that contained 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml selenite. The insulin, transferrin, and selenite were used rather than fetal bovine serum because serum contained 6–10 μ M Asc. After isolation, $56 \pm 6 \times 10^6$ and $46 \pm 5 \times 10^6$ cells/lung were obtained from control and scorbutic animals, respectively. The cells were then plated onto tissue culture plastic and were incubated for 24 h in 90% air–10% CO₂. After 24 h in culture, 22 ± 3 and $15 \pm 4\%$ of control and scorbutic cells, respectively, were adherent to tissue culture plastic. The alveolar type II cell purity was 94 ± 4 and $91 \pm 5\%$ for control and scorbutic cells, respectively, as determined by polychrome staining. Contamination was primarily due to alveolar macrophages.

Asc and Iso determination. To determine the lung Asc concentration, the tissue samples were frozen in liquid N₂, pulverized with a cold mortar and pestle, extracted with ice-cold 5% metaphosphoric acid, and stored at -70°C . After the type II cells were cultured for 24 h, the medium was removed, acidified with 5% metaphosphoric acid (final concentration), and stored at -70°C . The adherent cells (control or scorbutic) were washed three times, extracted with ice-cold 5% metaphosphoric acid, and stored at -70°C until analysis. Asc and Iso were determined by high-performance liquid chromatography analysis with electrochemical detection. Separation was achieved on a Waters C₁₈ Radial Pak column using isocratic elution (1 ml/min) with 0.6 mM hexadecyltrimethylammonium bromide, 0.5 mM sodium acetate, and 0.15 mM disodium EDTA (adjusted to pH 4.0 with acetic acid; see Ref. 17). Detection was achieved with an amperometric detector (Bio-Analytical Systems, West Lafayette, IN) equipped with a glassy carbon electrode with a 0.6-V applied potential and a silver-silver chloride reference electrode (16).

Asc transport. Na⁺-dependent Asc transport was demonstrated in cultured rat type II cells by Castranova et al. (4). In the current study, Asc transport was examined in the scorbutic guinea pig type II cell to determine if that transport mechanism was maintained and to determine if the scorbutic cell was functional. Media containing L-[1-¹⁴C]Asc (0.1 mM; 20 pCi/nmol) were applied to the cell, and at various time points the cells were washed three times with ice-cold media containing 1 mM Asc. Ice-cold 5% trichloroacetic acid was added to the well, and the radioactivity of the extract was determined. To determine if the transport was Na⁺ dependent, the cells were initially washed three times with Krebs

buffer (pH 7.4) in which choline chloride was substituted for sodium chloride, and then transport of [¹⁴C]Asc was measured in Krebs buffer with choline chloride.

Determination of cell necrosis. After the overnight adherence, wells were washed three times with DMEM-F-12 containing 3 mg/ml bovine serum albumin. To determine baseline viability after 24 h of culture, trypan blue (0.1 ml of 2 mg/ml) was added to the well (0.9 ml of medium). After 5 min, the number of attached cells excluding dye as well as those retaining dye was determined by microscopy using an ocular grid. For studies of H₂O₂ injury, the cells were challenged with 250 μ M H₂O₂ for 2 h, and then trypan blue exclusion was determined immediately. For studies of hyperoxic injury, the cells were exposed to 95% O₂ for 4 h, and then trypan blue exclusion was determined. To determine if exogenous Asc or Iso protected against oxidative injury, the cells were pretreated with Asc or Iso for 30 min, washed three times, and then exposed to H₂O₂ or 95% O₂.

Oxidant-induced apoptosis. After the scorbutic or control cells were cultured for 24 h, the wells were washed to remove nonadherent cells, and medium containing 100 μ M H₂O₂ was added. The cells were cultured for an additional 24 h to allow the cells to undergo apoptosis. In some experiments, cells were pretreated with 0.1 mM Asc or 0.1 mM Iso for 30 min, washed three times, and then treated with H₂O₂. For determination of apoptosis, the cells were washed, fixed, and stained by the TUNEL method with minor modifications (14). Briefly, adherent cells were fixed with freshly prepared paraformaldehyde (4% in phosphate-buffered saline; pH 7.4) for 30 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 (0.1% sodium citrate) for 2 min at 4°C and rinsed with the phosphate-buffered saline. The monolayer was treated with the TUNEL mixture and was incubated in a humidified chamber for 60 min at 37°C in the dark. The deoxynucleotidyl transferase labels single-strand breaks by labeling free 3'-COOH termini with fluorescein-labeled nucleotides. After the monolayers were rinsed three times with phosphate-buffered saline, the fluorescein incorporation into nucleotide polymers was detected by fluorescent microscopy (Zeiss Axioskop with filter set 10). The number of cells stained by the TUNEL method was expressed as a percentage of the total number of cells stained with DAPI, a dye that stains the AT regions of all DNA. The negative control was permeabilized cells treated with the fluorescent nucleotides without deoxynucleotidyl transferase. At least 1,000 nuclei were counted on each dish with 10–20 random viewing fields.

Statistical analysis. Each experiment was performed on cells divided into control and treatment groups from one animal. All points (means $\pm$ SE) were obtained from separate experiments with duplicates for each experiment. Statistical analysis was by analysis of variance, and comparisons were made using Student-Newman-Keuls test to determine probability. Statistical significance was accepted at $P \leq 0.05$.

RESULTS

Asc and Iso determinations. After 18 days on the respective diets, the Asc content of the lung tissue was decreased by 90% in the scorbutic guinea pig compared with the control (Table 1). In the cultured type II cells, the Asc content after 24 h of culture was decreased by 50% in the scorbutic cells compared with control cells (Table 1). With the assumption of an intracellular water space of 0.33 μ l for 10⁶ type II cells (12), the basal Asc concentration in type II cells was 1.8 ± 0.1 and

Table 1. Asc concentration in lung tissue and type II cells from guinea pigs fed control or Asc-deficient diets

	Lung Tissue, nmol/g tissue	Type II Cells, nmol/10 ⁷	Type II Cell Media, μ M
Control	1.85 $\pm$ 0.15	5.82 $\pm$ 0.31	1.25 $\pm$ 0.19
Scorbutic	0.16 $\pm$ 0.04*	2.72 $\pm$ 0.43*	0.28 $\pm$ 0.07*

Values are means $\pm$ SE of duplicate determinations of 6 independent preparations. After the animals were fed their respective diets for 18 days, type II cells were isolated. During the cell isolation procedure, the upper right lobe of the lung was ligated and removed to determine the ascorbate (Asc) concentration as described in METHODS. The remaining lung tissue was used for type II cell isolation. After the isolated type II cells were cultured in the absence of serum for 24 h, the Asc concentration was determined in the cellular and medium fractions. * $P \leq 0.05$ when compared with the control.

0.8 $\pm$ 0.1 mM for control and scorbutic cells, respectively ($P < 0.05$). Attempts were made to quantitate the oxidized form of Asc, dehydroascorbate, but none was detected in control or scorbutic tissue or cells (minimum detection level was 8 pmol).

Although no Asc was added to the medium of freshly cultured type II cells, Asc was present in the medium after 24 h of culture. For control cells, the Asc in the medium represented 23% of the total Asc pool (cells + medium; Table 1). For scorbutic cells, 13% of the total Asc pool was in the medium was decreased by 75% compared with the control.

After a 30-min incubation with 0.1 mM Asc, the cellular concentration increased to 6.3 $\pm$ 0.2 nmol/10⁷ cells (1.9 $\pm$ 0.2 mM) and 3.1 $\pm$ 0.1 nmol/10⁷ cells (1.0 $\pm$ 0.03 mM) for control and scorbutic cells, respectively ($n = 4$; $P \leq 0.05$ compared with the appropriate baseline value). The addition of reducing equivalents through Iso had no corresponding increase in basal Asc for either the control or the scorbutic cells (data not shown). Iso was transported into the cell and was increased from no detectable Iso to 4.7 $\pm$ 0.4 nmol/10⁷ cells (1.5 mM) in control cells. In scorbutic cells, Iso increased from not detectable to 2.2 $\pm$ 0.5 nmol/10⁷ cells (0.7 mM) after 30 min of incubation.

Asc transport. For both the control and scorbutic cells, there was an initial minor component of Asc uptake that was rapid (<15 min; Fig. 1). The uptake after 15 min was linear at a rate of 2.9 and 2.1 nmol $\cdot$ 10⁶ cells⁻¹ $\cdot$ h⁻¹ for control and scorbutic cells, respectively. When choline chloride was substituted for sodium chloride, the transport rate was decreased to 0.03 nmol $\cdot$ 10⁶ control cells⁻¹ $\cdot$ h⁻¹. As observed with control cells, the Asc transport rate was minimal when scorbutic cells were incubated with choline chloride (0.02 nmol $\cdot$ 10⁶ cells⁻¹ $\cdot$ h⁻¹).

H₂O₂-induced necrosis. After 24 h of culture, baseline cell viability was 97 $\pm$ 1% for control cells compared with 88 $\pm$ 2% for scorbutic cells ($P \leq 0.05$; Fig. 2). Incubation with 100 μ M Asc prevented the loss of viability in the scorbutic cell. Iso (100 μ M) similarly prevented loss of viability in the scorbutic cell. After exposure to 250 μ M H₂O₂, the viability of the control cells decreased $\sim$ 50% compared with baseline (Fig. 2). For scorbutic cells, viability was decreased to 35 $\pm$ 6%

after incubation with H₂O₂. If cells were pretreated with 100 μ M Asc for 30 min before incubation with H₂O₂, the control and scorbutic cells were completely protected from H₂O₂ injury. Pretreatment with 100 μ M Iso provided partial but incomplete protection from H₂O₂ injury in control and scorbutic cells.

H₂O₂-induced apoptosis. To determine if the scorbutic cells were more sensitive to oxidant-induced apoptosis, cells were cultured for 24 h after isolation, treated with 100 μ M H₂O₂, and cultured for an additional 24 h. After 48 h of culture, baseline apoptosis (no H₂O₂) for control cells was 10.6 $\pm$ 0.7% as determined by the TUNEL assay. The addition of H₂O₂ to control cells increased the percentage of shrunken and intensely fluorescent nuclei (apoptotic cells) by twofold ($P \leq 0.05$; Fig. 3). Pretreatment with Asc or Iso significantly decreased the H₂O₂-induced apoptosis in the control cells.

In scorbutic cells not treated with H₂O₂, baseline apoptosis was approximately twofold greater than baseline apoptosis for control cells ($P \leq 0.05$). Treatment of scorbutic cells with H₂O₂ increased apoptosis by 50% compared with H₂O₂-treated control cells. Pretreatment with Asc significantly decreased the H₂O₂-induced apoptosis in the scorbutic cell to 24.2 $\pm$ 0.9% ($P \leq 0.05$) but did not provide complete protection compared with basal values ($P \leq 0.05$). Pretreatment with Iso also significantly decreased the H₂O₂-induced

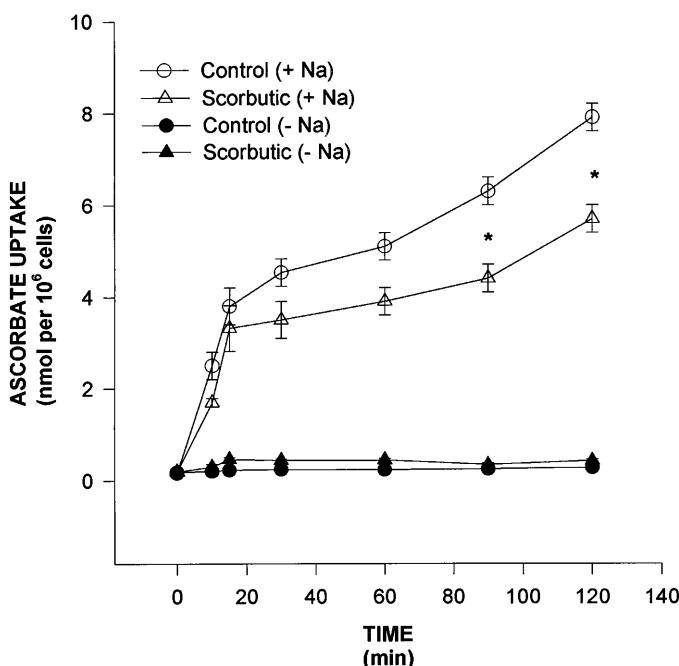


Fig. 1. Time course for ascorbate (Asc) uptake. After the control and scorbutic type II cells were cultured for 24 h, the cells were incubated in media containing 0.1 mM [1-¹⁴C]Asc (20 μ Ci/nmol). Where appropriate, buffer containing choline chloride rather than sodium chloride was applied to the cells before the [1-¹⁴C]Asc was added. To terminate the incubation, the buffer was aspirated, and the cells were washed with ice-cold media containing 1 mM Asc. Ice-cold trichloroacetic acid was added (5% final concentration), and the radioactivity of the extract was determined. Values represent means $\pm$ SE of triplicate determinations of 6 independent cell preparations. For some points, SE is contained within the symbol. * $P \leq 0.05$ when compared with control.

apoptosis, but the protection was less than that observed with scorbutic cells treated with Asc ($P \leq 0.05$).

Viability and 95% O_2 injury. To examine the effects of a more physiologically relevant oxidant, cells were exposed to 95% O_2 for 4 h. The PO_2 of the medium was 168 ± 6 and 247 ± 7 mmHg for cells exposed to room air and 95% O_2 , respectively (Blood Gas Monitor; Radiometer, Copenhagen, Denmark). There was no significant difference in the PO_2 of control and scorbutic cells. The control and scorbutic cells excluding trypan blue were 69 ± 4 and $59 \pm 6\%$, respectively, after hyperoxic exposure ($P \leq 0.05$; Fig. 4). With 100 μ M Asc pretreatment, hyperoxic-induced necrosis was significantly decreased and returned to baseline values. Iso pretreatment provided only partial protection.

To determine the concentration of Asc required to provide protection, cells were pretreated with 1–100 μ M Asc. At 100 μ M Asc (epithelial lining fluid concentration), there was complete protection in control and scorbutic cells (Fig. 5). Exogenous Asc concentrations of 1 and 10 μ M (plasma concentrations) provided partial protection against O_2 injury compared with basal cells or cells exposed to O_2 plus 100 μ M Asc. The effects of hyperoxia on apoptosis were also examined using the TUNEL and ELISA (Boehringer Mannheim) methods. Within the time frame examined, hyperoxia induced necrosis, but no apoptosis was observed in control or scorbutic cells.

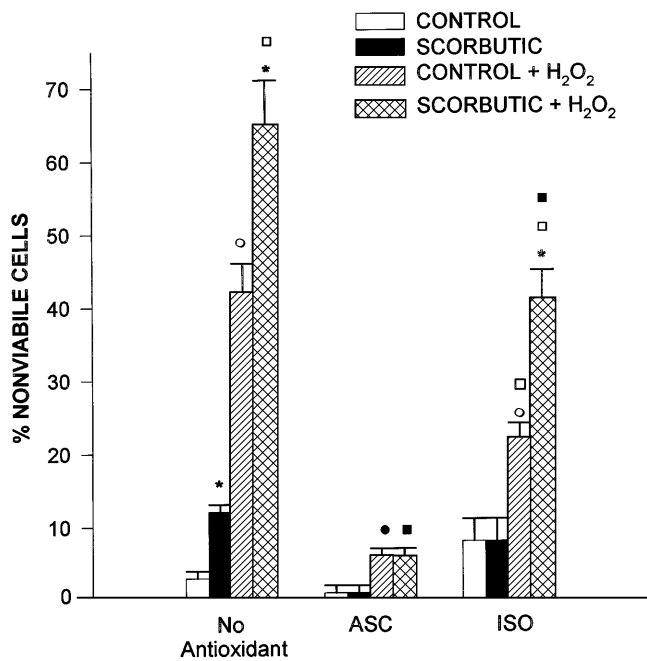


Fig. 2. H_2O_2 -induced necrosis. Control and scorbutic type II cells were cultured for 24 h and then were treated with or without 250 μ M H_2O_2 . Some cells were pretreated with 100 μ M Asc or isoascorbate (Iso) for 30 min and then were washed before H_2O_2 treatment. After incubation for 2 h, the percentage of nonviable cells was assessed by the inability of the cell to exclude trypan blue. Values represent the means $\pm$ SE of duplicate determinations of 9 independent cell preparations. * $P \leq 0.05$ when the scorbutic group was compared with the parallel control group. The following symbols denote $P \leq 0.05$ compared with the specified group: $\circ$, control; $\bullet$, control + H_2O_2 ; $\square$, scorbutic; and $\blacksquare$, scorbutic + H_2O_2 .

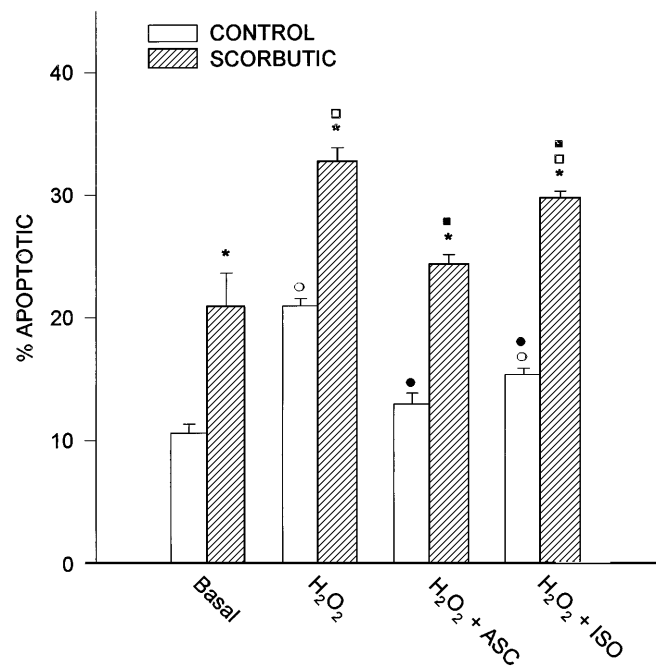


Fig. 3. H_2O_2 -induced apoptosis. Control and scorbutic type II cells were isolated and cultured for 24 h, 100 μ M H_2O_2 was added, and the cells were cultured for an additional 24 h. Where appropriate, cells were pretreated with 1 mM Asc or Iso for 30 min, washed, and then treated with H_2O_2 . After the attached cells were fixed and permeabilized, the monolayer was treated with the terminal transferase dUTP nick end labeling (TUNEL) mixture. Number of cells stained by the TUNEL method was expressed as a percentage of the total number of cells stained with 2,6-diamino-4-phenylindole (DAPI). Values represent means $\pm$ SE of duplicate determinations of 9 independent cell preparations. * $P \leq 0.05$ when the scorbutic group was compared with the parallel control group. The following symbols denote $P \leq 0.05$ compared with the specified group: $\circ$, control-basal; $\bullet$, control + H_2O_2 ; $\square$, scorbutic-basal; and $\blacksquare$, scorbutic + H_2O_2 .

DISCUSSION

Although significant gaps remain in our understanding of the cell biology of Asc, there is some evidence to indicate that Asc is a key component in the balance of free radical formation and propagation. Evidence continues to accumulate that adequate nutritional supply of Asc is important in protection against a variety of oxidative disease processes in the lung. Higher dietary intake and serum concentrations of Asc have been shown to maintain pulmonary function and protect against the development of chronic respiratory symptoms (21). Decreased Asc availability may be particularly relevant to smokers. Severe Asc deficiency (<23 μ mol/l of plasma) has been observed in 24% of active smokers and 12% of passive smokers (23). No severe Asc deficiency was observed in nonexposed nonsmokers.

A study by Willis and Kratzing (24) showed that lung tissue Asc dropped to 1% of the original level when guinea pigs were fed an Asc-deficient diet for 20 days. In the current study, the lung tissue Asc in the scorbutic animal was $\sim 10\%$ of the control lung after 18 days on the respective diets. In type II cells isolated from guinea pigs fed an Asc-deficient diet for 18 days, cellular Asc was decreased by 53%. Because the decrease in type II cells Asc concentration was not as

great as that observed in the lung tissue, this suggests that there was Asc sparing in the type II cell. This ability to concentrate Asc in the type II cell as the tissue concentration decreases may be one mechanism by which the type II cell remains relatively resistant to oxidant injury.

In the medium from control cells, there was 1.2 ± 0.2 μM Asc. This extracellular Asc could not be from an exogenous source because no serum or Asc was added to the medium. Rather, the extracellular Asc probably resulted from Asc released by healthy type II cells or from the release of cytoplasmic material from ruptured necrotic cells. Compared with control cells, the Asc in the medium from scorbutic cells was decreased by 76%. This decrease in extracellular Asc in the scorbutic cells was probably related to decreased intracellular Asc in scorbutic cells.

Castranova et al. (4) demonstrated that there were two components of Asc transport in freshly isolated rat type II cells [a minor rapid component (0–15 min) and a major component (15–120 min)]. In the current study, cultured type II cells from guinea pigs demonstrated a similar Asc transport profile. The transport rate of $2.9 \text{ nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ for control guinea pig type II cells was similar to the rate of $2.4 \text{ nmol} \cdot 10^6 \text{ cells}^{-1} \cdot \text{h}^{-1}$ reported for rat type II cells (4). This transport was dependent on the presence of extracellular Na^+ as

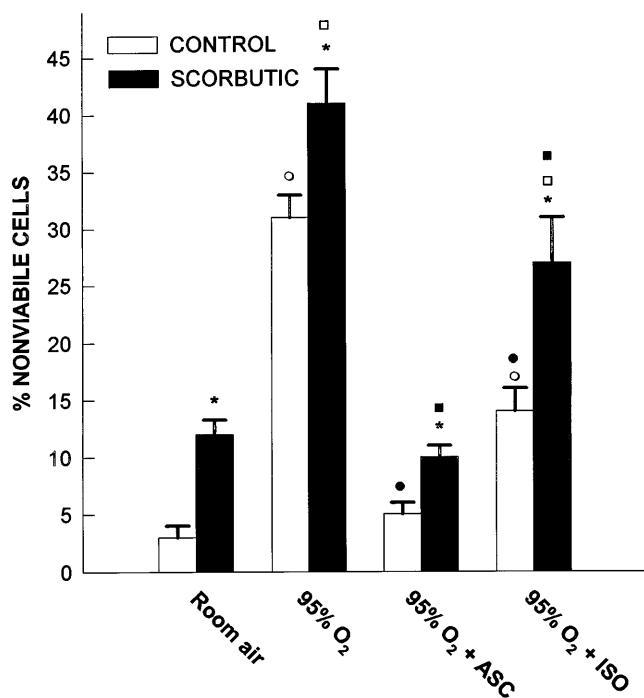


Fig. 4. Hyperoxic-induced necrosis. Control and scorbutic type II cells were isolated and were cultured for 24 h. Some cells were pretreated with $100 \mu\text{M}$ Asc or Iso for 30 min and were washed before exposure to 95% O₂. After exposure to hyperoxia for 4 h, the percentage of nonviable cells was assessed by an inability to exclude trypan blue. Values represent means $\pm$ SE of duplicate determinations of 9 independent cell preparations. * $P \leq 0.05$ when the scorbutic group was compared with the parallel control group. The following symbols denote $P \leq 0.05$ compared with the specified group: $\circ$, control-room air; $\bullet$, control + 95% O₂; $\square$, scorbutic-room air; and $\blacksquare$, scorbutic + 95% O₂.

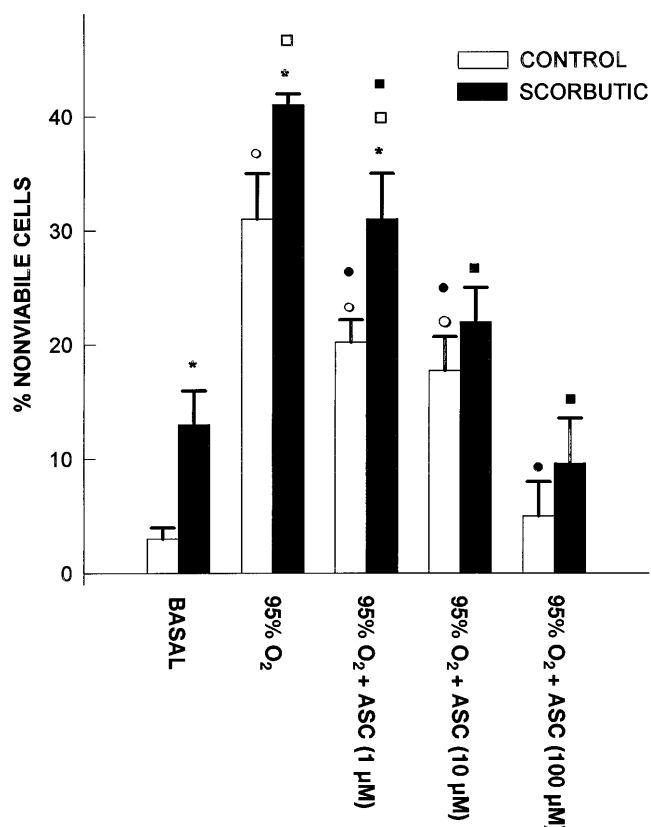


Fig. 5. Dose response to Asc protection. Control and scorbutic cells were pretreated with varying concentrations of Asc for 30 min, washed, and then exposed to 95% O₂ for 4 h. After treatment with O₂, the percentage of cells that were nonviable was assessed by an inability to exclude trypan blue. Values represent means $\pm$ SE of duplicate determinations of 6 independent cell preparations. * $P \leq 0.05$ when the scorbutic group was compared with the parallel control group. The following symbols denote $P \leq 0.05$ compared with the specified group: $\circ$, control; $\bullet$, control + 95% O₂; $\square$, scorbutic; and $\blacksquare$, scorbutic + 95% O₂.

demonstrated by limited transport when choline chloride was substituted for sodium chloride.

This transport mechanism was not lost during the period of limited Asc availability as evidenced by Na^+ -dependent Asc transport in the scorbutic cell, and the transport mechanism was not induced in the scorbutic cell when exogenous Asc was added. The minor rapid component of Asc transport by the scorbutic cells was not significantly different from that demonstrated by control cells; however, the major component of Asc transport by scorbutic cells was significantly lower than the control cells at 90 and 120 min. The mechanism by which chronic decreased Asc availability alters the major but not the minor component of Asc transport by the type II cell remains to be determined. However, the ability of the scorbutic cell to maintain a Na^+ and energy-dependent transport process suggested that the type II cell remained functional during the period of limited Asc availability.

In initial studies of cell viability after 24 h of culture (i.e., no oxidant stress), a greater percentage of the type II cells isolated from scorbutic guinea pigs were necrotic and apoptotic compared with control cells. Some

of the necrosis observed may have been secondary to apoptosis (22). The apoptotic cells observed after 24 h of culture probably represents the fraction of cells that were capable of attachment but were unable to completely repair from the damage caused by the enzymatic digestion necessary for cell isolation. Thus one would expect that a higher percentage of the scorbutic cells would be unable to repair and would become apoptotic.

When cells were treated with 250 μ M H_2O_2 , there was significant necrosis in type II cells isolated from control animals. When the necrosis initiated by H_2O_2 (Fig. 2) was compared with that of 95% O_2 (Fig. 4), the H_2O_2 caused greater cell death than hyperoxic exposure. This difference may be related to the low solubility of O_2 , which resulted in lower oxidant exposure in cells treated with 95% O_2 .

When Asc availability was limited, the cell was more sensitive to oxidant-induced necrosis. This was evidenced by a greater percentage of the scorbutic cells becoming necrotic after treatment with H_2O_2 or 95% O_2 . However, the oxidant-induced injury to the scorbutic cell was less dramatic than that expected from an organ that lost 90% of an important antioxidant. This limited injury may be related to the capacity of the type II cell to concentrate Asc or the upregulation of other defense mechanisms.

Although the percentage of apoptotic cells was higher in the scorbutic cells, both the control and the scorbutic cells were sensitive to 100 μ M H_2O_2 -induced apoptosis. In the current study, 21% of the control cells were apoptotic 24 h after treatment with 100 μ M H_2O_2 . This is contrast to the 0.6% of apoptotic cells observed in A549 cells exposed to 1 mM H_2O_2 and cultured for 48 h (14). This difference in oxidant-induced apoptosis may be a reflection of difference in sensitivity between primary cells and the A549 cell line. Alternatively, the majority of the apoptotic A549 cells may have lifted off the dish during the 48-h incubation after oxidant exposure. As observed in the study with A549 cells (14), apoptosis was observed with H_2O_2 exposure but not with hyperoxic exposure. Additional studies are needed to determine if the difference in mechanism of cell death between H_2O_2 and 95% O_2 is related to the different radicals produced or other factors.

Baseline cell viability as assessed through necrosis and apoptosis was restored by extracellular Asc or Iso. The ability of extracellular Asc or Iso to restore cellular viability suggested that the scorbutic cell was not injured beyond repair. In studies of oxidant-induced necrosis and apoptosis, Asc pretreatment provided greater protection than that observed with Iso. The inability of Iso to completely protect against injury was not due to a difference in Asc and Iso transport because both compounds reached similar intracellular concentrations during the 30-min preincubation. Iso, also known as erythorbic acid, chemically differs from Asc only in the spatial orientation of the C-5 hydrogen and hydroxyl group. Iso has chemical properties similar to Asc but has only 5% of the enzyme activation properties of Asc (3, 19). This suggested that a fraction of the

protection by Asc was due to chemical detoxification as represented by the Iso protection. However, the difference in protection between Asc and Iso suggests an enzyme-dependent activity specific for Asc.

In the scorbutic cells pretreated with Asc, the H_2O_2 -induced apoptosis was significantly decreased but the apoptosis was not completely inhibited as that observed in the control cells. The mechanism underlying the difference between the ability of the control and scorbutic cell to use Asc to protect against apoptosis is unclear. One possibility may be the scorbutic cell was unable to transport enough Asc during the 30-min period to protect against apoptosis. This scenario appears unlikely because the same preincubation period with Asc was able to protect against oxidant-induced necrosis. Alternatively, the extracellular matrix produced by the scorbutic cell may have modulated the sensitivity of the cell to oxidant-induced apoptosis. Asc is required for collagen processing (19), and, with decreased Asc availability, the scorbutic cells generate an altered extracellular matrix during the first 24 h of incubation (2). In other systems, an altered extracellular matrix is associated with increased sensitivity to apoptosis (10).

In summary, these studies demonstrated that during limited Asc availability the type II cell was able to maintain relatively high concentrations of Asc (50% loss) despite the decrease of the lung tissue to 10% of the original Asc concentration. The scorbutic cell was still functional as evidenced by the Na^+ -dependent Asc transport and the ability of Asc to restore cell viability, but the absence of this important nutritional component from the diet rendered a type II cell that was more sensitive to oxidant-induced necrosis and apoptosis. Asc pretreatment protected against oxidant-induced necrosis and apoptosis in both control and scorbutic cells. The Asc analog Iso, which has similar chemical properties, provided partial protection but not to the same extent as that observed with Asc. This suggested that some of the protection by Asc was chemical detoxification and that some of the protection was specific for Asc. Additional studies are needed to determine the other functions of the type II cell that are altered by decreased Asc availability and how the increased sensitivity to oxidant injury impacts on the ability of the type II cell to repair the denuded alveoli.

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