

α -Tocopherol Attenuates the Severity of *Pseudomonas aeruginosa*-induced Pneumonia

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Abstract

Pseudomonas aeruginosa is a lethal pathogen that causes high mortality and morbidity in immunocompromised and critically ill patients. The type III secretion system (T3SS) of *P. aeruginosa* mediates many of the adverse effects of infection with this pathogen, including increased lung permeability in a Toll-like receptor 4/RhoA/PAI-1 (plasminogen activator inhibitor-1)-dependent manner. α -Tocopherol has antiinflammatory properties that may make it a useful adjunct in treatment of this moribund infection. We measured transendothelial and transepithelial resistance, RhoA and PAI-1 activation, stress fiber formation, *P. aeruginosa* T3SS exoenzyme (ExoY) intoxication into host cells, and survival in a murine model of pneumonia in the presence of *P. aeruginosa* and pretreatment with α -tocopherol. We

found that α -tocopherol alleviated *P. aeruginosa*-mediated alveolar endothelial and epithelial paracellular permeability by inhibiting RhoA, in part, via PAI-1 activation, and increased survival in a mouse model of *P. aeruginosa* pneumonia. Furthermore, we found that α -tocopherol decreased the activation of RhoA and PAI-1 by blocking the injection of T3SS exoenzymes into alveolar epithelial cells. *P. aeruginosa* is becoming increasingly antibiotic resistant. We provide evidence that α -tocopherol could be a useful therapeutic agent for individuals who are susceptible to infection with *P. aeruginosa*, such as those who are immunocompromised or critically ill.

Keywords: vitamin E; pulmonary edema; type III secretion system; ExoY; antimicrobial resistance

Pseudomonas aeruginosa is a lethal pathogen that causes pneumonia in patients who are immunocompromised or critically ill (1, 2). The costs of treating nosocomial infections continues to climb, and the incidence of infection and antibiotic resistance is becoming more common (3, 4). One report specifically indicated that *P. aeruginosa* infection itself was an independent risk factor for mortality in nosocomial pneumonia (1). The increased mortality secondary to *P. aeruginosa* pneumonia is

due, in part, to the development of acute lung injury characterized by flooding of the airspaces with protein-rich edema. Specific contributors to *P. aeruginosa* pathogenesis include, but are not limited to, flagella, pili, LPS, elastase, alkaline phosphatase, exotoxin A, and components of the type III secretion system (T3SS) (5–7). In addition to the detrimental effects from the bacterium itself, there is an increase in multidrug-resistant *P. aeruginosa* strains that are developing at an alarming rate (8–10). Even more concerning

is the fact that there are very few antibiotics being discovered and tested by the pharmaceutical industry to combat these new multidrug-resistant strains (11). The T3SS plays a major role in distal lung injury caused by *P. aeruginosa* (12, 13), and four exoenzymes are currently known: ExoS, ExoT, ExoU, and ExoY. ExoY is a nucleotidyl cyclase that can generate cAMP, CGMP, and/or cUMP (14–16), and ExoU is a phospholipase that is correlated with high patient mortality (13, 17). ExoS and ExoT

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are N-terminal RhoGAPs that activate RhoA (18, 19) and cause ADP ribosylation of multiple cell proteins (20). Furthermore, we have previously published that ExoS and ExoT increase endothelial and epithelial paracellular permeability by a RhoA-dependent mechanism. The RhoA-mediated effects could be blocked both *in vitro* and *in vivo* by a specific RhoA inhibitor (21–23). Finally, clinical studies have shown elevated levels of PAI-1 in the BAL fluid of patients with *P. aeruginosa* pneumonia (24). Although knockout of PAI-1 in mice alleviates the *in vivo* effects of *P. aeruginosa* on lung permeability, PAI-1 deletion did not improve survival because of its inhibitory effects on neutrophil migration, endocytosis, and bacterial killing (23).

α -Tocopherol belongs to a family of lipid-soluble antioxidants including, but not limited to, α - and γ -tocopherols (25). In diseases such as asthma and allergic lung disease, α -tocopherol generally confers antiinflammatory effects, and γ -tocopherol mediates proinflammatory effects (26). For example, α -tocopherol can reduce airway hyperreactivity in mice (27) and block migration of leukocytes across endothelial cells (28). Furthermore, children and adults with asthma tend to have lower levels of α -tocopherol (29–31), and α -tocopherol is negatively associated with osteoarthritis (32).

Because *P. aeruginosa* pneumonia is often associated with multidrug resistance and there are few, if any, new antibiotic therapies in the discovery pipeline, new adjuncts that may attenuate the severity of *P. aeruginosa* pneumonia should be sought. Given the antiinflammatory properties of α -tocopherol and its salutary effects on lung inflammation in response to endotoxin (33) and sulfur and nitrogen mustards (34), we hypothesized that pretreatment of endothelial and epithelial cells with α -tocopherol would prevent increased paracellular permeability secondary to exposure to *P. aeruginosa*. We designed a series of experiments to test whether α -tocopherol may alleviate *P. aeruginosa*-mediated alveolar endothelial and epithelial paracellular permeability by inhibiting RhoA, in part, via PAI-1 activation (23), and increase survival in a mouse model of *P. aeruginosa* pneumonia. Furthermore, we tested the hypothesis that α -tocopherol decreases the activation of RhoA and PAI-1 by blocking the injection of T3SS exoenzymes into alveolar epithelial cells (35).

Methods

Reagents

All reagents were purchased from Fisher Scientific unless otherwise specified: Vital E-500 (α -tocopherol), Trolox, and γ -tocopherol (Stuart Products, Inc.); RhoA G-LISA (Cytoskeleton); plasminogen activator inhibitor (PAI)-1 ELISA (Innovative Research); mouse myeloperoxidase ELISA kit (Cell Sciences); ¹²⁵I-HSA (Jeanatope ISO-TEX Diagnostics); Myc-Tag and GAPDH monoclonal antibodies (Cell Signaling); IRDye 680RD anti-rabbit and 800CW anti-mouse antibodies (LI-COR Biosciences).

Rat Endothelial Cell Isolation

Rat microvascular endothelial cells (RMVECs) were isolated as described (36).

Cell Culture

RMVECs were cultured in DMEM, and L2 cells (ATCC) were cultured in Ham's F12-K medium with 10% FBS and 1% penicillin/streptomycin.

Preparation of *P. aeruginosa*

PAK and PAK-expressing GFP-fused or Myc-tagged ExoY were prepared as described (37).

Paracellular Resistance

Cellular barrier integrity was measured using Electric Cell-substrate Impedance Sensing as described (36).

Transepithelial Albumin Flux

L2 cells were assayed as described (23).

ELISA

RhoA and PAI-1 levels were measured according to manufacturer instructions.

Confocal Microscopy

Confocal microscopy was performed as described (37). Confluent cells were plated on coverslips, exposed to PAK, fixed, mounted, and viewed by fluorescence microscopy. RMVECs were additionally permeabilized and stained with rhodamine-phalloidin at 1:200.

Western Blotting

Western blotting was performed as described (38), with primary antibodies used at 1:1,000 and secondary antibodies used at 1:10,000.

Mice

C57BL/6 mice were purchased from Charles River Laboratories and housed in University of Alabama at Birmingham Animal Care Facilities. All use of animals was according to protocols approved by University of Alabama at Birmingham Animal Care and Use Committee.

Pneumonia

The mouse pneumonia model was performed as described (37).

In Vivo α -Tocopherol Levels

Mice were pretreated with α -tocopherol (3 units/kg). Six hours after PAK installation, mice were killed, desired organs were collected and homogenized, and α -tocopherol levels were measured by HPLC (39).

Lung Vascular Permeability

Lung endothelial permeability was measured as described (37).

Bacterial Lung Colony-Forming Units

Measurement of lung colony-forming units was performed as previously described (22).

BAL Protein Measurement and Cell Counts

Measurement of whole-lung protein was performed as previously described (40).

Lung Myeloperoxidase Measurements in Whole-Lung Tissue

Measurement of whole-lung myeloperoxidase (MPO) was performed as previously described (22).

Neutrophil Isolation

Peritoneal neutrophils were isolated as previously described (41).

Neutrophil Phagocytosis

Isolated neutrophils were pretreated with α -tocopherol and exposed to PAK, and phagocytosis was measured as previously described (41).

Statistical Analysis

All normal data are mean \pm SEM. Nonparametric data are mean \pm interquartile range. Normal distributions were verified using the Agostino-Pearson test. For normally distributed data, one-way ANOVA followed by Dunnett's test was used to compare three or more groups and Student's *t* test to compare two groups. Bonferroni

correction was used to adjust multiple comparisons. Nonparametric data were compared with a Kruskal-Wallis test followed by a Tukey *post hoc* test. A Kaplan-Meier analysis followed by a log rank (Mantel-Cox) test was used to compare the survival between the two groups. A *P* value of <0.05 was considered statistically significant.

Results

α -Tocopherol Attenuates *P. aeruginosa*-mediated Increases in Lung Endothelial Paracellular Permeability

To test the hypothesis that α -tocopherol may be a useful adjunct in treatment of

infection with *P. aeruginosa*, we first tested whether α -tocopherol (Vital E-500) could prevent PAK (wild-type strain of *P. aeruginosa*)-induced increases in lung endothelial permeability (Figure 1A). Although there was no change in permeability by α -tocopherol treatment alone, exposure to PAK increased paracellular permeability roughly fivefold. Pretreatment with 10 μ M α -tocopherol significantly decreased PAK-mediated increases in endothelial paracellular permeability. To understand whether the effects of α -tocopherol were specific to this isoform, we performed the same experiment using γ -tocopherol and Trolox (water-soluble analog of α -tocopherol) (Figure 1A). Neither of these treatments had a significant effect on endothelial

paracellular permeability. However, neither therapy was able to reduce PAK-mediated increases in permeability, indicating that the effects of α -tocopherol are both isoform- and fat soluble-specific.

α -Tocopherol Reduces *P. aeruginosa*-mediated Activation of RhoA and PAI-1 in Endothelial Cells and Prevents *P. aeruginosa*-mediated Stress Fiber Formation

Our previous work revealed that *P. aeruginosa* infection mediates its effects on lung permeability, in part, via the activation of RhoA and PAI-1, which can lead to actin stress fiber formation and increase in paracellular endothelial permeability (22, 23, 42). Therefore, we measured RhoA and PAI-1 levels in the

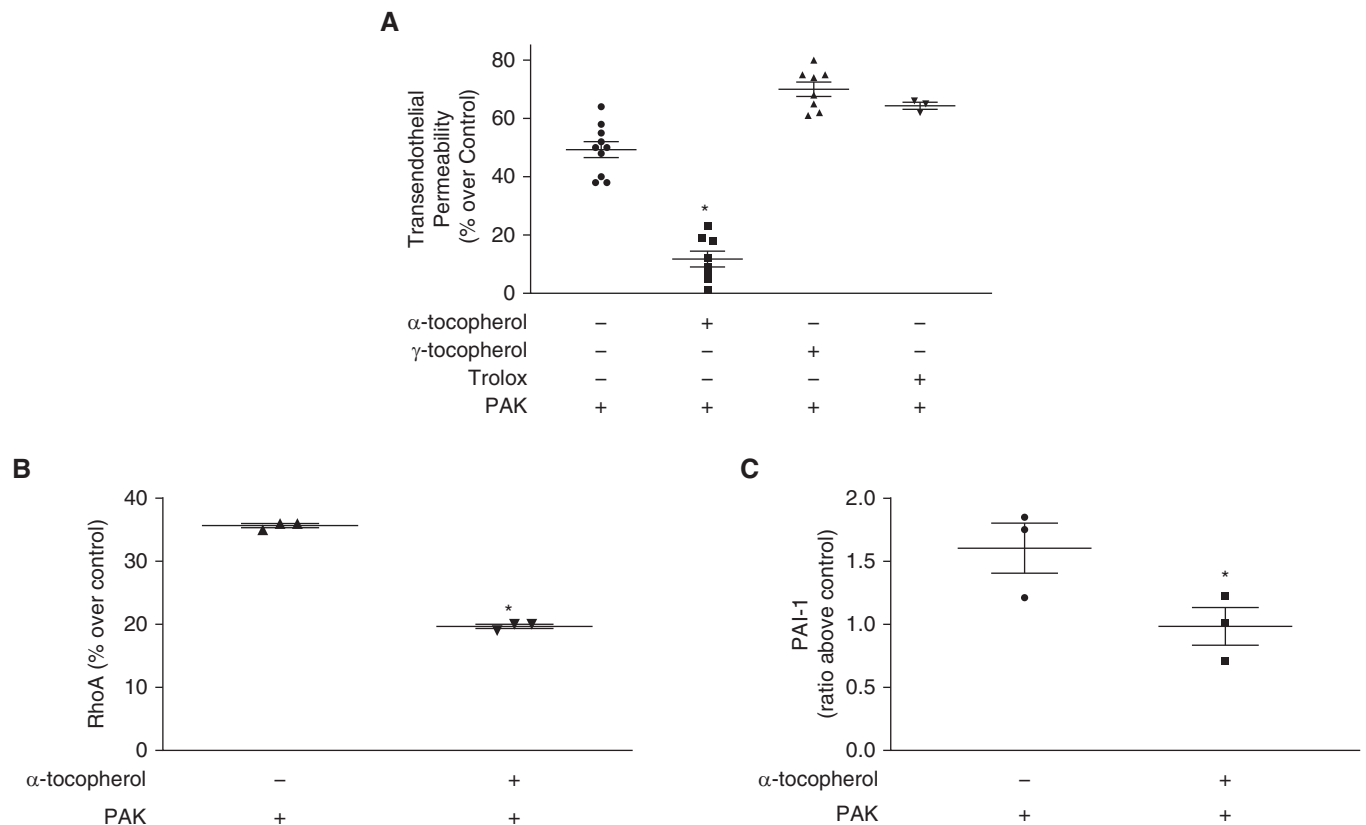


Figure 1. α -Tocopherol prevents endothelial paracellular permeability derangements and activation of RhoA and plasminogen activator inhibitor-1 (PAI-1) secondary to exposure to *Pseudomonas aeruginosa*. For all experiments, confluent rat microvascular endothelial cells were pretreated with 10 μ M α -tocopherol, γ -tocopherol, or Trolox (or vehicle), exposed to PAK (wild-type strain of *P. aeruginosa* at a multiplicity of infection of 40) as appropriate, and parameters were measured as described below. (A) Transendothelial paracellular permeability was measured by Electric Cell-substrate Impedance Sensing for 24 hours. Vehicle alone baseline was $5\% \pm 2\%$, α -tocopherol alone was $6\% \pm 2\%$, γ -tocopherol alone was $3\% \pm 2\%$, and Trolox alone was $4\% \pm 2\%$. (B) RhoA was measured in the supernatant after 6 hours by ELISA. Baseline optical density 490 for vehicle was 0.13 ± 0.01 and for α -tocopherol was 0.135 ± 0.01 . (C) PAI-1 was measured in the supernatant after 6 hours by ELISA. Baseline ng/ml for vehicle was 0.38 ± 0.1 and for α -tocopherol was 0.41 ± 0.2 . Data are expressed as the ratio of PAK-treated levels divided by non-PAK-treated levels (α -tocopherol or vehicle alone, percentage over control or ratio above control). Data are expressed as mean \pm SEM and are the result of three independent experiments. $P \leq 0.05$. *Significant difference compared with cells treated with PAK alone.

supernatants of RMVECs pretreated with 10 μ M α -tocopherol and exposed to PAK for 6 hours (Figures 1B and 1C). Interestingly, pretreatment with α -tocopherol was able to significantly reduce activation of both of these mediators of endothelial paracellular permeability. Furthermore, α -tocopherol was able to prevent formation of PAK-induced stress fibers in endothelial cells (Figure 2). Finally, the protective effect of α -tocopherol on the lung endothelial permeability and RhoA and PAI-1 activation was not associated with an increase in bacterial killing (data not shown).

α -Tocopherol Prevents Increases in Epithelial Paracellular Permeability and RhoA and PAI-1 Activation Induced by *P. aeruginosa*.

To determine whether α -tocopherol could prevent *P. aeruginosa*-induced derangements in alveolar epithelial cells as well, we exposed L2 cells to PAK after pretreatment with 10 μ M α -tocopherol. *P. aeruginosa*-induced increase in transepithelial permeability (Figure 3A), RhoA activation (Figure 3B), and increased PAI-1 levels (Figure 3C) were ameliorated by pretreatment with α -tocopherol. These results indicate that α -tocopherol may be

useful to protect both sides of the alveolar–capillary membrane.

α -Tocopherol Prevents Insertion of T3SS Exoenzymes into Alveolar Epithelial Cells

Previous studies have reported that T3SS exoenzymes are injected in parenchymal cells through insertion in lipid raft domains of the cell membrane (35). Furthermore, α -tocopherol has been shown to prevent accumulation of proatherogenic lipids in the lipid raft domains of the cell membrane (43). To further elucidate the mechanism by which α -tocopherol protects alveolar epithelial cells from *P. aeruginosa*-mediated injury, we examined whether α -tocopherol could prevent insertion of ExoY into L2 cells. ExoY is one of the exoenzymes of the T3SS of *P. aeruginosa* that plays an important role in mediating bacterial virulence (15, 44–46). Furthermore, wild-type strains of *P. aeruginosa* that express GFP-fused or Myc-tagged ExoY can be used to measure exoenzyme insertion into cells. PAK-Myc (PAK strain expressing Myc-tagged ExoY) and results of intracellular Myc (representing ExoY) was measured and quantified by Western blot (Figures 4A and 4B). α -Tocopherol prevented ExoY insertion by PAK-Myc. PAK-GFP (PAK

strain expressing GFP-fused ExoY) alone was able to insert significant amounts of ExoY into L2 cells (Figure 4C). Alternatively, when L2 cells were pretreated with α -tocopherol there was near complete abrogation of ExoY insertion into cells by PAK-GFP (Figure 4D). The mean intensity of immunofluorescence was quantified in L2 cells exposed to the aforementioned treatments (Figure 4E). In both cases, α -tocopherol was able to significantly reduce the levels of ExoY inserted into alveolar epithelial cells by *P. aeruginosa*.

α -Tocopherol Attenuates *P. aeruginosa*-mediated Lung Vascular Permeability, Reduces Bacterial Burden, and Prevents Mortality in a Murine Model of Pneumonia

We first sought to understand whether intraperitoneal administration of α -tocopherol to mice would achieve effective end-organ levels in a murine model of pneumonia. To this end, we treated mice with α -tocopherol with two separate injections 18 hours and 1 hour before PAK instillation. Mice were again injected with α -tocopherol 18 hours after PAK instillation. α -Tocopherol levels were measured in the plasma (see Figure E1A in the data supplement), lung (Figure E1B), kidney (Figure E1C), liver (Figure E1D), spleen (Figure E1E), and heart (Figure E1F) 6 hours after last α -tocopherol dose. In all organs measured, there was a 5- to 10-fold increase in α -tocopherol levels, indicating that α -tocopherol is found at therapeutic levels far exceeding control tissue concentrations in mice (47).

We next found that pretreatment with α -tocopherol was able to reduce lung permeability in a mouse model of *P. aeruginosa* pneumonia (Figure 5A). Interestingly, BAL protein was not decreased by α -tocopherol (Figure 5B). Lung bacterial burden, release of MPO in whole-lung tissue, and overall cells in the BAL were decreased by α -tocopherol (Figures 5C and 5E). Finally, isolated neutrophils did not have improved phagocytosis when pretreated with α -tocopherol (Figure 5F). To determine whether the decrease in lung permeability, reduced colony-forming units, MPO release, and cell influx could have clinically meaningful impact, we measured survival in our pneumonia model (Figure 6). Indeed, pretreatment with α -tocopherol significantly

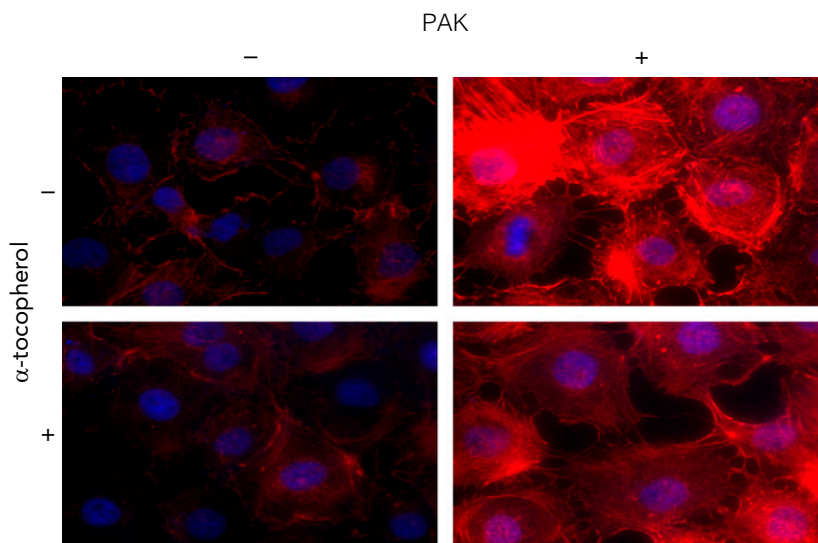


Figure 2. α -Tocopherol blocks *P. aeruginosa*-mediated stress fiber formation. Rat microvascular endothelial cells were pretreated with 10 μ M α -tocopherol and exposed to PAK. Six hours after PAK exposure, cells were fixed, permeabilized, stained with rhodamine-phalloidin, and mounted on slides with Heatshield/DAPI. Cells were imaged with confocal fluorescence microscopy. Experiments were performed in triplicate, and multiple areas of each slide were imaged for consistency. Representative images are shown.

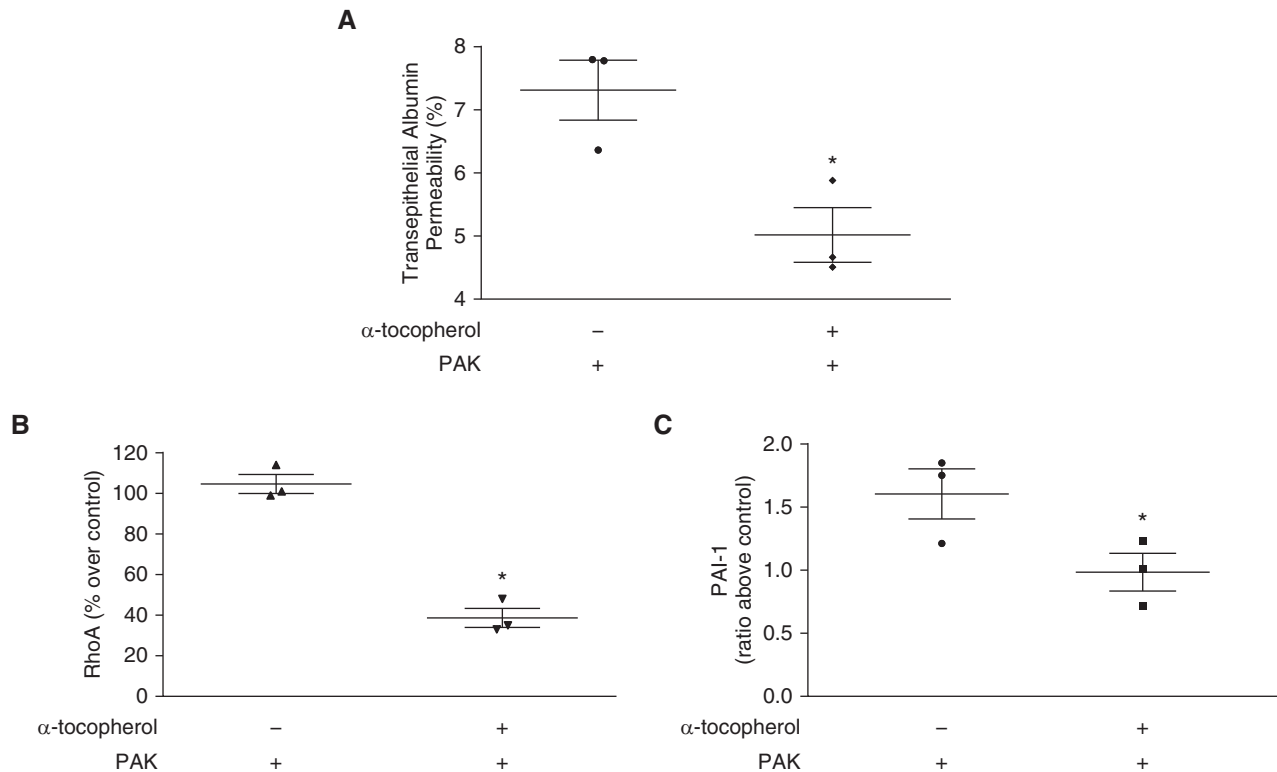


Figure 3. α -Tocopherol prevents epithelial paracellular permeability derangements and activation of RhoA and PAI-1 secondary to exposure to *P. aeruginosa*. (A) L2 (rat alveolar epithelial) cells were plated to Transwells and allowed to grow until they were polarized and could hold back media consistently. Transepithelial albumin permeability was performed as described, and cells were pretreated with 10 μ M α -tocopherol and exposed to PAK. Permeability was measured as the ratio of radioactive albumin levels in the basolateral side after 60 minutes divided by the level after 3 minutes. Baseline level for vehicle alone was 3.7 ± 0.4 and for α -tocopherol was 4.2 ± 0.5 . (B) Confluent L2 cells were pretreated with vitamin E and exposed to PAK. RhoA was measured in the supernatant after 6 hours by ELISA. Baseline OD490 for vehicle alone was 0.1 ± 0.01 and for α -tocopherol was 0.13 ± 0.01 . (C) Confluent L2 cells were pretreated with α -tocopherol and exposed to PAK. PAI-1 was measured in the supernatant after 6 hours by ELISA. Baseline level for vehicle was 3.2 ± 0.4 and for α -tocopherol was 2.8 ± 0.3 . Data are expressed as the ratio of PAK-treated levels divided by non-PAK-treated levels (α -tocopherol or vehicle alone, percentage over control or ratio above control). Data are expressed as mean \pm SEM and are the result of three independent experiments. $P \leq 0.05$. *Significant difference compared with cells treated with PAK alone.

decreased mortality from *P. aeruginosa* in our murine model of bacterial pneumonia (Figure 6A); however, when one dose of α -tocopherol was given immediately after PAK instillation, there was no beneficial effect on survival (Figure 6B).

Discussion

P. aeruginosa is an opportunistic pathogen that can cause lethal pneumonia in patients who are immunocompromised or critically ill (1, 2). Furthermore, multidrug resistance continues to be a clinical problem for those patients infected with *P. aeruginosa*, and there are few new antibiotics being discovered to combat the problem. α -Tocopherol is a lipid-soluble antioxidant compound that can attenuate inflammation in a variety of pathologic conditions,

including, but not limited to, asthma, allergic lung disease, sterile inflammation, and burns, by reducing levels of proinflammatory mediators such as IL-8, IL-6, TNF- α , and transforming growth factor- β_1 (25, 26, 48). The results of this study indicate that α -tocopherol significantly attenuates the severity of *P. aeruginosa*-induced pneumonia in mice by inhibiting the insertion of T3SS exoenzymes into the cytosol of lung parenchymal cells, thus decreasing lung vascular permeability, RhoA and PAI-1 activation, and stress actin fiber formation.

The first objective of our study was to test whether α -tocopherol could prevent *P. aeruginosa*-mediated increases in lung endothelial and alveolar epithelial paracellular permeability. We have previously demonstrated that *P. aeruginosa* increases *in vitro* and *in vivo* lung vascular

permeability and the formation of actin stress fibers via a mechanism that is toll-like receptor 4/RhoA/PAI-1 dependent (22, 23) and results in the activation of $\alpha_v\beta_5$ and $\alpha_6\beta_1$ integrins, respectively (21). Furthermore, a previous study has also shown that *P. aeruginosa* increases PAI-1 gene expression in alveolar epithelial cells, in part via a platelet-activating factor-dependent mechanism (49). In the present study, pretreatment with α -tocopherol attenuated the increase in paracellular permeability in both cell types. We then determined some of the mechanisms by which α -tocopherol reduced *P. aeruginosa*-mediated increases in paracellular permeability. Pretreatment with α -tocopherol prevented both RhoA and PAI-1 activation in lung endothelial and epithelial cells. This result is of importance, because increased PAI-1

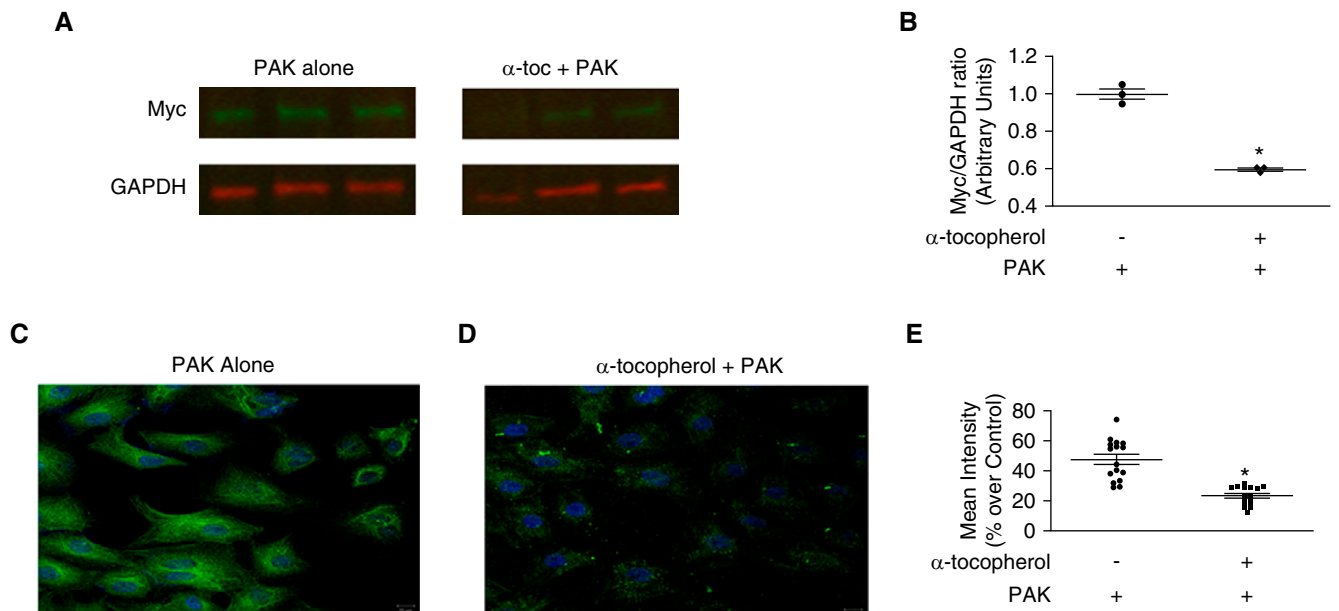


Figure 4. α -Tocopherol prevents insertion of myc- and GFP-tagged exoenzyme Y (ExoY) toxins into epithelial cells. Confluent L2 cells were pretreated with 10 μ M α -tocopherol and exposed to PAK-GFP (wild-type strain of *P. aeruginosa* that expresses GFP-fused ExoY toxin) or PAK-Myc (wild-type strain of *P. aeruginosa* that expresses Myc-tagged ExoY toxin) at a multiplicity of infection of 40 for 6 hours. Cells were washed and treated with gentamicin (150 μ g/ml) for 1 hour to eradicate extracellular PAK. (A and B) After appropriate treatment, cells were lysed and Western blotting using antibodies against Myc and GAPDH performed. (A) A representative blot is shown. (B) Quantification was performed using densitometry, and levels shown are Myc/GAPDH ratio. (C and E) After appropriate treatment with PAK-GFP, cells were fixed and mounted on slides with Vectashield/DAPI. Cells were imaged with confocal fluorescence microscopy. Experiments were performed in triplicate, and multiple areas of each slide were imaged for consistency. Representative images are shown. Quantification was performed by measuring mean intensity and is represented as its percentage over control (vehicle only). All quantified data are expressed as mean \pm SEM and are the result of three independent experiments. $P \leq 0.05$. *Significant difference compared with control.

concentrations in BAL fluids are associated with increased mortality in a cohort of patients with *P. aeruginosa* pneumonia (50). Furthermore, PAI-1 concentrations in BAL fluid can distinguish ventilator-associated pneumonia from colonization with *P. aeruginosa* in mechanically ventilated pediatric patients (24).

Our data also indicate that α -tocopherol, but not γ -tocopherol or Trolox, attenuated *P. aeruginosa*-induced increase in lung endothelial permeability, suggesting that this effect of α -tocopherol may not simply be due to its antiinflammatory properties. α -Tocopherol, but not γ -tocopherol, can accumulate in the lipid raft domains of the cell membrane (51, 52) and may prevent the incorporation of lipids in these domains (43). Furthermore, the T3SS is a complex molecular machinery used by *P. aeruginosa* to inject exoenzymes directly into eukaryotic cells (53–55). Three molecular partners (two hydrophobic and one hydrophilic) work together to make the T3SS needle that is capable of inserting

exoenzymes. The injection of T3SS exoenzymes of *P. aeruginosa* into host cells is localized to the lipid raft domains of the cell membrane (35). Thus, the second objective of our study was to determine whether α -tocopherol could prevent T3SS exoenzyme (ExoY) insertion by *P. aeruginosa* into the lipid raft domains of the cell membrane. We demonstrated by both confocal microscopy and Western blot that insertion of ExoY could be prevented in alveolar epithelial cells by pretreatment with α -tocopherol. Taken together, these data suggest that α -tocopherol may actually be able to prevent insertion of the *P. aeruginosa* T3SS needle and prevent exoenzyme injection that leads to improper cellular activation and disintegration of lung barrier function.

The third objective was to determine whether α -tocopherol would decrease the severity of lung injury and improve survival in a murine model of *P. aeruginosa* pneumonia. The results showed that pretreatment with α -tocopherol improved

survival after pretreatment, but α -tocopherol also improved lung permeability, decreased bacterial load, and reduced accumulation of neutrophils and other cells in the lung, as shown by the lower level of MPO in lung homogenates and decreased cells of mice with *P. aeruginosa* pneumonia. Pretreatment of neutrophils with α -tocopherol did not improve phagocytosis. However, α -tocopherol blocked the expression of intercellular adhesion molecule-1, which regulates the recruitment of neutrophils into the lung through PKC- α , and γ -tocopherol had the opposite effect (28, 56, 57). A follow-up study from the same research group suggested that α -tocopherol decreases neutrophil migration into the alveolar space, thereby reducing formation of radical oxygen species and preventing collateral damage to the intact barrier, but increases elastase activity of neutrophils and their ability to kill bacteria (58). Finally, BAL protein was not decreased by pretreatment with α -tocopherol. This would normally go against evidence that

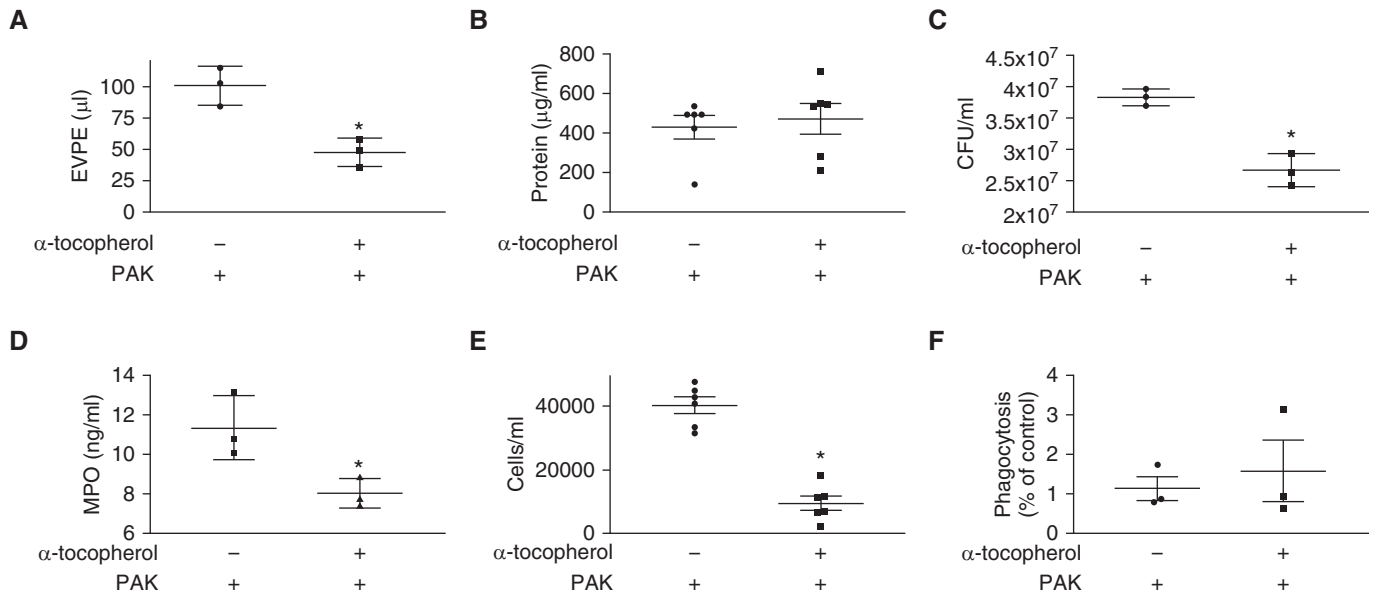


Figure 5. α -Tocopherol attenuates lung permeability and reduces lung bacterial burden in a murine model of pneumonia. For experiments A through E, C57BL/6 mice were intraperitoneally injected with α -tocopherol (3 units/kg) 18 and 1 hour before PAK (5×10^7 cfu) instillation and experiments were performed 6 hours after PAK installation. (A) Extravascular pulmonary edema (EVPE) was measured using ^{131}I -albumin as described in METHODS. Data are expressed as mean \pm SEM and are the result of three independent experiments. (B) BAL protein was measured as described in METHODS. Data are expressed as mean \pm SEM and are the result of six independent experiments. (C) Colony-forming units (cfu) were measured as described in METHODS. Data are expressed as mean \pm SEM and are the result of three independent experiments. (D) Myeloperoxidase (MPO) in lung homogenates was measured as described in METHODS. Measurements were made in duplicate, and data are expressed as mean \pm SEM and are the result of three independent experiments. (E) Influx of cells in the BAL was measured as described in METHODS. Data are expressed as mean \pm SEM and are the result of six independent experiments. (F) Murine neutrophil was measured as described in METHODS. Data are expressed as percentage of control, where vehicle or α -tocopherol groups were divided by cells infected with PAK to reduce variance between experiments. For all experiments, $P \leq 0.05$. *Significant difference compared with control.

cells and MPO were decreased regarding lung injury; however, previous studies have demonstrated that this increased protein may be a marker of increased net alveolar fluid transport (59, 60). Together with reduced permeability measurements (extravascular pulmonary edema), this

suggests that α -tocopherol may prevent epithelial injury and/or increase its rate of epithelial repair as a mechanism to reduce bacterial load and facilitate survival after bacterial pneumonia.

Does α -tocopherol protect against bacterial pneumonia in humans? One

study reported that α -tocopherol reduced the incidence of pneumonia in elderly males (61). Interestingly, a secondary analysis of the ATBC (Alpha-Tocopherol, Beta-Carotene Cancer Prevention) Study of Finnish male smokers aged 50 to 69 years showed that vitamin E

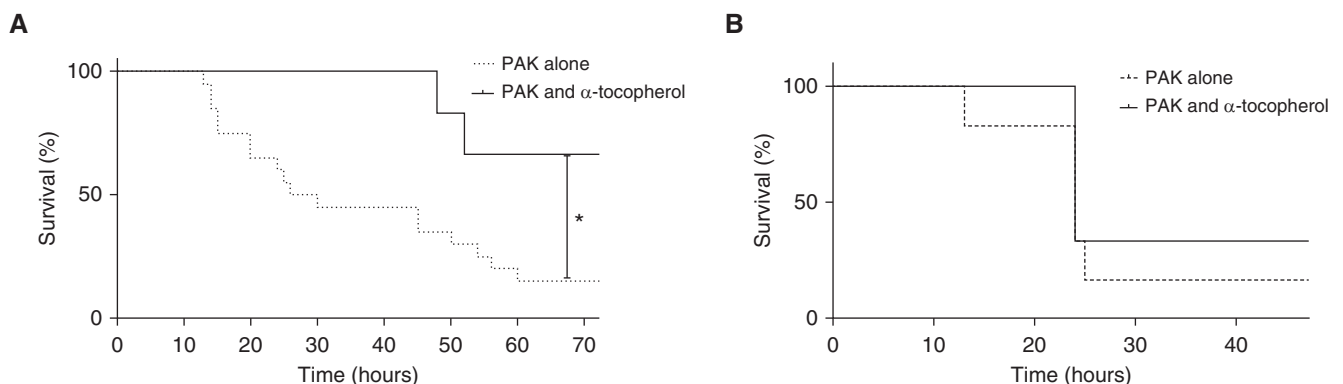


Figure 6. Pretreatment of mice with α -tocopherol, but not post-treatment, improves survival in a murine model of pneumonia. For experiments in A, C57BL/6 mice were intraperitoneally injected with α -tocopherol (3 units/kg) 18 and 1 hour before PAK (5×10^7 cfu) instillation and were given an additional dose of α -tocopherol 18 hours after PAK instillation. For experiments in B, C57BL/6 mice were intraperitoneally injected with α -tocopherol (3 units/kg) immediately after PAK instillation. (A) Survival was measured as described in METHODS ($n = 20$ for PAK only, $n = 8$ for PAK + α -tocopherol). (B) Survival was measured as described in METHODS ($n = 6$ for PAK only, $n = 6$ for PAK + α -tocopherol). $P \leq 0.05$. *Significant difference compared with control.

supplementation decreased pneumonia risk by 69% among participants who had the least exposure to smoking and exercised regularly, whereas vitamin E supplementation increased the risk of pneumonia in patients with the highest exposure to smoking and no exercise (62). Furthermore, it is important to point out that some studies suggest an increased rate of hemorrhagic stroke, elevated blood pressure, and increased all-cause mortality if >400 IU/d of vitamin E are used (63–68). The variable effectiveness of α -tocopherol in humans may be attributed to differences in patient population and doses of α -tocopherol used in these studies. In addition, there are polymorphisms in the genes implicated in vitamin E metabolism that include apolipoprotein E, lipoprotein lipase, CD36 scavenger receptor, scavenger receptor class B, and α -tocopherol transfer protein that may influence the end-organ level and effect of vitamin E supplementation (69). Importantly, these results do not mean that α -tocopherol cannot be a useful adjunct to antibacterial therapy, but dosing must be judicious and patients must be selected appropriately. Furthermore, although post-treatment did not protect mice from pneumonia in our study, it has been well described that certain populations, such as those who have experienced trauma with hemorrhage and resuscitation or severe neurologic injury, have an increased risk of secondary bacterial pneumonia. These populations may be significant targets for adjunct α -tocopherol therapy to prevent secondary bacterial pneumonia after these injuries.

Finally, our results have another important implication. We showed that α -tocopherol prevented the ExoY insertion into alveolar cells. These results suggest that α -tocopherol would also inhibit the intracellular injection of all T3SS exoenzymes of *P. aeruginosa*. Our recent data indicate that a complex of cytotoxic and prion-like proteins containing tau and β -amyloid are released from lung endothelial and alveolar epithelial cells in an ExoY- or ExoU-dependent manner (70). These cytotoxic proteins are present *in vivo* in the BAL fluid and plasma of patients with *P. aeruginosa* pneumonia. Experimental data showed that these cytotoxic proteins cause both lung and brain injury in mice. Therefore, it is possible that α -tocopherol might inhibit the

release of these cytotoxic proteins by preventing the intracellular insertion of T3SS exoenzymes; this question is currently the focus of future investigations in our laboratory.

We have created a model for the findings of our present study (Figure 7). In this model, *P. aeruginosa* comes into contact with cells and can inject toxins (exoenzymes) of the T3SS into host cells. ExoS/T activates RhoA, leading to the release of PAI-1. In concert, these activators lead to increased formation of actin stress fibers. Injection of ExoU leads to increased phospholipase activity, and injection of ExoY leads to increases in cyclic nucleotides. All of the secondary consequences of exoenzyme injection can lead to increases in paracellular permeability and destruction of alveolar–capillary membrane integrity. Our model suggests that α -tocopherol prevents injection of T3SS exoenzymes by *P. aeruginosa*, thereby preventing the downstream effects of this bacterial toxin.

There are limitations to our study. First, we used labeled ExoY as a tool to demonstrate that α -tocopherol prevents

injection of T3SS exoenzymes into the cytosol of alveolar epithelial cells. We are aware that, although PAK does express ExoY to intoxicate cells, there is no current evidence to suggest that host cell intoxication with ExoY and subsequent nucleotidyl cyclase formation activates the RhoA/PAI-1 pathway. This pathway has been classically activated by ExoS/T. However, previous studies have shown that the all T3SS exoenzymes are injected into the parenchymal cells by the same mechanism (reviewed in Reference 45). Second, in our *in vivo* studies, we demonstrate that there is decreased bacterial burden after infection with *P. aeruginosa* and pretreatment of mice with α -tocopherol. However, our data (not shown) also indicate that α -tocopherol has no direct antibacterial activity. Hence, another mechanism to decrease bacterial burden exists. As described above, α -tocopherol can activate certain neutrophil killing functions, although it decreases neutrophil influx into the alveolar airspace.

In summary, we report a novel mechanism by which α -tocopherol inhibits *P. aeruginosa*-mediated lung permeability,

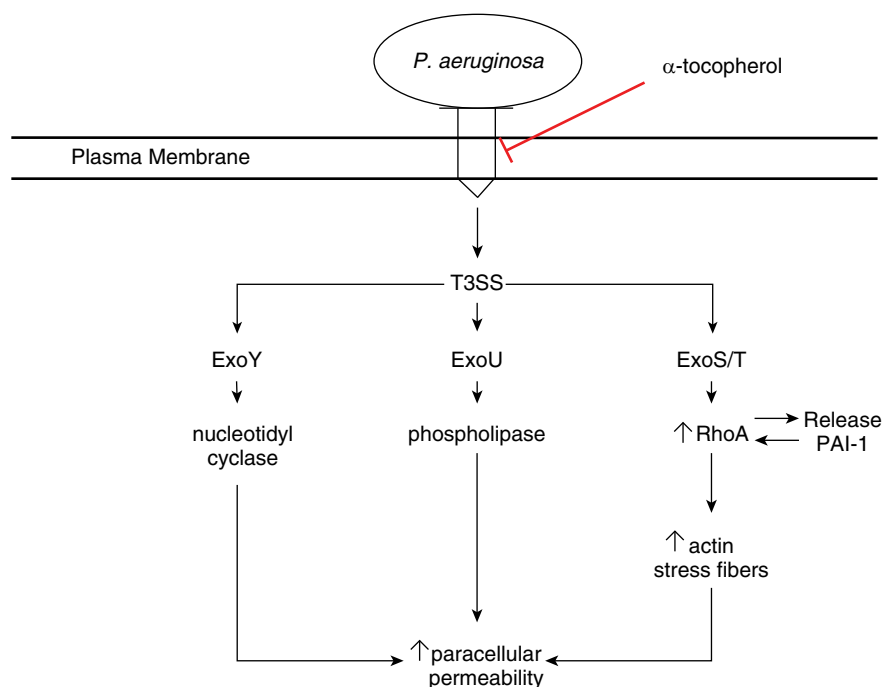


Figure 7. Model of host cell intoxication by *P. aeruginosa* exoenzymes and α -tocopherol mechanism. Briefly, *P. aeruginosa* injects toxins (exoenzymes) into host cells. These exoenzymes activate pathways that lead to increased paracellular permeability. α -Tocopherol prevents increases in paracellular permeability by preventing injection of exoenzymes and activation of subsequent, deleterious pathways. T3SS = type III secretion system.

RhoA and PAI-1 activation, stress fiber formation, and insertion of the T3SS exoenzyme into alveolar epithelial cells. Given that *P. aeruginosa* typically infects critically ill and immunocompromised patients, administration of α -tocopherol could be a novel therapy for patients suffering from trauma, burns, cystic fibrosis,

and other pathologies that leave a patient temporarily or permanently immunocompromised. Clinical trials should be considered in these patient populations to test whether α -tocopherol could attenuate the severity of lung infection with this lethal pathogen that is becoming resistant to extended-spectrum antibiotics,

which can cause undesired complications of long-term antibiotic therapy. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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