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Mechanisms of attenuation of abdominal sepsis induced acute lung injury by ascorbic acid

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[1]*Division of Pulmonary Disease and Critical Care Medicine, Department of Internal Medicine, Virginia Commonwealth University, Richmond, Virginia;* [2]*Department of Pharmacotherapy and Outcomes Science, Virginia Commonwealth University, Richmond, Virginia;* [3]*Department of Biostatistics, Virginia Commonwealth University, Richmond, Virginia;* [4]*Department of Emergency Medicine, Virginia Commonwealth University, Richmond, Virginia; and* [5]*Virginia Commonwealth University Reanimation Engineering Science Center, Richmond, Virginia*

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Fisher BJ, Kraskauskas D, Martin EJ, Farkas D, Wegelin JA, Brophy D, Ward KR, Voelkel NF, Fowler AA 3rd, Natarajan R. Mechanisms of attenuation of abdominal sepsis induced acute lung injury by ascorbic acid. *Am J Physiol Lung Cell Mol Physiol* 303: L20–L32, 2012. First published April 20, 2012; doi:10.1152/ajplung.00300.2011.— Bacterial infections of the lungs and abdomen are among the most common causes of sepsis. Abdominal peritonitis often results in acute lung injury (ALI). Recent reports demonstrate a potential benefit of parenteral vitamin C [ascorbic acid (AscA)] in the pathogenesis of sepsis. Therefore we examined the mechanisms of vitamin C supplementation in the setting of abdominal peritonitis-mediated ALI. We hypothesized that vitamin C supplementation would protect lungs by restoring alveolar epithelial barrier integrity and preventing sepsis-associated coagulopathy. Male C57BL/6 mice were intraperitoneally injected with a fecal stem solution to induce abdominal peritonitis (FIP) 30 min prior to receiving either AscA (200 mg/kg) or dehydroascorbic acid (200 mg/kg). Variables examined included survival, extent of ALI, pulmonary inflammatory markers (myeloperoxidase, chemokines), bronchoalveolar epithelial permeability, alveolar fluid clearance, epithelial ion channel, and pump expression (aquaporin 5, cystic fibrosis transmembrane conductance regulator, epithelial sodium channel, and Na^+-K^+-ATPase), tight junction protein expression (claudins, occludins, zona occludens), cytoskeletal rearrangements (F-actin polymerization), and coagulation parameters (thromboelastography, pro- and anticoagulants, fibrinolysis mediators) of septic blood. FIP-mediated ALI was characterized by compromised lung epithelial permeability, reduced alveolar fluid clearance, pulmonary inflammation and neutrophil sequestration, coagulation abnormalities, and increased mortality. Parenteral vitamin C infusion protected mice from the deleterious consequences of sepsis by multiple mechanisms, including attenuation of the proinflammatory response, enhancement of epithelial barrier function, increasing alveolar fluid clearance, and prevention of sepsis-associated coagulation abnormalities. Parenteral vitamin C may potentially have a role in the management of sepsis and ALI associated with sepsis.

barrier integrity; epithelial ion channels; coagulopathy

BACTERIAL INFECTIONS OF THE lungs and abdomen are among the most common causes of sepsis (3). One of the most frequent complications of sepsis is acute lung injury (ALI). Sepsis that is not caused by pneumonia is associated with the highest risk of progression to ALI (20). A significant feature of ALI is tissue infiltration of polymorphonuclear neutrophils (PMN). In response to bacterial infection, neutrophil activation in the lungs leads to ALI (53) and PMN infiltration represents a primary mechanism for sepsis-induced pulmonary dysfunction and injury (10).

In the normal lung, continuous fluid clearance by the lung lymphatics is essential for the maintenance of dry alveolar surfaces (60). Ion pumps and channels positioned on alveolar epithelial cell surfaces generate transepithelial osmotic gradients that drive water movement from the alveolar space into the lung interstitium. The key pumps and channels involved in alveolar fluid transport include aquaporin 5 (Aqp5), cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channel (ENaC), and Na^+-K^+-ATPase (37). In ALI, the lung endothelial barrier is damaged, resulting in abnormal capillary permeability and pulmonary edema as the lymphatic clearance is overwhelmed (60). In contrast to the endothelium, the alveolar epithelium is often spared in sepsis-induced ALI, and therefore active ion and fluid clearance is preserved (56). Recent reports, however, suggest that ion pump and channel functions are affected early during sepsis (37).

To maintain a "dry" alveolar space and normal lung function, it is essential that the milieu within the alveolar space remain distinct from that of the subepithelial compartment (30). The maintenance mechanism depends on the formation and proper functioning of specialized molecular structures between adjacent cells comprising the epithelial sheet, the so-called tight junctions (TJ). The alveolar epithelial TJ is a complex of integral membrane proteins that firmly interact with the epithelial cytoskeleton (27). TJs serve as a regulated semipermeable barrier that limits passive diffusion of solutes across paracellular pathways between adjacent cells (1). Han et al. (30) recently showed that ALI was associated with diminished expression and function of TJ proteins in lung epithelium.

Emerging evidence indicates that inflammation and coagulation are connected (34). This is especially important in sepsis as inflammatory cytokines activate the coagulation cascade and inhibit fibrinolysis, thereby shifting normal hemostasis toward a prothrombotic state. Sepsis-driven coagulation induces consumption of coagulation factors leading to disseminated intravascular coagulation (DIC), a phenomenon frequently associated with sepsis-induced ALI. Indeed, impairment of capillary blood flow during sepsis has been observed in human tissues by orthogonal polarization spectral imaging and sidestream

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dark-field imaging (17). It is currently estimated that as many as 50% of all sepsis patients develop DIC (25). Bastarache et al. (6) have recently reported that in ALI, the alveolar compartment contains high levels of tissue factor (TF) procoagulant activity that favor fibrin deposition in the air spaces. TF activation results in thrombin formation, which augments permeability and enhances inflammation (14).

Vitamin C is a small, water-soluble molecule that readily acts as a one- or two-electron reducing agent for many radicals and oxidants. Vitamin C is bioavailable equally as either dehydro-L-ascorbic acid (DHA) or L-ascorbic acid (AscA). Specialized cells can take up reduced vitamin C (AscA) through Na^+-dependent ascorbate cotransporters (SVCT1 and SVCT2). Most other cells take up vitamin C in its oxidized form (DHA) via facilitative glucose transporters (48). Sepsis lowers plasma AscA concentrations (57) and, importantly, low vitamin C levels correlate inversely with multiple organ failure and directly with survival (9). Studies using animal models show that vitamin C prevents endotoxin-induced hypotension and improves arteriolar responsiveness, arterial blood pressure, capillary blood flow, liver function, and survival in experimental sepsis (4, 59).

We recently showed that vitamin C, administered after the onset of endotoxemia, attenuates proinflammatory and procoagulant states that induce lung vascular injury and improved survival in an animal model of sepsis (23). In the present study we show that vitamin C attenuates sepsis-induced ALI by enhancing alveolar epithelial barrier integrity. Furthermore, vitamin C induced the expression of ion channels and pumps, which play critical roles in improving alveolar fluid clearance. In addition, we also observed marked changes in the viscoelastic clot properties of septic mice blood. Vitamin C infusion completely normalized the coagulopathy in septic mice.

EXPERIMENTAL PROCEDURES

Epithelial cell culture. The A549 human lung epithelial cell line (ATCC CCL-185) was obtained from American Type Culture Collection, Manassas, VA. Cells were cultured under sterile conditions and maintained in medium F12-K medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under a 5% CO_2 atmosphere at 37°C.

Experimental protocols of sepsis. Male C57BL/6 mice (weight 25–30 g, age 8 wk) were obtained from Jackson Laboratory (Bar Harbor, ME). Polymicrobial sepsis (peritonitis) was induced by intraperitoneal (i.p.) introduction of fecal stem solution into the peritoneum (FIP). Fecal stem solution was prepared by a modification of the method reported by Bernardshaw et al. (8). Dry fecal pellets were randomly collected from all the mice-containing cages 1 day before the challenge. Weighed pellets (70 pellets ~1 g) were pooled and suspended in saline (180 mg feces/1 ml saline). The solution was capped tightly, vortexed for 5 min, and stored overnight at 4°C. The mixture was then allowed to return to room temperature and solids were compacted by low-speed centrifugation (50 *g*) for 5 min. The recovered supernatant was termed fecal stem solution (the recoverable liquid was ~20% of the starting volume). Thirty minutes after i.p. fecal challenge, vitamin C-treated mice received i.p. injection of either ascorbic acid (AscA) or dehydroascorbic acid (DHA) (200 mg/kg in saline). Untreated mice received i.p. saline instead of vitamin C. Blood was collected 4–16 h later by cardiac puncture and lungs were harvested. Mice were divided into four groups: *1*) control mice received saline alone (0.4 ml i.p.); *2*) FIP mice received fecal stem solution (0.4 ml i.p.) followed 30 min later by saline (0.1 ml i.p.); *3*) FIP-AscA mice received fecal stem solution (0.4 ml i.p.) followed 30 min later by AscA (0.1 ml i.p.); *4*) FIP-DHA mice received fecal stem solution (0.4 ml i.p.) followed 30 min later by DHA (0.1 ml i.p.). To prevent volume depletion, sterile saline (0.5 ml) containing the analgesic buprenorphine (4 μg/ml) was injected subcutaneously at 6-h intervals. All animal studies were performed in accordance with protocols approved by the Virginia Commonwealth University Animal Care and Use Committee. Details of the *Escherichia coli* endotoxin model of sepsis are provided elsewhere (23).

Histological assessment of lung injury. Lungs were intratracheally infused with low-melting agarose, immersed in 10% formalin for 24 h, then paraffin embedded. Four-micrometer sections were stained with hematoxylin and eosin. Lung architecture was evaluated by bright-field microscopy (×10 and ×40 magnification) with an AXIO imager A1 microscope, Axiocam HRc camera, and Axiovision software (Zeiss, Jena, Germany). Two random tissue sections from four different lungs in each group were examined by a blinded investigator. For each subject, a five-point scale was applied: 0 = minimal (little) damage, 1+ = mild damage, 2+ = moderate damage, 3+ = severe damage and 4+ = maximal damage. Points were added up and are expressed as median ± SE.

Immunohistochemistry for PMN and claudin-4. Lungs were harvested from anesthetized mice, inflated with 10% formalin at a pressure of 20 cmH_2O, and fixed for 24 h. Transversal sections (4 μm) were deparaffinized and rehydrated, and antigen retrieval was performed with 0.01 M sodium citrate (pH 6.0) for 20 min. After blocking of endogenous peroxidase with 3% H_2O_2/PBS for 5 min, sections were incubated with blocking buffer (1% normal swine serum/PBS) for 15 min. Sections were incubated with primary antibody for PMN (rat monoclonal to LY-6G/-6C neutrophil marker, Hycult Biotechnology, 1:25) or claudin-4 (rabbit polyclonal antibody, Santa Cruz Biotechnology, 1:50) in blocking buffer overnight at 4°C. They were then incubated with biotinylated secondary antibody, anti-rat (1:500) for PMN and anti-rabbit (1:1,000) for claudin-4, for 1 h in blocking buffer at room temperature, followed by an amplification step with streptavidin-horseradish peroxidase (1:200) for 45 min at room temperature in blocking buffer. Negative controls with nonspecific Ig were run in parallel. They were used at identical concentrations and staining conditions as the target primary antibodies. Slides were developed with 3,3′-diaminobenzidine chromogen substrate and counterstained with Mayer's hematoxylin.

W/D ratios. Wet-to-dry (W/D) ratios were measured as described by Eltzschig et al. (18). In short, 16 h following initiation of sepsis, lungs were excised en bloc. The weight was obtained immediately to prevent evaporative fluid loss of the tissues. Lungs were then dried in an oven at 60°C to constant weight and the dry weight was measured. W/D ratios were then calculated as milligrams water per milligram of dry tissue.

BAL fluid analysis. Following euthanasia, the trachea was cannulated with a 20-gauge flexible catheter and the lungs were lavaged with three 1.0-ml washes of sterile saline (room temperature). Bronchoalveolar lavage (BAL) fluid was centrifuged (400 *g* for 10 min at 4°C) and the protein content of BAL supernatant was determined by use of the BCA protein assay kit (Pierce).

Measurement of bronchoalveolar epithelial permeability. Epithelial barrier function to small solutes was assessed by measuring leakage of FITC-dextran (average molecular mass 4 kDa; FD4) from the plasma compartment into the alveolar space as described previously (30). Briefly, following treatments, mice were injected via the tail vein with 0.1 ml of FD4 solution in PBS (10 mg/kg). Ten minutes later animals were anesthetized and the lungs were lavaged as described above. The aliquots of BAL fluid were pooled. Blood was obtained by cardiac puncture, and the serum was collected. Ten microliters of serum were mixed with 200 μl of PBS. The concentrations of FD4 in the BAL fluid and dilute serum were determined with a SPECTRAmax GEMINI XS Microplate Spectrofluorometer (Molecular Devices, Sunnyvale, CA) by using an excitation filter of 492 nm and an emission wavelength of 515 nm. The BAL fluid-to-

serum fluorescence ratio was calculated and used as a measure of pulmonary epithelial permeability.

AFC. Alveolar fluid clearance (AFC) was measured as described by Fukuda et al. (24) with minor modifications. Mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). A tracheostomy was done, and the lungs were mechanically ventilated by a small animal ventilator (Harvard Apparatus) with an inspired oxygen fraction of 1.0, tidal volume of 9–10 ml/kg, and a respiratory rate of 90 breaths/min. Body temperature was kept constant at 37–38°C by a heating pad. AFC was measured by instilling 300 μl of 0.9% NaCl solution containing 5% acid-free BSA (Sigma-Aldrich). Half of the instillate volume was delivered to both lungs every 5 min over 30 s via the trachea with a small catheter (PE-10) and a syringe to prevent backward flow into the tracheostomy tube followed by injection of 100 μl of air to achieve complete deposition of all fluid into the alveolar space. We used the end of first instillation as *time 0* in this model. At the end of the experiment the chest was opened to produce bilateral pneumothoraces to facilitate aspiration of the remaining alveolar fluid via the tracheal catheter. The aspirate was centrifuged at 3,000 *g* for 10 min, and the supernatant of the fluid was used to measure the total protein concentration via a BCA assay (Pierce). AFC was calculated by the following equation: AFC = 1 − (C0/C30), where C0 is the protein concentration of the instillate before instillation and C30 is the protein concentration of the sample obtained after 30 min of mechanical ventilation. Clearance is expressed as a percentage of total instilled volume (% instilled volume/30 min).

Western blot analysis. Cell fractionation was performed as described previously by Bapat et al. (5). Briefly, following treatments, A549 cells were suspended in ice-cold membrane separation buffer containing 0.25 m sucrose, 5 mM $MgCl_2$, 2 mM EGTA, and 2 mM EDTA in Tris buffer plus protease/phosphatase inhibitors. Cells were disrupted with brief sonication and centrifuged at 1,000 *g* for 5 min to remove unbroken cells and nuclei. Supernatant was collected and spun at 100,000 *g* for 1 h; the supernatant constituted the cytosolic fraction and membrane fraction was the pellet, which was resuspended in membrane separation buffer with 1% Triton X-100. Protein concentrations were determined and equal amounts separated by 4–20% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membrane (0.2-μm pore size). Na^+-K^+-ATPase β_1 in membrane and cytosolic fractions was then detected by chemiluminescence. Optical densities of antibody-specific bands were determined by use of Quantity One acquisition and analysis software (Bio-Rad). Whole cell extracts were isolated as described previously (23). Proteins were resolved by SDS polyacrylamide gel electrophoresis (4–20%) and electrophoretically transferred to polyvinylidene fluoride membranes (0.2-μm pore size). Immunodetection was performed by chemiluminescent detection. Blots were stripped by using the Restore Western Blot Stripping Buffer as described by the manufacturer.

RNA isolation and QPCR analysis. Isolation of total RNA and real-time quantitative (QPCR) analysis were performed as described previously (23). Total RNA was extracted and purified by using QIAshredders and RNeasy columns according to the manufacturer's specifications (Qiagen). Lungs were snap frozen in liquid nitrogen and powdered with a Biopulverizer (RPI) prior to RNA extraction. Total RNA (1 μg) was reverse transcribed into cDNA by use of the High Capacity cDNA Reverse Transcription kit. Complementary DNA (cDNA) was diluted (1:500) and real-time QPCR was performed with POWER SYBR Green QPCR Master Mix. Primers were designed to anneal to sequences on separate exons or to span two exons. Cycling parameters were 95°C, 10 min, 40 cycles of 95°C, 15 s; 60°C, 1 min. A dissociation profile was generated after each run to verify specificity of amplification. All PCR assays were performed in triplicate. No template controls and no reverse transcriptase controls were included. 18S rRNA was used as housekeeping gene against which all the samples were normalized for differences in the amount of total RNA added to each cDNA reaction and for variation in the reverse transcriptase efficiency among the different cDNA reactions. Automated gene expression analysis was performed by using the Comparative Quantitation module of MxPro QPCR Software (Agilent).

Fluorescence microscopy. Fluorescence microscopy for actin and Na^+-K^+-ATPase in A549 cells was performed as described previously (38). Briefly, A549 epithelial cells were grown on Ibidi six-channel IbiTreat μ-slide VI. Following treatments culture media was aspirated and cells were fixed in 3.7% paraformaldehyde in PBS for 10 min at 4°C. Cells were permeabilized by exposure to 0.15% Triton X-100 in PBS for 5 min at 4°C, washed with Triton/PBS and blocked with 0.5% BSA in PBS for 30 min at room temperature. Cells were incubated with mouse anti-Na^+-K^+-ATPase β_1 monoclonal antibodies (Millipore). For detection, cells were subsequently incubated with Alexa Fluor 546-conjugated goat anti-mouse IgG (H + L). For detection of cellular actin, A549 cells were incubated with Alexa Fluor 568 phalloidin. Fluorescence imaging of A549 was performed by use of an Olympus model IX70 inverted phase microscope (Olympus America, Melville, NY) outfitted with an IX-FLA fluorescence observation system equipped with a TRITC filter cube (530–560 nm excitation, 590–650 nm emission, Chroma Technology, Brattleboro, VT) through an Uplan FI objective (×40). Fluorescence images were digitized and captured by a MagnaFire digital camera (Optronics, Goleta, CA).

Ascorbic acid assays. A549 cells were loaded with DHA in Hanks balanced salt solution for 30 min at 37°C. Intracellular AscA levels were estimated spectrophotometrically from a standard curve (Ascorbic Acid Quantification Kit, BioVision, Mountain View, CA) as described previously (23) and the intracellular concentrations derived from the measured amount of AscA and the known A549 cell volume (13).

Analysis of F-actin-to-G-actin ratio. The concentration of F-actin and G-actin in A549 epithelial cells was measured as described previously (45, 29). Briefly, cells were homogenized in F-actin stabilization buffer (50 mM PIPES, pH 6.9, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 1 mM ATP with protease inhibitors). Supernatants were collected after centrifugation at 150,000 *g* for 60 min at 37°C. Pellets were resuspended in ice-cold distilled H_2O plus 1 μM cytochalasin D and then incubated on ice for 1 h to dissociate F-actin. The resuspended pellets were gently mixed every 15 min. The supernatant of the resuspended pellets was collected after centrifugation at 5,000 rpm for 2 min at 4°C. Equal volumes of the first supernatant (G-actin) or second supernatant (F-actin) were subjected to immunoblot analysis using anti-actin antibody. The amount of F-actin and G-actin was determined by scanning densitometry using Quantity One acquisition and analysis software (Bio-Rad).

TEG. Thromboelastography (TEG; TEG 5,000, Haemoscope, Niles, IL) by recalcification (10 mM final calcium concentration) was performed in whole blood at 37°C 30 min after blood draw as described previously (54). TEG parameters measured included clot onset time (R), clot formation (or kinetics) time (K), clotting angle (Angle), maximal clot strength (MA), and shear elastic modulus (G). TEG 5000 was calibrated as directed by the manufacturers.

Statistical analysis. Mean values were calculated from data obtained from four to six animal studies in each group and at least three separate in vitro experiments. Statistical analysis was performed via SigmaStat 3.1 (SPSS, Chicago, IL). Results were compared by one-way ANOVA and the post hoc Tukey test or Newman-Keuls test to identify specific differences between groups. Statistical significance was confirmed at a P value < 0.05. Lung injury score is given as median ± SE. Kaplan-Meier survival curves were produced by log-rank analysis.

RESULTS

Fecal peritonitis promotes pulmonary inflammation and PMN infiltration of the lungs. The fecal stem solution, as described here, contains greater than 40 different aerobic and anaerobic species of bacteria, with *E. coli*, *Enterococcus*, and *Staphylococcus* predominating among the aerobic bacteria (35). Introduction of bacteria into the abdomen (FIP) induced lung histological changes consistent with ALI (Fig. 1, *A*, *C*, *E*), a robust proinflammatory response (Fig. 1, *H–K*), and thickened alveolar walls infiltrated with intramural PMNs (Fig. 1, *F* and *K*). Mortality was negligible in all groups (FIP, FIP-AscA, FIP-DHA) at 16 h after induction of peritonitis. However, mortality in the FIP group increased dramatically with 100% mortality occurring within 26 h of administration of fecal stem solution. In contrast, mortality remained negligible in the FIP-AscA and FIP-DHA groups ($P = 0.002$ vs. FIP, Fig. 1*L*). Therefore, all our assessments were performed at or before 16 h following peritonitis induction. Infusion of parenteral vitamin C 30 min following peritonitis significantly reduced the expression of pulmonary proinflammatory chemokines, decreased PMN sequestration in the lung, and demonstrated a lower degree of histological tissue injury (Fig. 1).

Vitamin C attenuates FIP-induced excess lung water and alveolar epithelial permeability. We next performed lung W/D ratio to assess for excess lung water 16 h following induction of peritonitis. As depicted in Fig. 2*A*, the W/D lung weight ratio was significantly increased in the FIP group compared with controls ($P < 0.05$). Infusion of parenteral vitamin C 30 min following peritonitis significantly reduced excess lung water to levels seen in control lungs ($P < 0.05$).

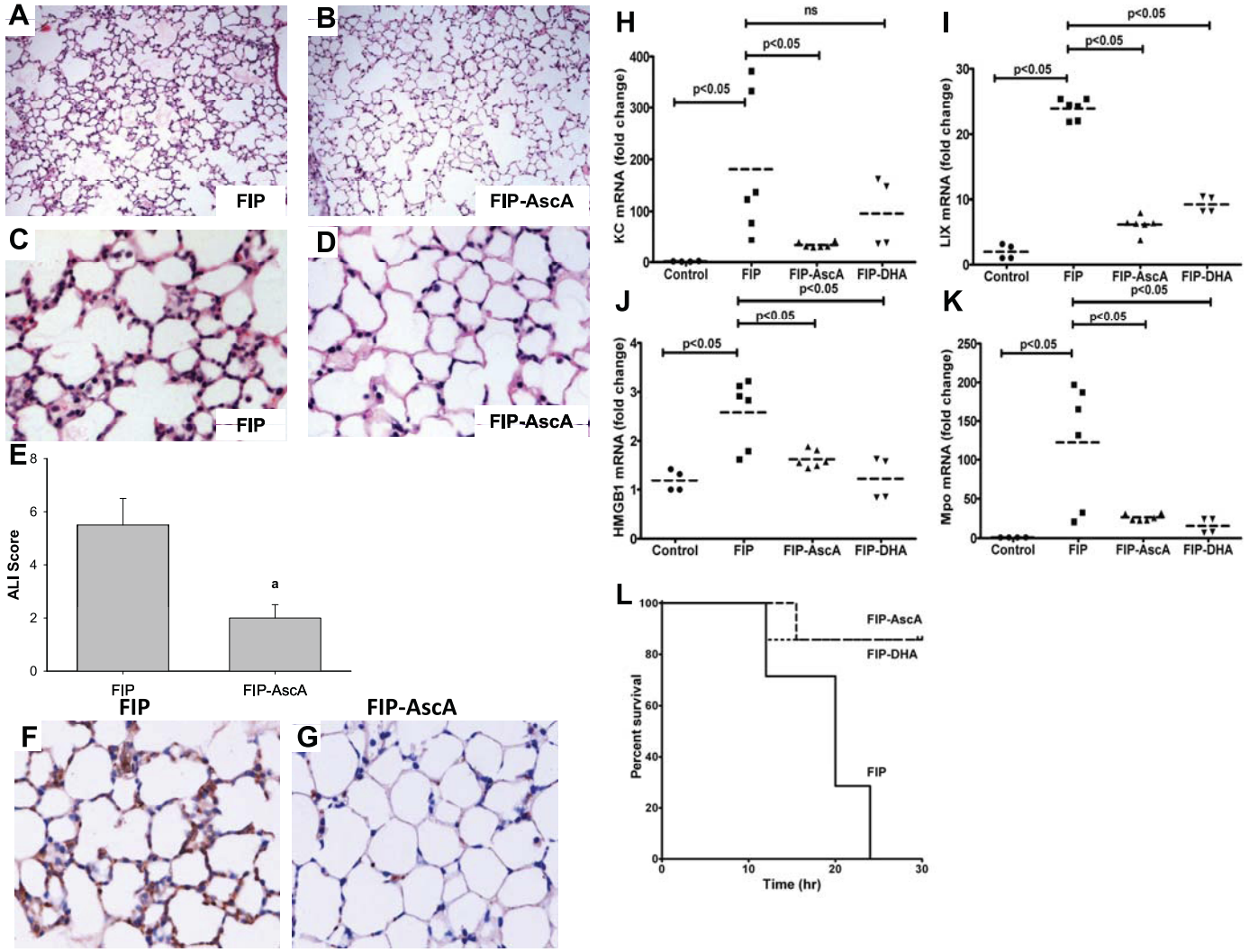

Fig. 1. Fecal peritonitis promotes pulmonary inflammation and polymorphonuclear neutrophil (PMN) infiltration while increasing mortality. Mice were injected intraperitoneally (i.p.) with saline or fecal stem solution (FIP, 180 mg/ml). Ascorbic acid (AscA; 200 mg/kg) or dehydroascorbic acid (DHA; 200 mg/kg) was administered i.p. in saline 30 min after FIP. *A–D*: representative hematoxylin and eosin (H&E) staining of lung sections from mice 16 h after FIP (*A* and *B*, ×10 magnification; *C* and *D*, ×40 magnification) ($N = 3$ for each group). *E*: for quantification of tissue damage due to sepsis, a histological score for acute lung injury (ALI) was assessed based on H&E staining. Results are displayed as median ± SE ([a]$P < 0.025$ vs. FIP; $N = 4$ for each group). *F* and *G*: representative immunohistochemistry for PMN from lungs of mice 16 h after FIP (×40 magnification) ($N = 3$ for each group); *H–K*: real-time quantitative analysis (QPCR) for cytokine-induced neutrophil chemoattractant factor (KC), LPS-induced CXC chemokine (LIX), high-mobility group box 1 (HMGB1), and myeloperoxidase (Mpo) from lungs of mice 16 h after FIP ($N = 4–6$ for each group). *L*: Kaplan-Meier survival curves of septic mice following induction of peritonitis (FIP). AscA (200 mg/kg) or DHA (200 mg/kg) was administered i.p. in saline 30 min after FIP ($P = 0.002$, log-rank analysis, $N = 7$ for each group). ns, Not significant.

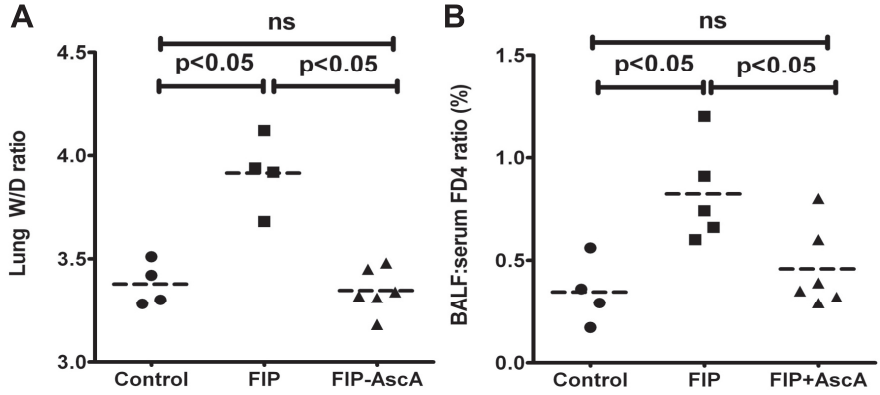

Fig. 2. Vitamin C attenuates FIP-induced excess lung water and alveolar epithelial permeability. Mice were injected i.p. with saline or fecal stem solution (FIP, 180 mg/ml). AscA (200 mg/kg) was administered i.p. in saline 30 min after FIP. *A*: 16 h following initiation of sepsis, lungs were excised, patted dry, and weighed. Lungs were dried in an oven at 60°C to constant weight and the dry weight was measured. Wet-to-dry ratios were then calculated as milligrams water per milligram of dry tissue ($N = 4–6$ per group). *B*: following treatments, mice were injected via the tail vein with 0.1 ml of FITC dextran 4 (FD4) solution in PBS (10 mg/kg). Bronchoalveolar lavage (BAL) was performed 4 h after FIP and the ratio of FD4 in BAL fluid and serum of mice was estimated ($N = 4–6$ per group).

Using a small molecule fluorescent tracer FD4, we found significant increases in the ratio of BAL fluid FD4 content to serum FD4 content within 4 h of peritonitis onset (Fig. 2*B*). Vitamin C infusion significantly diminished tracer leakage into the alveolar space indicating preservation of epithelial barrier function by vitamin C. However, the total BAL protein was not different (data not shown) between the three groups, signifying that the alveolar capillary permeability to large solutes was unchanged in this model of sepsis.

Vitamin C augments epithelial ion channel and transporter expression and alveolar fluid clearance in septic mice. FIP significantly decreased the expression of Aqp5, but not of ENaC or Na^+-K^+-ATPase (Fig. 3, *A–D*). Although CFTR expression was considerably reduced, it did not reach statistical significance. Of major importance, infusion of septic mice with vitamin C significantly induced the expression of Aqp5, CFTR, ENaC, and Na^+-K^+-ATPase (Fig. 3, *A–D*). Similar results were produced in vitro by using human alveolar epithelial cells exposed to LPS (data not shown) and in vivo in mice exposed to endotoxin (data not shown).

To examine the physiological significance of these changes in mRNA levels, we measured AFC rates in our model of sepsis. Consistent with studies by others, basal mean AFC was 32%. Four hours after the induction of peritonitis, AFC decreased significantly in the FIP group compared with controls (Fig. 3*E*, $P < 0.05$). Infusion of parenteral vitamin C 30 min following induction of peritonitis significantly increased AFC rates to levels observed in the control group (Fig. 3*E*, $P < 0.05$).

Vitamin C prevents sepsis-mediated disassembly of the Na^+-K^+-ATPase pump. Sepsis impairs AFC by downregulation or endocytosis of the Na^+-K^+-ATPase pump (7). Therefore we examined the subcellular localization of Na^+-K^+-ATPase in human alveolar epithelial cells (A549) activated by endotoxin. In the absence of exogenous vitamin C, A549 intracellular ascorbate levels were below the level of detection (Fig. 4*A*). Incubation with exogenous DHA rapidly increased intracellular ascorbate concentrations to levels similar to those reported for other cell types (11). Following DHA loading, cells were exposed to endotoxin (LPS) for 1, 2, and 4 h. Cells were subjected to subcellular fractionation and the β_1-subunit of Na^+-K^+-ATPase was detected by Western blots. As seen in Fig. 4*B*, endotoxin exposure induced a rapid translocation of Na^+-K^+-ATPase to the cytosolic compartment. This translocation was prevented by DHA loading (Fig. 4*B*). These results were confirmed by immunofluorescence microscopy (Fig. 4*C*).

Vitamin C modulates TJ protein expression in septic mice lungs. We found that FIP induced the expression of claudin-2 (Cldn2) and claudin-4 (Cldn4), while significantly decreasing expression of TJ proteins claudin-18 (Cldn18) and occludin (Ocln), as well as the cytoskeletal connector protein zona occludens-1 (ZO-1) (Fig. 5). In contrast, vitamin C infusion induced Cldn18, occludin, and ZO-1 expression while preventing the enhanced expression of Cldn2 and Cldn4 (Fig. 5). Similar changes in the protein expression of ZO-1 and Cldn4 were observed by Western blot analysis (Fig. 5, *F–H*) and by immunohistochemistry (Fig. 5, *I–L*). These results were reproduced in vitro, in A549 cells exposed to endotoxin (data not shown).

Vitamin C prevents cytoskeletal rearrangements in alveolar epithelium exposed to proinflammatory stimuli. Since tight junctional complexes examined above are likely interacting with the actin cytoskeleton, we examined actin cytoskeletal rearrangements in A549 cells activated by proinflammatory stimuli. A549 cells exposed to endotoxin (LPS) or cytomix (CM) demonstrated significant cell contraction with emergence of intercellular gaps (arrows, Fig. 6, *C* and *E*). These changes were prevented by DHA exposure. Furthermore, we found significant actin polymerization as documented by increased cellular F-actin content following endotoxin or CM challenge (Fig. 6*G*). DHA loading decreased F-actin polymerization in activated A549 cells.

Vitamin C prevents changes in viscoelastic properties of septic mouse blood. We recently showed that vitamin C infusion corrected endotoxin-induced coagulopathy in mice restoring prothrombin and activated partial thromboplastin times to near normal (23). We extended these observations in our FIP model by examining the viscoelastic properties of mouse blood using TEG, a whole blood functional clotting assay that assesses all plasma and cellular components that participate in the formation kinetics, strengthening, and fibrinolysis of clotting. Blood from septic mice displayed profound changes in the rate of clot formation, clot strength, and clot stability including increased reaction time (R, although this did not reach statistical significance), reduced speed of clotting (K and Angle), as well as decreased clot stiffness and firmness (MA and G) (Table 1). Vitamin C infusion restored hemostasis in septic mice as observed by the normalization of these viscoelastic properties (Table 1).

Vitamin C alters expression of key procoagulant, anticoagulant, and fibrinolytic proteins in septic mice lungs. We recently reported that lung mRNA and protein expression of the procoagulant protein TF was significantly induced following

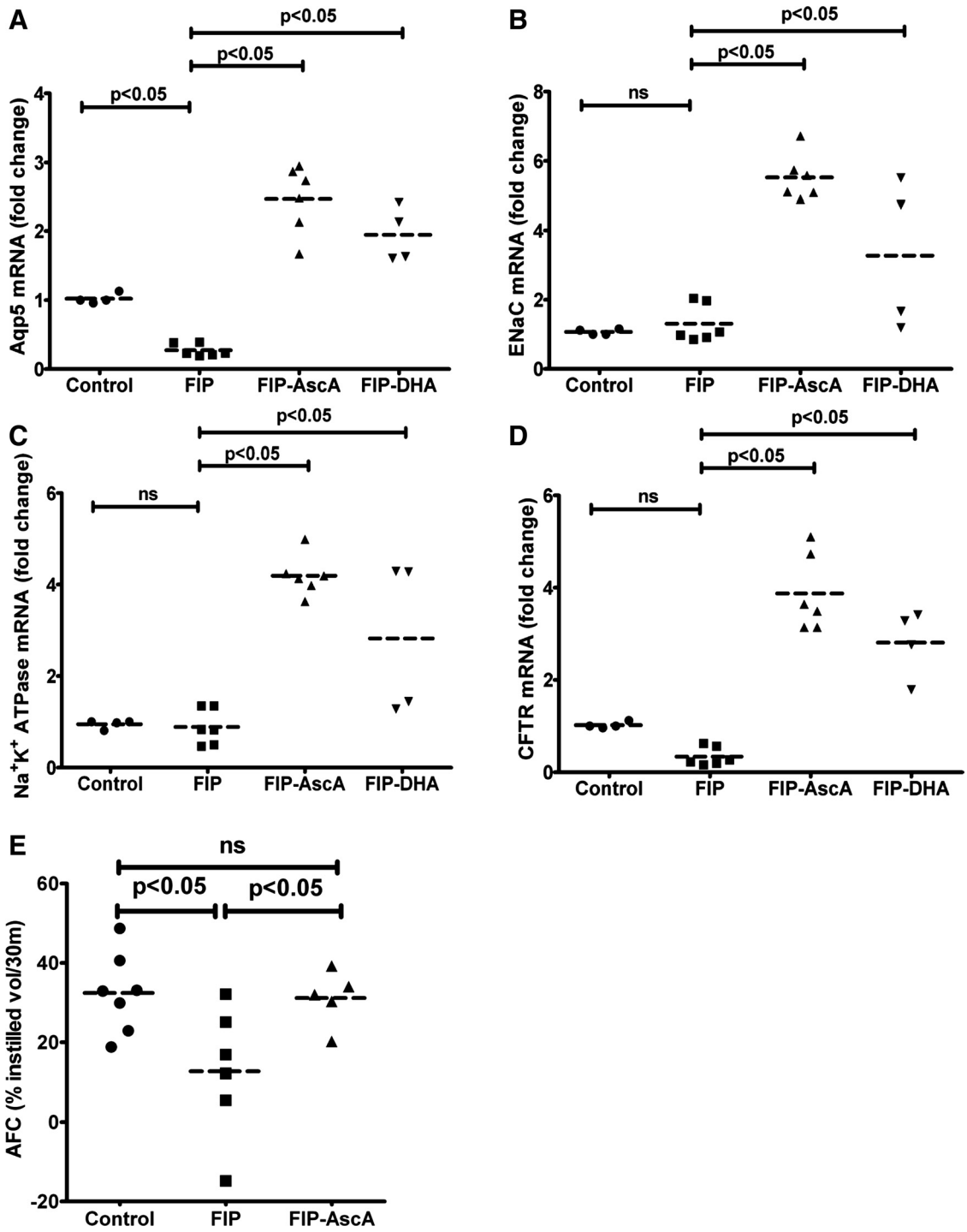

Fig. 3. Vitamin C augments epithelial ion channel/transporter expression and alveolar fluid clearance in septic mice. Mice were injected i.p. with saline or fecal stem solution (FIP, 180 mg/ml). AscA (200 mg/kg) or DHA (200 mg/kg) was administered i.p. in saline 30 min after FIP. Real-time QPCR for aquaporin 5 (Aqp5; *A*), epithelial sodium channel (ENaC; *B*), Na^+-K^+-ATPase (*C*), and cystic fibrosis transmembrane conductance regulator (CFTR; *D*) from lungs of mice 16 h after FIP ($N = 4–6$ per group). *E*: to determine whether vitamin C infusion affects pulmonary fluid transport, we measured alveolar fluid clearance (AFC) using a ventilated live mouse model. Four hours after the induction of peritonitis AFC was measured by instilling 300 μl of 0.9% NaCl solution containing 5% acid-free BSA. AFC was measured by the equation AFC = 1 − (C0/C30), where C0 is the protein concentration of the instillate before instillation and C30 is the protein concentration of the sample obtained after 30 min of mechanical ventilation. Clearance is expressed as a percentage of total instilled volume (% instilled volume/30 min).

endotoxin infusion (23). In the present study, we observed significant TF expression in septic lung (Fig. 7*A*) that was attenuated by the vitamin C intervention. Expression of the anticoagulant protein thrombomodulin (TM) was significantly reduced following FIP (Fig. 7*D*). Vitamin C infusion significantly induced TM expression. Expression of the anticoagulant endothelial protein C receptor (EPCR) and the fibrinolytic tissue plasminogen activator (t-PA) was not significantly al-

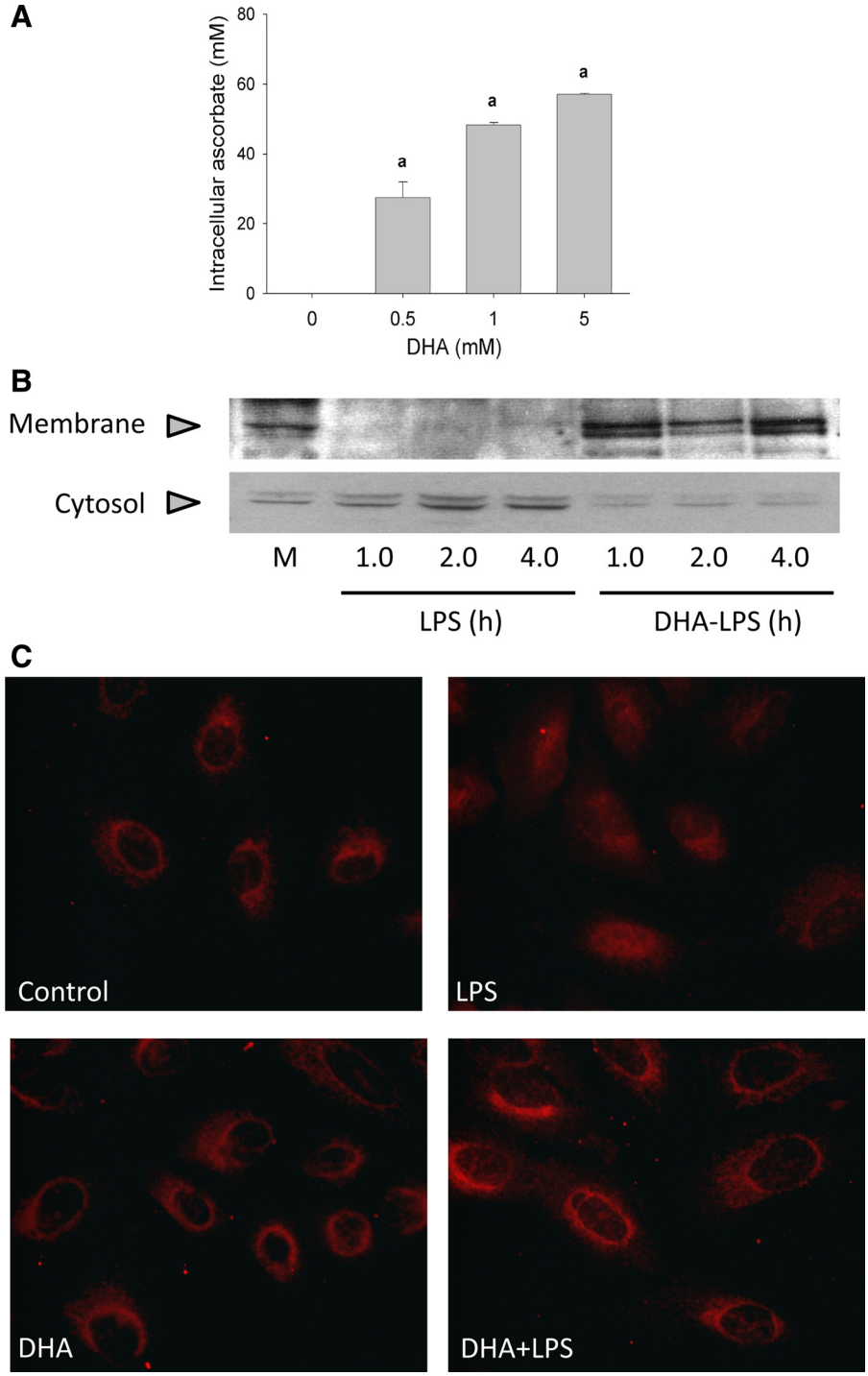

Fig. 4. Vitamin C prevents downregulation of the Na^+-K^+-ATPase pump. *A*: A549 epithelial cells were exposed to media alone or loaded with DHA in Hanks' balanced salt solution for 30 min at 37°C. Intracellular AscA concentrations were determined as described in EXPERIMENTAL PROCEDURES ($^aP < 0.05$, $N = 3$ for each group). *B*: representative Western blot for Na^+-K^+-ATPase in A549 alveolar epithelial cells. A549 cells were exposed to medium (M) or loaded with DHA (5 mM, 30 min) prior to exposure to endotoxin (LPS, 10 μg/ml) for 1, 2, and 4 h. Cells were subjected to subcellular fractionation as described in EXPERIMENTAL PROCEDURES, and the β_1-subunit of Na^+-K^+-ATPase in the membrane and cytosolic fractions was detected by Western blotting. *C*: representative immunofluorescence images for Na^+-K^+-ATPase in A549 alveolar epithelial cells. A549 cells were exposed to media alone or loaded with DHA for 30 min followed by exposure to endotoxin (LPS, 10 μg/ml) for 2 h. Cells were fixed and processed for detection of Na^+-K^+-ATPase by immunofluorescence microscopy as described in EXPERIMENTAL PROCEDURES.

tered by FIP. However, vitamin C significantly induced expression of both EPCR (Fig. 7*B*) and tissue factor pathway inhibitor (TFPI) (Fig. 7*C*). Finally, vitamin C induced expression of the fibrinolytic protein t-PA in septic lung (Fig. 7*E*). Similar observations were made in the endotoxin model of sepsis-induced ALI (data not shown).

DISCUSSION

The lungs are the most frequently injured remote organ following onset of bacterial peritonitis (55). In this study, we show that parenterally infused vitamin C, administered following induction of polymicrobial peritonitis, attenuates the subsequent development of lung injury and significantly increases survival. With sepsis-induced lung injury involving impairment of multiple pathways (i.e., proinflammatory, coagulation), our initial findings of attenuated lung injury led us to hypothesize that vitamin C exerted pleiotropic effects at multiple levels.

Using a murine model of polymicrobial sepsis, we provide new insight into the multiple mechanisms whereby vitamin C

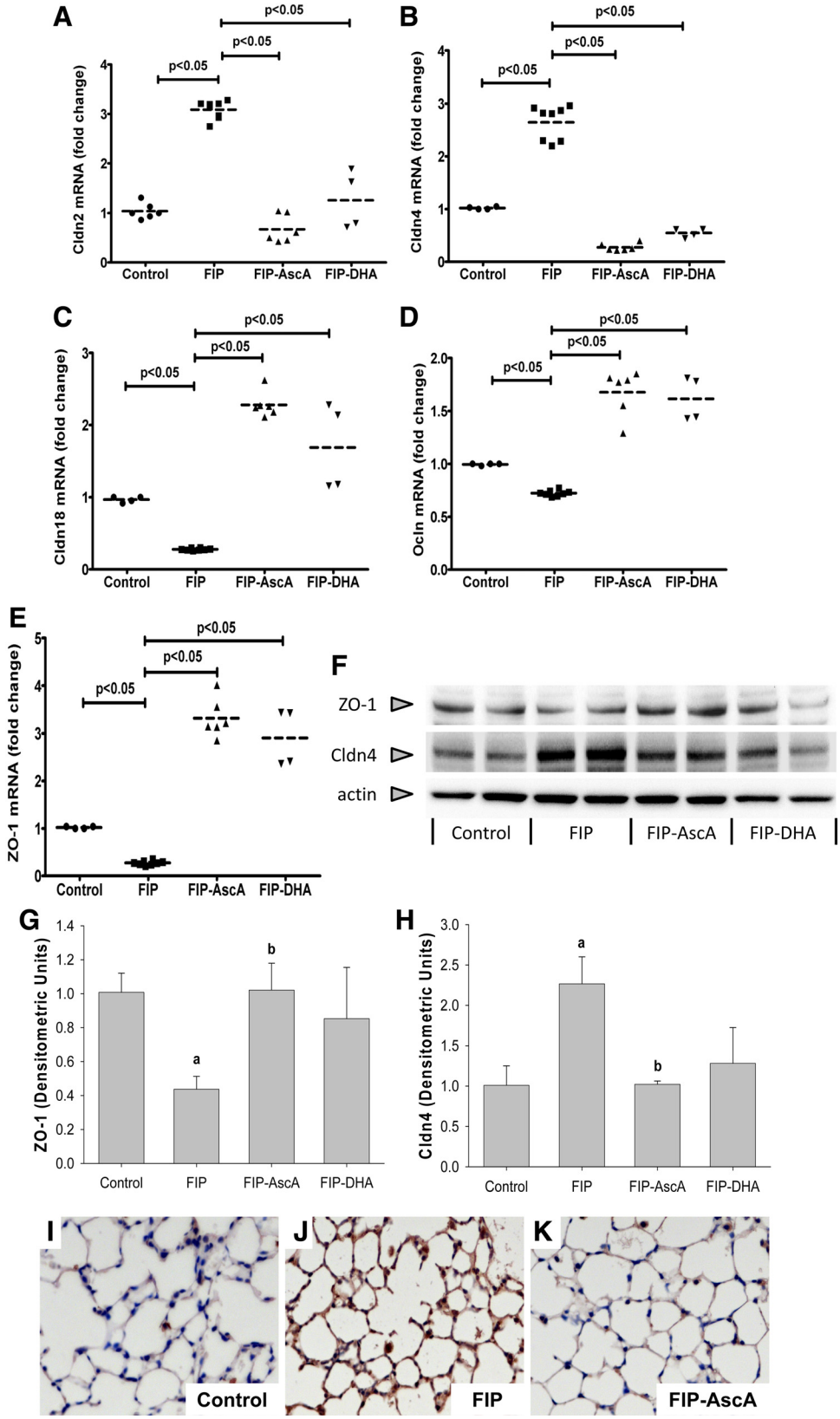

Fig. 5. Vitamin C modulates alveolar barrier function in septic mice lungs. Mice were injected i.p. with saline or fecal stem solution (FIP, 180 mg/ml). AscA (200 mg/kg) or DHA (200 mg/kg) was administered i.p. in saline 30 min after FIP. Real-time QPCR for claudin2 (Cldn2; *A*), claudin4 (Cldn4; *B*), claudin18 (Cldn18; *C*), occludin (Ocln; *D*), and zona occludens-1 (ZO-1; *E*) from lungs of mice 16 h after FIP ($N = 4–6$ per group). *F*: representative Western blot for Cldn4, ZO-1, and actin from lungs of mice 16 h after FIP ± AscA/DHA ($N = 4$ per group). *G* and *H*: densitometry for normalized expression of ZO-1 and Cldn4 was performed with Quantity One analysis software ($^{a}P < 0.05$ vs. control; $^{b}P < 0.025$ vs. FIP, $N = 4$ for each group) *I–K*: representative immunohistochemistry for Cldn4 from lungs of mice 16 h after FIP ± AscA (×40 magnification) ($N = 3$ for each group).

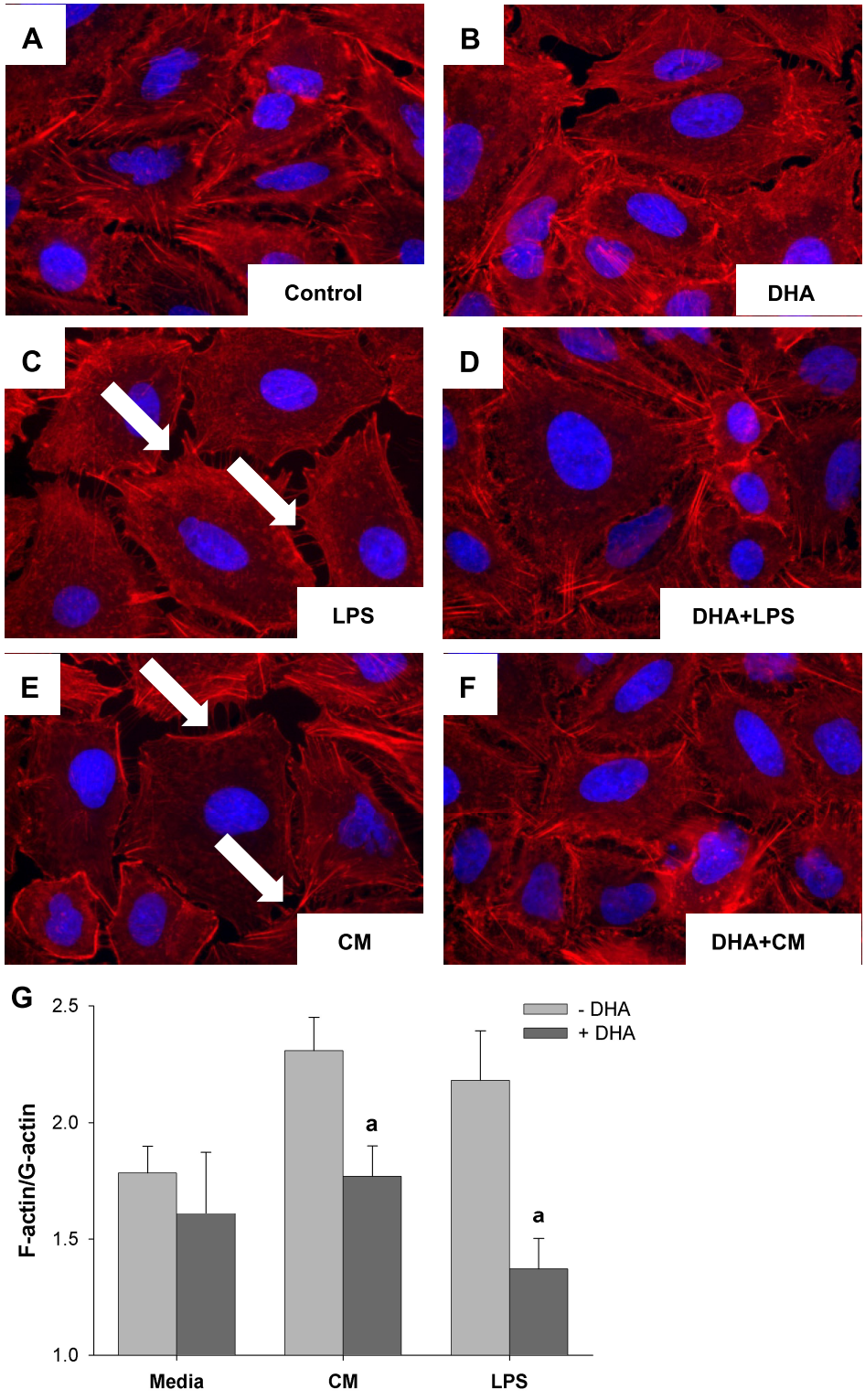

Fig. 6. Vitamin C prevents cytoskeletal rearrangements in alveolar epithelial cells exposed to proinflammatory stimuli. *A–F*: A549 epithelial cells were exposed to media (control) or loaded with DHA (5 mM, 30 min) prior to exposure to endotoxin (LPS, 10 μg/ml) or cytomix (CM; 10 ng/ml of TNF-α, IL-1β, and IFN-γ) for 4 h. Cells were processed for detection of F-actin by fluorescent microscopy as described in EXPERIMENTAL PROCEDURES. Representative images for each treatment group are shown (×60 magnification) (arrows indicate cell contraction with emergence of intercellular gaps, $N = 3$ per group). *G*: the concentration of F-actin and G-actin from the A549 epithelial cells exposed to identical conditions as above was measured as described in EXPERIMENTAL PROCEDURES and depicted as the ratio of F-actin to G-actin ($^aP < 0.05$, $N = 3$ per group).

attenuates ALI and improves survival. A common pathological feature of sepsis induced ALI is an acute proinflammatory response coupled with PMN infiltration in lung. Vitamin C infusion significantly attenuated both the lung proinflammatory response as well as PMN sequestration (Fig. 1). Loss of lung barrier function follows quickly in the setting of sepsis-induced neutrophilic capillaritis. Our peritonitis model showed excess lung water (Fig. 2) and a significant leakage of the tracer FD4 into BAL fluid, suggesting loss of both endothelial and epithelial barrier function (at least to small solutes) (Fig. 2). However, the movement of proteins across the alveolar epithelial barrier was not significantly different in septic mice (data not shown), indicating that the alveolar epithelial barrier to large solutes was still intact in this model of abdominal peritonitis.

Table 1. *Vitamin C prevents changes in viscoelastic properties of septic mouse blood*

Parameters	Control	FIP	FIP-AscA
R, min	5.9 ± 2.6	12.97 ± 12.93	6.8 ± 3.23
K, min	1.9 ± 0.9	14.4 ± 3.75*	2.0 ± 0.69†
Angle,°	59.88 ± 12.19	12 ± 13.36*	63.1 ± 9.73†
MA, mm	64.23 ± 4.43	9.97 ± 10.11*	67.57 ± 7.81†
G, dyn/cm^2	9.15 ± 1.67	0.6 ± 0.7*	11.0 ± 3.67†

Mice were injected intraperitoneally (i.p.) with saline or fecal stem solution (FIP, 180 mg/ml). Ascorbic acid (AscA; 200 mg/kg) was administered i.p. in saline 30 min after FIP. Thromboelastography from citrated mouse blood was performed as described in EXPERIMENTAL PROCEDURES (N =3 per group). R, clot onset time; K, clot formation (or kinetics) time; Angle, clotting angle; MA, maximal clot strength; G, shear elastic modulus. *$P < 0.05$ vs. control; †$P < 0.05$ vs. FIP.

Thus vitamin C infusion preserved lung barrier function in septic mice. In support of this observation, we found that expression of critical ion channels and pumps were induced in septic lung treated with vitamin C (Fig. 3), in endotoxin-exposed alveolar epithelial cells and also in an endotoxin model of ALI (data not shown). Increased expression of these ion channels and pumps was associated with increased AFC in septic mice that received vitamin C (Fig. 3). We further found that vitamin C prevented disassembly of the Na^+-K^+-ATPase pump in endotoxin challenged alveolar epithelial cells (Fig. 4). Beyond restoration of ion channel and pump expression in A549, vitamin C preserved epithelial paracellular permeability to ions and small molecules by normalizing expression of TJ proteins and by preventing actin-cytoskeletal rearrangements (Figs. 5 and 6). Finally, vitamin C infusion abolished the coagulation abnormalities present in septic mouse blood (Table 1). This normalized coagulopathy was associated with attenuation of TF expression and induction of TFPI, EPCR, TM, and t-PA expression, which corrected the procoagulant-anticoagulant-fibrinolytic imbalance in septic mouse lungs (Fig. 7).

Increased AFC can be achieved by increased synthesis and/or activity of key ion channels and pumps located specifically on the apical and basolateral surfaces of alveolar epithelium (16, 36, 41). In our study vitamin C infusion increased AFC by significantly upregulating expression of ENaC and Na^+-K^+-ATPase (Fig. 3). Recent studies also show that diminished Na^+-K^+-ATPase function during ALI results from endocytosis of Na^+-K^+-ATPase into intracellular pools (33). Here, endotoxin exposure of pulmonary epithelial cells induced a rapid translocation of Na^+-K^+-ATPase to the cytosolic compartment. In contrast, incubation with vitamin C prior to endotoxin exposure promoted Na^+-K^+-ATPase localization to the membrane (Fig. 4). Fang et al. (22) have suggested that AFC may be indirectly achieved by chloride transport through the CFTR. We found decreased pulmonary expression of CFTR in mice with abdominal sepsis. Vitamin C intervention significantly increased CFTR expression in septic lung (Fig. 3). Since posttranslational damage to CFTR by reactive oxygen and nitrogen species decreases CFTR function, it is also possible that vitamin C, by virtue of its antioxidant properties, activates CFTR directly by modifying its redox state. Finally, in our model lung Aqp5 expression was significantly decreased. In contradistinction, Aqp5 expression was significantly induced following vitamin C infusion (Fig. 3). The significance of this observation remains to be determined since it has been reported that although osmotically driven water permeability between the air space and capillary compartments is reduced by AQP deletion, loss of the AQP channels, per se, does not decrease AFC (50). Therefore, based on these observations we conclude that vitamin C has profound effects on multiple mechanisms that positively regulate AFC in septic mice.

Paracellular fluid movement between cells is directly mediated by the claudin family of transmembrane proteins (2). Paracellular permeability is determined by the pattern of claudin expression in a particular epithelial cell type (32, 58). In particular, cldn4 was induced in a ventilator-induced lung injury model and manipulations of claudins reduced pulmonary edema. In our studies, sepsis significantly up regulated lung cldn2 and cldn4 expression (Fig. 5). In contrast, sepsis significantly decreased cldn18, occludin, and ZO-1 expression. Vitamin C reversed these changes (Fig. 5). We speculate that vitamin C-induced increases in cldn18, occludin, and ZO-1 expression compensates for diminished expression of cldn2 and 4. Whether vitamin C exerts direct effects on claudin expression remains to be investigated.

Tight junction proteins do not operate in isolation but rather bind cytoskeletal proteins to ensure that junctional forces and cell shape are maintained (26). New evidence suggests that ion channels and pumps are functionally associated with cytoskeletal elements. These studies have documented changes in cellular F-actin content that associate with cellular shrinkage and increased intercellular gap formation (49). In our studies, proinflammatory stimuli significantly induced actin polymerization in alveolar epithelial cells (Fig. 6). F-actin content correlated visually with cell shrinkage and increased intercellular gap formation, changes that were prevented by loading cells with vitamin C. These studies suggest that vitamin C is a key factor in maintenance of alveolar epithelial barrier integrity in the face of proinflammatory stimuli.

Clinical ALI is characterized by profound imbalances in systemic and intra-alveolar coagulation and fibrinolysis. Inflammation-induced activation of the coagulation cascade and impairment of fibrinolysis leads to fibrin deposition in the air spaces and the lung microvasculature (6). Activation of coagulation amplifies the inflammatory cascade by driving proinflammatory gene expression. Recent evidence from human studies suggests that ALI is characterized by activation of procoagulants such as TF, downregulation of the anticoagulant and anti-inflammatory protein C, and increased production of the antifibrinolytic protein plasminogen activator inhibitor-1 (PAI-1) (52). These abnormalities combine to shift the intra-alveolar environment from anticoagulant and profibrinolytic to procoagulant and antifibrinolytic. In addition, viscoelastic tests of clot formation, such as TEG or rotational thrombelastometry, have identified reduced clot strength, prolonged clot initiation times, and increased fibrinolysis in sepsis patients (42, 15). We have previously shown that vitamin C restored prothrombin time and activated partial thromboplastin time and corrected virtually all signs of sepsis-induced coagulopathy in an endotoxin model of sepsis (23). In this study we observed significant normalization of viscoelastic properties of septic mouse blood by vitamin C infusion (Table 1). At a molecular level, this normalization was characterized by attenuation of TF expression; induction of EPCR, TFPI, and TM, which favor anticoagulation; and increased expression of t-PA, which pro-

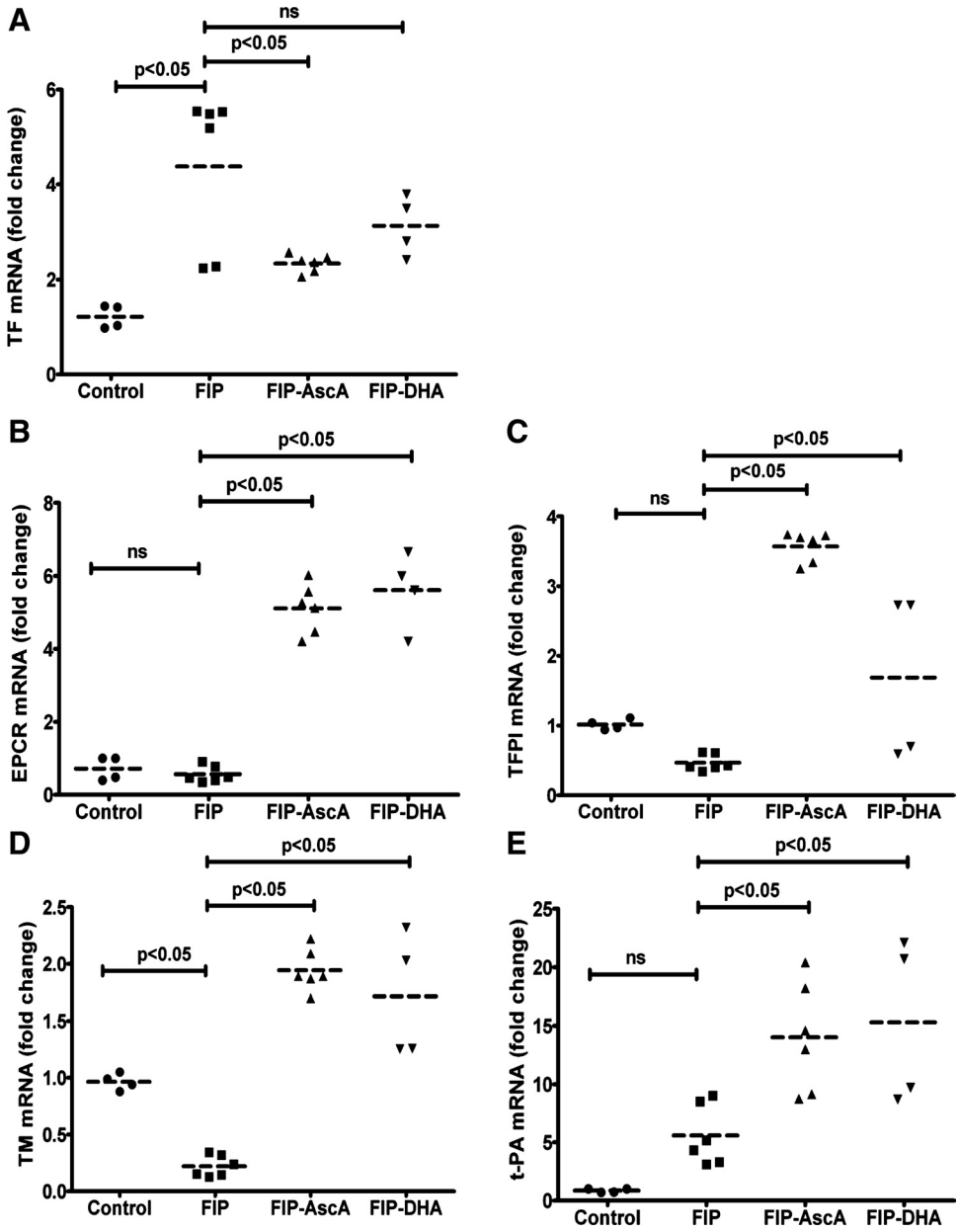

Fig. 7. Vitamin C infusion restores coagulation homeostasis in septic mice. Mice were injected i.p. with saline or fecal stem solution (FIP, 180 mg/ml). AscA (200 mg/kg) or DHA (200 mg/kg) was administered i.p. in saline 30 min after FIP. Real-time QPCR for tissue factor (TF; *A*), endothelial protein C receptor (EPCR; *B*), tissue factor pathway inhibitor (TFPI; *C*), thrombomodulin (TM; *D*), and tissue plasminogen activator (t-PA; *E*) from lungs of mice 16 h after FIP (N = 4–6 per group).

motes fibrinolysis (Fig. 7). Although TF is the pivotal mediator for initiation of extrinsic coagulation pathway, its procoagulant activity is balanced by its natural inhibitor, TFPI. Recombinant TFPI was shown to attenuate lung injury in a rat model of *E. coli* sepsis (19) and also to improve survival in a lethal baboon model of *E. coli* sepsis (12). The receptors EPCR and TM play critical roles in activated protein C (APC) generation on the endothelial cell surface. Protein C is transformed to its active form on the endothelial cell surface by the TM-thrombin complex with EPCR further enhancing protein C activation by binding to the TM-thrombin complex (44). APC proteolytically destroys coagulation factors Va and VIIIa, thus suppressing further thrombin formation (21). Activated protein C also neutralizes PAI-1, thereby promoting fibrinolysis (39). Thus modulation of the procoagulant, anticoagulant, and fibrinolytic pathways could account for the key regulatory effects of vitamin C on coagulation abnormalities associated with peritonitis-mediated ALI.

In addition to these mechanisms, recent reports suggest that parenteral vitamin C could exert beneficial effects by several other mechanisms. Vitamin C is quickly taken up by microvascular endothelial cells in millimolar quantities where it could potentially enhance microvascular functions including capillary blood flow and microvascular permeability barrier (57). Because of the retention of high levels of intracellular ascorbate, the protective effects of vitamin C such as scavenging of reactive oxygen species, increased tetrahydrobiopterin content in platelets and endothelial cells and increased endothelial nitric oxide synthase-derived nitric oxide in these cells could also last for long durations (31, 43). Furthermore, increased intracellular vitamin C concentrations could alter redox-sensitive signaling pathways to diminish septic induction

of NADPH oxidase and inducible nitric oxide synthase (46). Adhesion of leukocytes and platelets to endothelium lead to formation of blood clots and blood flow stoppage during sepsis. Intravenous vitamin C could potentially reverse adhesion by increasing bioavailable nitric oxide, reducing P-selectin protein expression and reducing platelet adhesion (47). Finally, apoptosis is inhibited in vitamin C-deficient neutrophils (51). Intravenous vitamin C could potentially restore neutrophil ascorbate levels to ensure efficient and safe resolution of inflammation and prevent the release of cytotoxic and hydrolytic neutrophil granule enzymes, which can cause tissue damage (40).

As a clinical problem, sepsis is a leading cause of death in the critically ill with more than 750,000 new cases diagnosed in the United States annually, of which 40% of septic patients eventually develop ALI (28). In the aggregate our experimental data illustrate that parenterally administered vitamin C accumulates intracellularly and augments lung barrier cell function and structure. This protective effect may require normalization of gene and protein expression, cell membrane remodeling, and the normalization of several signaling mechanisms. To what degree these molecular events are controlled by oxidative stress and mitochondrial dysfunction remains to be investigated.

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AUTHOR CONTRIBUTIONS

Conception and design: B.J.F., A.A.F., R.N.; analysis and interpretation: B.J.F., D.K., E.J.M., D.F., K.R.W., D.B., R.N.; drafting the manuscript for important intellectual content: B.J.F., J.A.W., D.B., K.R.W., N.F.V., A.A.F., R.N.

DISCLOSURES

J. A. Wegelin: Tri-Continental-Part of my retirement is in this mutual fund. Asset Manager-My stocks are managed by a professional asset manager. I do not make decisions what stocks to buy or sell.

D. Brophy: NovoNordisk A/S-Consultancy; National Institutes of Health, Department of Defense, Entegrion, LLC, NovoNordisk A/S-Grants/grants pending.

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