Supercritical fluid-aerosolized vitamin E pretreatment decreases leak in isolated oxidant-perfused rat lungs

BROOKS M. HYBERTSON, ROGER P. KITLOWSKI, ERIC K. JEPSON, AND JOHN E. REPINE
Weed-Waring Institute for Biomedical Research, Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

Hybertson, Brooks M., Roger P. Kitlowski, Eric K. Jepson, and John E. Repine. Supercritical fluid-aerosolized vitamin E pretreatment decreases leak in isolated oxidant-perfused rat lungs. J. Appl. Physiol. 84(1): 263–268, 1998.—We hypothesized that direct pulmonary administration of supercritical fluid-aerosolized (SFA) vitamin E would decrease acute oxidative lung injury. We previously reported that rapid expansion of supercritical CO2 formed respirable particles of vitamin E and that administering SFA vitamin E to rats increased lung vitamin E levels and decreased neutrophil-mediated lung leak. In the present investigation, we found that pretreatment with SFA vitamin E protected isolated rat lungs against the oxidant-induced lung leak caused by perfusion with xanthine oxidase (XO) and purine, an enzyme system that generates superoxide anion (O2·−) and hydrogen peroxide. SFA vitamin E droplets were 0.7–3 µm in diameter, and inhalation of the airborne droplets for 30 min deposited ~55 µg of vitamin E in rat lungs. Isolated rat lungs perfused with XO (0.02 U/ml) and purine (10 mM) gained more weight (1.75 ± 0.12 g, n = 8), retained more Ficoll (11.5 ± 1.2 mg/ left lung, n = 7), and accumulated more Ficoll in their lung lavages (700 ± 146 µg/ml, n = 8) than control lungs (0.25 ± 0.06 g (n = 10), 6.2 ± 1.2 mg/ left lung (n = 9), and 141 ± 31 µg/ml (n = 8), respectively, P < 0.05). In contrast, isolated lungs from rats that were pretreated with SFA vitamin E had decreased (P < 0.05) weight gains (0.32 ± 0.06 g, n = 7), Ficoll retentions (3.3 ± 1.1 mg/ left lung, n = 7), and lung lavage Ficoll concentrations (91 ± 26 µg/ml, n = 6) after perfusion with XO and purine compared with isolated lungs from control rats perfused with XO and purine. This protective effect was not observed in rat lungs given sham treatments (CO2 alone or vitamin E acetate aerosolized with supercritical CO2). Our results suggest that direct pulmonary supplementation of vitamin E decreases susceptibility to vascular leakage caused by XO-derived oxidants.

oxidants; antioxidants; acute respiratory distress syndrome

THE PATHOGENESIS OF ACUTE lung injury, such as that seen in patients with acute respiratory distress syndrome (ARDS), appears to involve alterations in oxidant-antioxidant balance (6, 21, 29). Patients with ARDS have increased blood lipid peroxidation products (27, 30), breath hydrogen peroxide concentrations (1, 20), serum ferritin levels (7), plasma oxidized protein levels (26), and plasma oxidized leukotriene B4 levels (31), as well as blood and lung lavage oxidized (inactivated) antiprotease levels (6). In addition, ARDS patients have decreased plasma vitamin E levels (3, 30) and lung lavage glutathione levels (24).

Because of the increased oxidative stress in lungs of ARDS patients, it seemed reasonable that increasing intrapulmonary levels of vitamin E, an antioxidant that scavenges free radicals and inhibits lipid peroxidation, would decrease oxidant-induced lung leak. Vitamin E is an effective antioxidant in vivo, and vitamin E deficiency increases pulmonary susceptibility to oxidative stress (5). Nonetheless, a major concern regarding vitamin E administration to protect against acute oxidative insults has been related to the slow process by which enterally delivered vitamin E is absorbed and systemically distributed.

In the present investigation we increased lung vitamin E levels by allowing rats to inhale fine, airborne particles of α-tocopherol, the major component of vitamin E, formed by supercritical fluid aerosolization, a process that forms drug aerosols with fine particle size that can be directly administered to the lungs via inhalation (16, 18). We found that supercritical fluid-aerosolized (SFA) vitamin E decreased leak in a simplified, isolated, perfused rat lung model in which oxidants were generated selectively in the perfusate by XO.

MATERIALS AND METHODS

Source of reagents. Heparin sodium was obtained from Elkins-Sinn (Cherry Hill, NJ). Pentobarbital sodium was obtained from Abbott Laboratories (Chicago, IL), ketamine hydrochloride from Parke-Davis (Morris Plains, NJ), and xylazine from Haver (New York, NY). High-performance liquid chromatography (HPLC)-grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ). CO2 was purchased from General Air Service and Supply (Denver, CO). Purine (7H-imidazo[4,5-d]pyrimidine), xanthine oxidase (XO grade III, from buttermilk, chromatographically purified, 1.4 U/mg protein), (+)-α-tocopherol (vitamin E), (+)-α-tocopherol acetate (vitamin E acetate), and all other reagents were purchased from Sigma Chemical (St. Louis, MO).

SFA vitamin E administration. An exposure chamber was constructed by using an acrylic chamber and an aluminum oven with a hinged top outfitted with cartridge heaters and a temperature controller (Omega Engineering, Stamford, CT; Fig. 1). The oven was filled with water and used to heat a stainless steel supercritical fluid extraction vessel (Keystone Scientific, Bellafonte, PA) to 45°C. Before each experiment the extraction vessel was loaded with 0.5 g of α-tocopherol (vitamin E) and then filled with supercritical CO2 using a syringe pump (model 260D, Isco, Lincoln, NE). The efflux from the extraction vessel passed through a nozzle (model 15–12AF1 stainless steel valve, High Pressure Equipment, Erie, PA), and the pressure drop across the nozzle caused expansion of supercritical CO2, loss of solvent strength, and precipitation, which formed airborne vitamin E droplets. The spray from the nozzle was directed through an opening in the top of the exposure chamber. Air (12.5 l/min) was added to the exposure chamber to dilute the CO2 gas in the chamber to 3–4%. Male Sprague-Dawley rats (300–400 g; Sasco, Omaha, NE) were placed in a cage inside the exposure chamber and allowed to inhale the SFA vitamin E droplets for 30 min. In control experiments, rats were placed inside the chamber and exposed ether to SFA vitamin E acetate handled in a manner...
identical to that described for vitamin E or to CO₂ and air without any vitamin E or vitamin E acetate. Vitamin E acetate was used to control for microparticulate deposition in rat lungs, because it has physicochemical properties similar to vitamin E (but it is not an antioxidant), and supercritical CO₂ alone was used to control for rat handling and gas exposure.

SFA vitamin E particle size measurement. Airborne droplets of SFA vitamin E were analyzed using a laser light-scattering particle counter (Lasair model 310, Particle Measuring Systems, Boulder, CO) (15). The sampling inlet was positioned inside the exposure chamber, and the aerosol was sampled at a rate of 1 ft³/min. Exposure chamber background counts were determined without aerosol generation and were subtracted to determine the vitamin E droplet size distribution.

Measurement of pulmonary deposition of SFA vitamin E and vitamin E acetate. In separate experiments, rats were subjected to SFA vitamin E or vitamin E acetate for 30 min, anesthetized with ketamine (90 mg/kg ip) and xylazine (7 mg/kg ip), and then ventilated with room air via tracheostomy. After the chest was opened and the lungs were perfused blood free with phosphate-buffered saline, the lungs were removed, dissected free from the heart, connective tissue, and major airways, gently blotted, and then frozen. Subsequently, lungs were assayed for vitamin E and/or vitamin E acetate content by HPLC. Briefly, lung samples were weighed and then homogenized in 1.5 ml of absolute ethanol and 1.5 ml of 10% ascorbic acid solution with a Virtishear tissue homogenizer (Virtis, Gardiner, NY) at maximum speed for two 30-s bursts. Samples were kept on ice during homogenizing. Three milliliters of hexane were added to the homogenized samples with 0.037% butylated hydroxytoluene added to prevent oxidation and increase vitamin E recovery. The samples were mixed by vortex, and the resultant emulsions were centrifuged (9,000 g, 5–10°C, 10 min). Two milliliters of the hexane (upper) phase were withdrawn and transferred to a new tube. Hexane extracts were evaporated to dryness under flowing N₂ gas and then redissolved in 500 µl of methanol. Methanol solutions were filtered into sample vials through 0.45-µm pore-size fluoropolymer syringe filters (ACRO LC13, Gelman Sciences, Ann Arbor, MI). Subsequently, 10-µl aliquots of the filtered samples were injected for HPLC analysis (Varian 9095 Autosampler). Reverse-phase HPLC separation was performed using a C₁₈ column (Nova Pak C₁₈, 15 cm x 3.9 mm, 5-µm particle size, Waters, Milford, MA) and a short precolumn (Guard-Pak Resolve C₁₈, Waters). A 99% methanol-1% water mobile phase was used with a flow rate of 1.0 ml/min (model 510 HPLC pump, Waters). The detector was a Waters model 481 variable-wavelength LC spectrophotometer with the absorption wavelength set at 292 nm (for vitamin E) or 285 nm (for vitamin E acetate), and the data were collected and analyzed using chromatography software (EZChrom Chromatography Data System, San Ramon, CA). Calibration curves were generated by using standard solutions of α-tocopherol and α-tocopherol acetate in methanol and used to calculate concentrations in the lung samples.

Isolation and perfusion of rat lungs. After pretreatment, rats were anesthetized with pentobarbital sodium (60 mg/kg ip), the trachea was cannulated, and then mechanical ventilation (95% air-5% CO₂) was initiated (3 ml tidal volume, 60 breaths/min, 2 cmH₂O positive end-expiratory pressure). After a midline thoracotomy was performed, heparin (200 U, 0.2 ml) was injected into the right ventricle, and the right and left ventricles were cannulated to allow inflow and efflux of perfusate. Next, the lungs and heart were excised and hung from a force transducer in a heated, humidified chamber. Lungs were then perfused with Earle's balanced salt solution (37°C, pH 7.4) with sodium bicarbonate (2.2 g/l) and Ficoll-70 (40 g/l, a 70,000 mol wt sucrose polymer used to maintain osmotic balance and as a tracer for measurement of fluid leakage from the vasculature into the lungs). After the first 30 ml of left ventricular effluent were removed to flush out blood cells, the flow circuit was closed so that 30 ml of perfusate were allowed to recirculate (40 ml/kg body wt⁻¹·min⁻¹). Pulmonary arterial pressures and lung weights were monitored continuously with pressure and force transducers.

Lung injury protocol. After isolated perfused rat lungs stabilized for 20 min, XO (0.02 U/ml) and/or purine (10 mM) was added to the recirculating perfusate solution and weight gain was monitored for 60 min (11). In a separate experiment (data not shown), this mixture of XO and substrate was confirmed to generate oxidants for the duration of the isolated lung experiment. At the conclusion of the experiment, the lung vasculature was perfused with 10 ml of saline and the
lungs were lavaged via the trachea with 5 ml of saline. Subsequently, lung lavage fluid supernatants (centrifuged at 15,000 g, 10 min) and lung tissue were frozen (−70°C) and saved for Ficoll assays. In control experiments, adding either XO alone (20 mU/ml) or purine alone (10 mM) to the perfusate did not cause lung leak: weight gains were 0.10 ± 0.05 g (n = 4) and 0.49 ± 0.13 g (n = 4), respectively, compared with 0.25 ± 0.06 g (n = 10) in buffer-perfused control lungs. Adding allopurinol (50 µM, an XO inhibitor) or the combination of catalase (50 U/ml) and copper-zinc superoxide dismutase (10 µM) decreased lung weight gain caused by XO and purine by 95 or 94%, respectively (n = 3–6 each group), indicating that the lung leak was dependent on XO activity, decreased by antioxidants, and mediated by oxidants (2).

Measurement of Ficoll levels in lung tissue and lung lavage. Samples of lung lavage fluid or lung tissue homogenate supernatant were mixed with 0.05% anthrone in sulfuric acid, incubated at room temperature for 20 min, and then analyzed spectrophotometrically for Ficoll at 627 nm (23).

**RESULTS**

Size distribution of SFA vitamin E. The size distribution of SFA vitamin E droplets was analyzed using a laser light-scattering particle-size analyzer (Fig. 2). Particle counts were mass weighted for each particle diameter range and expressed as mass percent. The size distribution indicates that particle diameters of 0.7–3 µm were most prevalent, with nearly all droplets being ~1 µm in diameter. This size range is considered appropriate for drug delivery into the lungs (13).

Effect of SFA vitamin E treatment on lung vitamin E levels. Rats exposed to SFA vitamin E for 30 min had increased (P < 0.05) lung tissue vitamin E levels (52.8 ± 7 µg/g, n = 3) compared with untreated control rats (14.8 ± 0.8 µg/g, n = 6), indicating a deposited aerosol dose of ~55 µg of vitamin E per two lungs. Similarly, rats exposed to SFA vitamin E acetate for 30 min had increased (P < 0.05) lung tissue vitamin E acetate levels (33.7 ± 5 µg/g, n = 3) compared with untreated control rats (0.9 ± 0.4 µg/g, n = 3), indicating a deposited aerosol dose of ~50 µg of vitamin E acetate per two lungs.

Effect of SFA vitamin E pretreatment on leak in isolated rat lungs perfused with XO and purine. Isolated rat lungs perfused with XO and purine had increased (P < 0.05) weight gain (Fig. 3), lung tissue Ficoll retention (Fig. 4), and lung lavage Ficoll levels (Fig. 5) compared with buffer-perfused control lungs. In contrast, isolated lungs taken from rats pretreated for 30 min with SFA vitamin E and then perfused with XO and purine had decreased (P < 0.05) weight gain, lung tissue Ficoll retention, and lung lavage Ficoll levels compared with lungs from control (non-vitamin E-pretreated) rats perfused with XO and purine. Moreover, the values measured in rats pretreated with SFA vitamin E and then perfused with XO and purine were not different (P > 0.05) from values obtained for control lungs perfused with buffer without addition of XO and
Pulmonary arterial pressures did not change after phil recruitment (37, 41), and increase pulmonary circulating XO can cause tissue injury, mediate neutrophil recruitment per se. The reasons for this impression were based in part on observations that instilling IL-1 intratracheally causes a neutrophil-dependent, oxidant-mediated lung leak and that SFA vitamin E treatment did not decrease the neutrophil accumulation in the lungs after IL-1 instillation. To further characterize the beneficial effects of aerosol-delivered vitamin E against oxidative lung injury and to further define the mechanism, we chose to use the simplified isolated, perfused rat lung model and to challenge the lungs with oxidants generated by a chemical source (XO) circulating through the pulmonary vasculature rather than by activated neutrophils within the lungs. Because this approach uses buffer perfusion, all blood elements are removed and, accordingly, the effects of oxidants in the lungs can be addressed specifically. In addition, the design has relevance with respect to ARDS, since XO levels and XO substrates, xanthine and hypoxanthine, are increased in the plasma of ARDS patients (14, 28). Moreover, circulating XO can cause tissue injury, mediate neutrophil recruitment (37, 41), and increase pulmonary permeability (2, 19, 39). Circulating XO has also been implicated as a possible contributor to decreased survival in ARDS patients (28) and as a mediator of a variety of oxidative insults (12, 35).

In the present investigation, we found that administering vitamin E as an inhaled aerosol from supercritical CO2 increased lung vitamin E levels and decreased lung leak caused by perfusion of isolated rat lungs with oxidants generated by XO and purine. In contrast, inhalation administration of SFA vitamin E acetate, a precursor to active vitamin E that is not an antioxidant until it is hydrolyzed to form the free alcohol, increased lung tissue vitamin E acetate levels, did not increase lung tissue vitamin E levels (data not shown), and did not protect isolated rat lungs against oxidative injury, indicating that protection was due to the antioxidant properties of vitamin E and not to nonspecific effects of microparticulate deposition in the lungs. The results resemble previous findings that vitamin E treatment not only inhibits lipid peroxidation but also protects lungs against various oxidative insults in vivo (5, 8–10, 38). In addition, the work is consistent with and supported by observations that vitamin E deficiency potentiates oxidative damage in many forms of oxidative lung injury, including exposure to ozone, hyperoxia, nitrogen dioxide, smoke, and paraquat (4, 5, 10, 36).

Two noteworthy aspects of this investigation are 1) the rapidity with which inhalation of SFA vitamin E increased vitamin E levels in the lung and made isolated rat lungs less susceptible to injury caused by perfusion with XO and purine and 2) the fact that vitamin E delivered to the lungs by deposition in the air spaces was protective against an oxidative insult that originated within the pulmonary vasculature. This contrasts with dietary or intravenous approaches for administering vitamin E; these predictably take many hours or days to increase lung vitamin E levels (22, 25). Indeed, in previous work, vitamin E supplementation has been proposed as a means to protect the lungs in the setting of ARDS (8, 40). It has been determined, however, that enteral administration of α-tocopherol to patients with acute respiratory failure was largely ineffectual at increasing serum vitamin E levels (32), likely as a consequence of poor gastrointestinal absorption. Although vitamin E can be rapidly delivered directly and effectively to lungs by intratracheal instillation of a liposomal formulation containing α-tocopherol (33, 34), SFA vitamin E inhalation may be advantageous, because it is less invasive than intratracheal instillation and may distribute vitamin E more homogeneously.

The significance of our results includes the following possibilities. First, because ARDS is a highly fatal condition for which no specific therapy is available and because some ARDS patients have decreased vitamin E levels, our results suggest that pulmonary antioxidant supplementation with SFA vitamin E may benefit patients with ARDS. Second, our results suggest that supercritical fluid aerosolization may have potential for...
prompt, effective delivery of vitamin E and other lipid materials to the lung. Pulmonary delivery of SFA vitamin E has promise, since it is rapid and lung specific, two features that are important in critically ill patients with oxidative lung injury who likely would not benefit in optimal ways from oral or intravenous delivery of SFA vitamin E to the lungs might be not benefit in optimal ways from oral or intravenous delivery of SFA vitamin E to the lungs might be beneficial for the treatment or prevention of oxidative lung injury that follows smoke inhalation, hyperoxia exposure, or pulmonary inflammation before and after the development of ARDS.

We gratefully acknowledge the assistance of Jacqueline Smith Evans with manuscript preparation.

B. M. Hybertson was supported with a Parker B. Francis Foundation Fellowship in Pulmonary Research. In addition, the work was supported by National Heart, Lung and Blood Institute Specialized Center of Research Grant HL-40784 and National Institute of Diabetes and Digestive and Kidney Diseases Grant 2-T35-DK-07496-11, the Swan Foundation, and Newborn Hope.

Address for reprints requests: B. M. Hybertson, Webb-Waring Biomedical Research, 4200 East Ninth Ave., Box C-322, Denver, CO 80262.

Received 10 April 1997; accepted in final form 16 September 1997.

REFERENCES


