B. anthracis Lethal Toxin but not Edema Toxin, Increases Pulmonary Artery Pressure and Permeability in Isolated Perfused Rat Lungs

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Abstract

Introduction: Although lethal (LT) and edema toxin (ET) contribute to lethality during *Bacillus anthracis* infection, whether they increase vascular permeability and the extravascular fluid accumulation characterizing this infection is unclear. We employed an isolated perfused Sprague-Dawley rat lung model to investigate LT and ETs effects on pulmonary vascular permeability.

Methods and Results: Lungs ($n \geq 6$ per experimental group) were isolated, ventilated, suspended from a force transducer and perfused. Lung weight and pulmonary artery (Ppa) and left atrial pressures were measured over 4h after which vascular permeability coefficients (Kf.c) and lung wet-to-dry weight ratios (W/D) were determined. Compared to controls, LT increased Ppa over 4h and Kf.c and W/D at 4h ($p<0.0001$). ET decreased Ppa in a significant trend ($p=0.09$) but did not significantly alter Kf.c or W/D ($p \geq 0.29$). Edema toxin actually blocked LT's increases in Ppa but not LT's increases in Kf.c and W/D. When Ppa was maintained at control levels, LT still increased K.fc and W/D ($p \leq 0.004$). Increasing each toxin's dose five times significantly increased and a toxin-directed monoclonal antibody decreased the toxins' effects ($p \leq 0.05$). Two rho-kinase inhibitors (GSK269962 and Y27632) decreased LT increases in Ppa ($p \leq 0.02$) but actually increased Kf.c and W/D in LT and control lungs ($p \leq 0.05$). A vascular endothelial growth factor receptor inhibitor (ZM323881) had no significant effect ($p \geq 0.63$) with LT.

Conclusion. Thus, LT but not ET can increase pulmonary vascular permeability independent of increased Ppa and could contribute to pulmonary fluid accumulation during anthrax infection. However, pulmonary vascular dilation with ET could disrupt protective hypoxic vasoconstriction.

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Key Words: anthrax; lethal toxin; edema toxin; lung or pulmonary dysfunction

New and Noteworthy: The most important findings from the present study are that *Bacillus anthracis* lethal toxin increases pulmonary artery pressure and pulmonary permeability independently in the isolated rat lung while edema toxin decreases the former and does not increase permeability. Each effects could be a basis for organ dysfunction in patients with this lethal infection. These findings further support the need for adjunctive therapies that limit the effects of both toxins during infection.

Abbreviations:

cAMP: cyclic adenosine monophosphate

EF: edema factor, the toxic moiety of edema toxin

ET: edema toxin, a binary type toxin combining EF and PA

Kf.c: permeability coefficient

LF: lethal factor, the toxic moiety of edema toxin

LT: lethal toxin, a binary type toxin combining LF and PA

MAP: mean systemic arterial blood pressure

PA: protective antigen

Pla: left atrial pressure

Ppa: pulmonary artery pressure

Ppc: pulmonary capillary pressure

PBS: phosphate buffer saline

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W/D: Wet to dry weight ratio

WT: lung block weight

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Introduction

While invasive bacterial infection and sepsis are associated with reductions in vascular endothelial integrity and extravasation of proteinaceous fluid, these changes are pronounced with *Bacillus anthracis* infection. Pathology from patients dying with inhalational anthrax in Sverdlovsk, Russia in 1979 was notable for extravasation of fluid, protein and blood cells (1, 20). Recurrent pleural effusions have been prominent in patients with inhalational anthrax and respiratory failure in the US and Europe (7, 24). Cutaneous and soft tissue anthrax infection produces marked tissue edema (15, 55). Finally, gastrointestinal anthrax is associated with intestinal and retroperitoneal edema (49, 55). Since loss of vascular integrity during *B. anthracis* infection could contribute to organ dysfunction directly as well as the resistant shock patients demonstrate, understanding its basis may improve management.

B. anthracis produces two toxins, edema and lethal toxin (ET and LT), consisting of protective antigen (PA), the component necessary for host uptake of each of the toxin's toxic moieties, edema and lethal factor (EF and LF)(22, 55). Selective inhibition of either toxin is protective in bacteria challenged animal models and administration of each toxin alone in animals produces hypotension, organ injury and lethality (2, 11, 12, 23, 33, 37, 38, 53). Despite their pathogenic importance, whether ET or LT contribute to increased vascular permeability during infection is unclear. While ET produces localized tissue edema when injected subcutaneously in animals, EF has potent adenylate-cyclase activity that increases intracellular cAMP levels (32), an action potentially increasing endothelial barrier function (47). By contrast, *in vitro* studies now suggest that LT but not ET increases the permeability of vascular endothelial and lung epithelial cell monolayers (6, 14, 29, 31, 52, 57).

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In order to investigate the effects of ET and LT on vascular permeability at the organ level, we employed an isolated perfused rat lung model. Sprague-Dawley rats which are sensitive to LT's lethal effects were used as lung donors for most experiments. Studies examined the effects of each toxin alone or together and in low or high doses; LT under perfusion conditions of either constant flow or pressure; a PA directed monoclonal (PA-mAb) when combined with either toxin alone; two Rho-kinase inhibitors (GSK269962 and Y27632) and a VEGFR inhibitor (VEGFR-I, ZM323881) when combined with LT. The choice of agents for these later investigations was based in part on a recent review of in *in vitro* and zebra fish embryo studies that implicated Rho-kinase and vascular endothelial growth factor pathways in LTs permeability effects (6, 44, 63). In a final study, we also examined LT in lungs prepared from other rat strains sensitive or insensitive to LT's lethal effects.

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Materials and Methods

Animal Care:

The protocol for this study (CCM 16-02) was reviewed and approved by the Animal Care and Use Committee at the Clinical Center, National Institutes of Health.

Sprague-Dawley Rat Isolated Perfused Lung Model Studies

Table 1 summarizes challenge and treatment doses and numbers of lungs from Sprague-Dawley (Study 1 to 9), Wistar, Brown-Norway and Lewis (Study 10) rats (all males weighing 300-350g) tested in 10 studies. Figure 1 shows a general timeline for studies. Study 1 examined the effects of: protective antigen (PA) alone (control, 2μg/ml, n=10); LT alone [lethal factor (LF) 1μg/ml + PA 2μg/ml, n=17)]; ET alone [edema factor (EF) 1μg/ml + PA 2μg/ml, n=11]; or the same doses of LT and ET together (LT+ET, n=19). Additional LT (n=7) and LT+ET (n=8) lungs employed to measure effluent cAMP levels were included in Study 1 analysis. Toxin concentrations were based on the total recirculating volume in the perfusion system and were comparable to ones reported in live *B. anthracis* challenged animals and human patients (36, 40, 46, 61). After lungs were isolated and equilibrated (see *Lung Isolation and Perfusion* below), PA alone or toxin was added to the perfusion system and lungs were perfused at a constant flow rate for 4h while lung weight (LW), pulmonary artery pressure (Ppa) and left atrial pressure (Pla) were monitored. At 30 min intervals, perfusion and effluent samples were removed for analysis. After 4h, Pla was increased 7cmH_2O and change in lung weight was assessed over 15min and a lung permeability coefficient (Kf.c) calculated (see *Measurements and Calculations* below)(16, 59, 60). Before and following the increase in Pla, pulmonary capillary pressure (Ppc) was

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154 calculated. Lungs were then removed for wet-to-dry weight ratio determination (W/D, see
155 below). Measures in subsequent studies were similar to Study 1.

156 The next six studies investigated LT alone. Study 2 compared a dose of LT (5μg/ml)
157 (n=6) five times greater than in Study 1 to PA only (n=6). Study 3 examined whether LT would
158 increase Kf.c if Ppa was kept constant throughout. After PA (n=9) or LT (n=14) was added to the
159 perfusion circuit in doses similar to Study 1, flow rate was adjusted every 1-3min starting at
160 90min to maintain Ppa at baseline levels (see *Lung Isolation and Perfusion* below). Pressure in
161 PA lungs remained constant throughout and flow rate was not altered. For comparison,
162 additional lungs (n=8) were perfused with LT at a constant flow rate. Study 4 examined whether
163 prevention of LT uptake by host cells with a PA directed monoclonal antibody (PA-mAb, dose
164 of 10×the molar PA dose included in LT), would block LT's lung effects (see *Toxin and*
165 *Treatment Preparation* section below). Lungs were perfused under constant flow with PA only
166 (n=10), LT and nonspecific mAb (NS-mAb; n=10), or LT and PA-mAb (n=8). Study 5
167 compared the effect of GSK2969629 (a rho-kinase inhibitor, n=6) versus diluent (control, n=6)
168 and Study 6 compared the effect of Y27632 (another rho-kinase inhibitor, n=8) versus diluent
169 (n=8), both in LT challenged lungs. Each agent was also compared to diluent in lungs not
170 challenged with LT (3 diluent versus 3 GSK269962 lungs and 3 diluent versus 3 Y-27632 lungs).
171 Study 7 compared the effect of ZM323881 (VEGFR inhibitor, n=9) versus diluent control (n=7)
172 in LT challenged lungs.

173 Two studies evaluated ET further. Study 8 compared an ET dose (5μg/ml, n=12) five
174 times greater than in Study 1 to PA only (10μg/ml, n=12) under constant flow. Study 9 examined
175 the effects of a PA-mAb (dose of 10×the molar PA dose included in ET) with ET. Lungs were

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perfused with PA only (n=12), ET and NS-mAb (n=9) or ET and PA-mAb (n=9). The ET and PA doses were similar to Study 8.

Wistar, Brown-Norway and Lewis Rat Isolated Lung Studies

A tenth study (Study 10) examined whether LT had similar effects on lungs from Wistar and Brown-Norway rats sensitive to LTs lethal effects and from Lewis rats that are resistant (42). Lungs were prepared and challenged and measures made as in Study 1.

Lung Isolation and Perfusion

Animals were anesthetized with ketamine 60mg/kg and xylazine 5 mg/kg. The trachea was cannulated with a 14ga blunt needle and the animal was mechanically ventilated (Model 683 Animal Ventilator, Harvard Apparatus, Holliston, MA) with a 1.2ml tidal volume (TV), 2 cmH_2O positive end expiratory pressure (PEEP), 60 breaths per minute (BPM) respiratory rate and gas mixture of 95% O_2 and 5% CO_2. Two minutes after a midline laparotomy and administration of 0.2 ml of 10,000 u/ml heparin via the inferior vena cava, animals were exsanguinated after inferior vena cava and descending aorta transection. The lung and heart were exposed with a midline sternotomy, the pulmonary artery (PA) and left atrium (LA) were cannulated and the lung was perfused free of blood with 20 ml of 4°C perfusate (Pulmonary Arterial and Left Atrial Cannulas, Harvard Apparatus, Holliston, MA). The PA and LA cannulas were immediately connected to the perfusion system and the lung and heart block (termed lung-block) were dissected free and suspended from a force transducer (MLT0201, Colorado Springs CO) in a heated water-jacket (37°C) and humidified chamber while ventilation continued. Perfusion was immediately started with a closed perfusion circuit and variable speed peristaltic

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pump (MasterFlex L/S, Vernon Hills, IL). Perfusate was circulated from a closed reservoir to the PA and then returned from the LA to the reservoir. The perfusate consisted of a modified Krebs-Henseleit (KH) buffer containing 118.5mM NaCl, 25mM $NaHCO_3$, 4.7mM KCl, 1.5mM $MgSO_4$, 1.5 KH_2PO_4, 1.92mM $CaCl_2$, 5.74mM Glucose, with BSA (4%), dextran (1.67%), and (13 uM) 21 amino acids. Pulmonary artery pressure (Ppa) (i.e. the perfusion pressure) and LA pressure (Pla) were continuously measured via pressure transducers. Perfusion rate was set at 3 ml/min/100g body-weight, TV at 8ml/kg, and PEEP at 2 cmH_2O, and Pla at 3-4 cmH_2O. Lungs were then equilibrated for 15min. The perfusate pH was maintained at 7.35 to 7.45 with adjustment of gas flow rate. Any lung with an increase in either Ppa $\geq$1 cmH_2O or lung block weight $\geq$0.05 g during this equilibration period was excluded from study.

Measurements and Calculations

Lung-block weight, Ppa, and Pla measures were recorded every 15min. In studies with constant flow the perfusion flow rate was not changed during the experiment. In the constant pressure study (Study 3), the flow rate was reduced 1 rpm (0.167 ml/min) every 1 to 3min when Ppa increased higher than the pressure at 90 min, the earliest time Ppa was observed to increase with LT under constant flow. Perfusate and effluent samples (0.5 ml) were drawn at 30min intervals to assess pH, gas tensions, electrolytes and lactate. In some lungs challenged with LT, ET or LT+ET (n= 8, 11, 8 lungs respectively) and lungs challenged with PA or ET with either PA-mAb or NS-mAb (n=12, 9 and 9 lungs respectively), effluent was collected at 60, 120, 180 and 240 min for cAMP measurements.

After 240 min of perfusion, pulmonary capillary permeability was measured as the capillary filtration coefficient (Kf.c, ml/min/cmH_2O/100g). After 10 seconds of baseline lung-

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block weight measures, Pla was increased 7 cmH_2O by raising the LA reservoir and maintained at that level for 15 min. During this period, lung weight (WT) was recorded every minute. As previously described, lung weight gain during the initial 5 min was attributed to filling of the pulmonary vasculature, while weight gain during the final 10 min was attributed to extravasation of perfusate (16, 59, 60). Pulmonary capillary pressure (Ppc) was measured before and after Pla was increased by occluding both perfusion inlet and outlet tubing for 4 seconds and recording the pressure after Ppa and Pla had become equal. Kf.c was calculated using the formula: $Kf.c=[(dWt/dt)/\Delta Ppc / WT_e \times 100)]$, where; dWt/dt is the slope of the lung weight (WT) change per min from 5 to 15min; ΔPpc is the change in Ppc related to the increase in Pla; and WT_e is the estimated baseline lung weight (0.00472 x animal's body weight)(60). Following Kf.c measures, lungs were removed and wet-to-dry weight ratios subsequently calculated.

A FITC-albumin assay was used to further assess pulmonary vascular permeability in six lungs challenged with LT and six challenged with PA using toxin doses and perfusion methods similar to Study 1. At 120 min after starting perfusion, FITC-albumin (Sigma-Aldrich, St. Louis, MO) was added to the perfusate at a final concentration of 0.16 mg/ml. At 240 min, rather than calculating Kf.c, lung uptake of FITC-albumin was measured. After a sample of perfusate was collected, lungs were perfused with 20 ml 1× phosphate buffered saline (PBS, pH = 7.4) to remove labelled albumin from the pulmonary circulation. The lung block was removed from the perfusion circuit and the right lower lung lobe was collected for wet-dry-weight ratio determination and the right middle lobe was weighed and homogenized in 0.5 ml PBS. The left lobe was lavaged through the tracheal cannula 3 times with 1.5 ml PBS and the return volume was measured. The lung homogenate and lavage were then centrifuged at 8000rpm for 10min and the supernatants collected. The FITC-albumin concentrations in perfusate, and the

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supernatants of the lung homogenate and lavage were then measured by fluorescence (excitation 485 nm and emission 520 nm wavelengths) with a plate reader (Biotek, Winooski, VT). The lung tissue and BAL FITC-albumin concentrations were then normalized based on the concentration of perfusate FITC-albumin concentration and expressed as either (mg/g lung tissue)/(mg/ml perfusate) or (mg/ml lung lavage)/(mg/ml perfusate) respectively.

Perfusate and effluent PO_2, PCO_2, sodium, potassium, chloride, glucose and lactate levels were measured using the Stat Profile Critical Care Express System (NOVA Biomedical, Waltham, MA). cAMP was measured using a chemiluminescent direct immunoassay (Arbor Assays, Ann Arbor, MI).

Toxin and Treatments

Toxin components (PA, LF and EF) were recombinant proteins prepared from *Escherichia coli* as previously described(11). LT and ET consisted of LF and EF respectively with PA in ratios of 1:2 on the basis of weight. The lipopolysaccharide (LPS) content of the PA, LF and EF was 0.001, 0.002 and 0.006ng/μg respectively with the Limulus Amoebocyte Lysate Chromogenic endotoxin assay (Clongen Inc, Gaithersburg, MD). The PA-mAb was Raxibacumab (Human Genome Sciences) and the control was an inactive, non-specific monoclonal antibody(38). Rho-kinase inhibitors GSK269962 and Y27632 and VEGFR-inhibitor ZM323881 (Selleck Chemicals, Houston, TX) were endotoxin free and diluted in dimethyl sulfoxide (DMSO) (final concentrations of DMSO in the perfusion circuit were 0.03, 0.0125 and 0.02% respectively). Compared to perfusion over 4h with buffer alone, perfusion with buffer with protective antigen or with DMSO alone in the high and low concentrations employed did not have significantly different effects on any of the lung parameters measured ($p \geq 0.10$ for all).

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Statistics

Data were analyzed using PROC Mixed in SAS Version 9.4 software (SAS Institute, Inc., Cary, NC). Two-way repeated measure ANOVAs accounting either for challenge (toxin vs PA), or treatment type (treatment vs diluent) and time after challenge or treatment were conducted to assess the effects of challenge or treatment over time respectively on pulmonary arterial pressure, perfusion rate and effluent cAMP level. Two-way ANOVAs were used to determine the dose effect (low vs high) of LT or ET (toxin vs PA). One-way ANOVAs were then used to assess the effects of challenge or treatment on other parameters. Serial changes from baseline and the final change from baseline for Ppa and perfusion rate were used for analysis and presentation. The Kf.c was analyzed after log-transformation due to its non-normal distribution. Throughout, data are presented as mean ± SEM. A p-value ≤ 0.05 was considered significant. Lungs were randomized to a particular challenge and/or treatment prior to preparation and lung numbers vary in comparison groups in part related to exclusion of lungs during the equilibration based on criteria outlined above.

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Results

Sprague-Dawley Rat Isolated Lung Model Studies

Compared to control lungs (group study sizes shown in figures) perfused with protective antigen (PA) only, LT (lethal factor and PA) progressively increased serial changes from baseline in pulmonary artery pressure (Ppa, cmH_2O) starting midway through the 240min observation period ($p<0.0001$ for the time interaction) (Figure 2). By 240min, LT increased the final mean change (±SEM) in Ppa (5.16±0.78 vs 1.15±1.02 cmH_2O) and the mean (±SEM) pulmonary capillary pressure prior to permeability measure (Ppc, 7.31±0.21 vs 5.99±0.11 cmH_2O), permeability coefficient [Kf.c, 4.48±0.78 vs 2.48±0.07 log(ml/min/cmH_2O/100g)] and wet-to-dry weight ratio (W/D) (8.24±0.53 vs 5.53±0.08) ($p\leq0.004$). Edema toxin produced small reductions in Ppa over time approaching significance ($p=0.09$ for the time interaction) but did not significantly alter the final mean change in Ppa (-0.23±0.49 cmH_2O, $p=0.22$) or the Ppc (6.13±0.18 cmH_2O), Kf.c (2.36±0.08 [log(ml/min/cmH_2O/100g)]) or W/D (5.58±0.10) (all $p\geq0.29$). When combined, LT and ET (LT+ET) did not significantly alter Ppa over time ($p=0.44$ for the time interaction) or the final Ppa change (-0.08±0.29cmH_2O) or the Ppc (6.11±0.10 cmH_2O) ($p\geq0.15$) but did increase Kf.c [4.31±0.32 log(ml/min/cmH_2O/100g)] and W/D (7.78±0.35) ($p\leq0.0004$). Thus, ET counteracted LT's pulmonary vascular constrictor effects but not its permeability effects. Additional studies were then conducted of LT (Studies 2 to 7) and ET (Studies 8 and 9) with Sprague-Dawley lungs.

In Study 2, compared to PA, a dose of LT (5μg/ml) five times greater than in Study 1, also produced progressive increases in Ppa over time ($p<0.0001$ for the time interaction) and at 240min increased the final change in Ppa (13.10±2.38 vs 1.09±1.29 cmH_2O) and the Ppc (9.79±0.67 vs 6.52±0.20 cmH_2O), Kf.c [4.33±0.15 vs 2.55±0.12 log(ml/min/cmH_2O/100g)]) and

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311 W/D (7.68±0.32 vs 5.61±0.10) (all p≤0.001) (Figure 3). Compared to the low dose LT, the high
312 dose significantly increased Ppa and Pcp (p≤0.003), but not Kf.c and W/D (p≥0.56).
313 To further exclude the possibility that increases in Ppa with LT contributed to this toxin's
314 permeability effects, Study 3 investigated LT while Ppa was maintained at constant baseline
315 levels (see methods). Compared to PA controls also perfused under constant pressure (but which
316 didn't require changes in flow to maintain constant pressure), lungs perfused under constant
317 pressure and challenged with LT required progressive reductions in flow (p<0.0001) to maintain
318 pressures not different from baseline or controls (Figure 4). At 4h, compared to the overall
319 change from baseline in Ppa in control lungs (-1.14±0.38 cmH_2O), this change was not
320 significantly different in LT lungs perfused under constant pressure (-0.66±0.15 cmH_2O, p=0.18)
321 but was increased in lungs perfused under constant flow (3.94±1.49 cmH_2O, p=0.003) as seen
322 previously. By contrast, compared to Kf.c and W/D in control lungs [2.86±0.12
323 log(ml/min/cmH_2O/100g) and 5.95±0.12)], with LT both parameters were increased in lungs
324 perfused either under constant pressure [3.95±0.09 log(ml/min/cmH_2O/100g) and 7.37±0.24,
325 p≤0.004] or constant flow [4.67±0.45 log(ml/min/cmH_2O/100g) and 7.78±0.45, p≤0.001]. Thus,
326 increases in Kfc and W/D with LT did not appear related to increasing Ppa with LT.
327 In Study 4 (Figure 5), compared to PA, perfusion with LT and treatment with a
328 nonspecific monoclonal antibody (NS-mAb), produced progressive increases in Ppa (p<0.0001
329 for the time interaction) and increases in the final change in Ppa (4.73±1.43 vs 1.15±1.02 cmH_2O,
330 p=0.02), Ppc (7.88±0.35 vs 6.40±0.48 cmH_2O, p=0.0002), Kf.c [4.04±0.18 vs 2.48±0.07
331 log(ml/min/cmH_2O/100g), p<0.0001)] and W/D (7.05±0.42 vs 5.53±0.08, p=0.001). In contrast,
332 perfusion with LT when combined with PA-mAb to inhibit host cell uptake of toxin, produced
333 small but progressive decreases in Ppa over time (p=0.02 for the time interaction) and did not

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significantly alter the final change in Ppa (-0.75±0.29 cmH_2O), Ppc (6.81±0.45 cmH_2O), Kfc [2.81±0.16 log(ml/min/cmH_2O/100g)] or W/D (5.61±0.23)($p \geq 0.09$). Compared to LT with NS-mAb, LT with PA-mAb progressively lowered Ppa ($p < 0.0001$ for the time interaction) and decreased the final change in Ppa and in Ppc, Kf.c and W/D ($p \leq 0.03$).

Studies 5 and 6 examined whether either of two rho-kinase inhibitors, GSK269962 or Y27632 would alter LT's effects. In LT challenged lungs, compared to diluent, both agents reduced increases in Ppa ($p \leq 0.03$ for the time interaction for both) and the final change in Ppa at 240min significantly or in a trend approaching significance ($p = 0.004$ and 0.07) (Figure 6) but neither altered Ppc significantly ($p \geq 0.21$). Surprisingly however, both agents actually increased Kf.c and W/D with LT in patterns that were or approached significance ($p \leq 0.07$). These agents were also compared to diluent in normal lungs (six Y-27632 versus six diluent lungs and two GSK269962 versus two diluent lungs). The effects of the two agents did not differ significantly for any parameter ($p \geq 0.15$) and these were combined for analysis. Compared to diluent, the rho-kinase inhibitors did not alter Ppa or Ppc significantly ($p \geq 0.44$) but did increase Kf.c [4.30±0.66 vs 2.95±0.0.17 log(ml/min/cmH_2O/100g)] and W/D (8.58±1.16 vs 6.52±0.42) in trends approaching significance ($p = 0.08$ and 0.09 respectively) (data not shown). In Study 7 in LT challenged lungs, compared to diluent, the VEGFR-inhibitor ZM323881 did not produce significant changes in any measured parameter ($p \geq 0.77$, data not shown).

In Study 8 (Figure 7), compared to PA, perfusion with an ET dose (5μg/ml) five times greater than in Study 1 produced progressive decreases in Ppa over time ($p = 0.0004$ for time interaction) and decreased the final change in Ppa (-1.12±0.36 vs -0.39±0.24 cmH_2O, $p = 0.07$) and in Ppc (5.32±0.11 vs -5.86±0.12 cmH_2O, $p = 0.003$). The higher ET dose also produced a decrease in Kf.c that approached significance [2.40±0.27 vs 2.79±0.13 log(ml/min/cmH_2O/100g),

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p=0.14] but did not alter W/D (5.86±0.22 vs 5.90±0.14) (p≥0.86). The effects of the high and low dose of ET did not differ significantly for any of parameters (p≥0.44).

In Study 9 (Figure 8), compared to PA, perfusion with ET combined with NS-mAb treatment, progressively decreased Ppa (p<0.0001 for the time interaction) and decreased the final change in Ppa in a significant trend (-0.97±015 vs -0.39±0.24 cmH_2O, p=0.10) and Kf.c [2.40±0.06 vs -2.79±0.13 log(ml/min/cmH_2O/100g), p=0.03] but did not alter Ppc (5.90±0.19 vs 5.86±0.13 cmH_2O, p=0.84) or W/D (5.95±0.14 vs 5.90±0.0.14, p=0.82). Compared to PA control, perfusion with ET combined with PA-mAb did not alter Ppa over time (p=0.94 for the time interaction) or the final change in Ppa (-0.45±0.23 cmH_2O), Ppc (5.86±0.09 cmH_2O), W/D (5.73±0.18) or Kf.c [2.27±0.08 log(ml/min/cmH_2O/100g) (p≥0.09)]. Compared to ET with NS-mAb, ET with PA-mAb produced progressively higher Ppa (p<0.04 for the time interaction) but did not significantly alter the final change in Ppa (p=0.09) or in Ppc, Kf.c or W/D (p≥0.35).

FITC-albumin Determinations in Lung Tissue and Lavage Following LT or PA Challenge

Compared to PA challenged lungs (n=6), LT challenge (n=6) significantly increased the lung tissue FITC-albumin concentration normalized to the perfusate concentration [0.62±0.13 vs 0.25±0.03 (mg/g)/(mg/ml), p=0.03] (Figure 9). The lung lavage FITC-albumin was not detectible in either PA or LT challenged lungs. Similar to other studies, LT in these experiments also increased W/D (p≤0.01) (data not shown).

cAMP Level Determinations in Perfusate Following ET, LT or PA Challenge in Lungs From Studies 1, 8 and 9

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In effluent samples collected at 60, 120, 180 and 240 min from studies 1 and 8, compared to lungs perfused with PA alone, LT alone did not increase cAMP levels ($p=0.85$) while ET alone and ET combined with LT increased cAMP levels although these increases varied over time ($p<0.0001$ for the time interaction) (Figure 10). In study 9, compared to PA alone, ET with NS-mAb also produced significant increases in cAMP that varied over time ($p=0.0002$ for the time interaction) but ET with PA-mAb did not. cAMP levels were also significantly increased with ET with NS-mAb compared to ET with PA-mAb ($p=0.005$ for the time interaction).

Wistar, Brown-Norway and Lewis Rat Isolated Lung Model Studies

In study 10, in Wistar and Brown-Norway rats sensitive LT's lethal effects, compared to control lungs, LT produced progressive increases in serial mean (±SEM) changes from baseline in Ppa ($p<0.0001$) and increased the final change in Ppa at 240min and in mean Ppc, Kf.c and W/D ($p\leq0.06$) (Figure 11). In Lewis rats resistant to LT's lethal effects, the toxin did not produce significant changes in any of the parameters measured ($p\geq0.35$).

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Discussion

B. anthracis LT and ET had very different effects on pulmonary vascular pressures and permeability in isolated perfused Sprague-Dawley rat lungs. Lethal toxin produced pulmonary vascular constriction evidenced by increases in both pulmonary artery and capillary pressures (Ppa and Pcp) and it increased the measured lung permeability coefficient (Kf.c) and wet-to-dry weight ratios (W/D). Increases in Kf.c and W/D with LT were not related to the toxin's effects on increasing vascular pressure. Increased lung permeability with LT was also noted when assessed with a FITC-albumin assay. Although the effects of ET were less pronounced, in contrast to LT, ET reduced Ppa and Pcp and produced small but nonsignificant reductions in permeability. Edema toxin strongly blocked LT's pulmonary artery vasoconstrictor effects but did not alter its permeability ones. Inhibition of host cell uptake of LF or EF with PA-mAb negated each toxin's effects.

Findings here add to others suggesting that LT can increase vascular permeability. In prior studies, lethal toxin increased permeability across monolayers constructed from human pulmonary, brain and umbilical vein microvascular endothelial cells and rat pulmonary microvascular endothelial cells using either electrical resistance or labeled protein or dextran measurements (52, 63). These changes with LT in rat pulmonary microvascular cells occurred in as little as 1h, a time consistent with the permeability increases noted at 4h with LT in the present study (35). Injection of LT *in vivo* increased extravasation of fluorescent microspheres in zebra fish embryos and fluorescein labeled dextran in mouse lungs (5, 6). Different from LT's effects on permeability, this is the first report we are aware of showing that this toxin can cause direct pulmonary artery constriction, effects that were dose related and dependent on PA mediated host cell uptake of LF.

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On the one hand, the effects of LT on increasing both pulmonary vascular permeability and pressures are consistent with an effect of LF on activating intracellular actin-myosin elements in either endothelial or smooth muscle cells (52). Lethal toxin has been shown to stimulate endothelial actin-myosin elements and in so doing, to disrupt adherens-junctional complex proteins and inhibition of ERK1/2, a known action of LF, has been associated with this disruption (26, 52). No study has examined LTs effects on pulmonary artery smooth muscle cells. But ERK-1/2 activation has been associated with vascular smooth muscle relaxation related to some stimuli (51, 56) and LT's ERK-1/2 inhibitory effects would counter this relaxation. Also consistent with the present findings, in another study we conducted in aortic rings, LT increased the maximal contractile force rings developed with phenylephrine (34).

On the other hand, some mechanisms leading to increased permeability and vascular pressures in this lung model appear to differ. Two different rho-kinase inhibitors negated the effects of LT on Ppa but did not alter Ppa in lungs challenged with diluent alone. Involvement of rho-kinase in LT's pulmonary vasoconstrictor effects are very consistent with the role rho-kinase plays in regulating pulmonary vascular tone and producing pulmonary artery smooth muscle contraction related to stimuli such as hypoxia and endothelin, actions which Y27362 has been shown to inhibit (3, 27, 41). However, neither rho-kinase inhibitor decreased the toxin's permeability effect and both increased permeability in LT challenged as well as in normal lungs. These findings differ from *in vitro* ones with LT. In one of the first studies to suggest that LT increased endothelial permeability, two different rho-kinase inhibitors (H1152 and Y27632) both decreased LT stimulated myosin-light chain phosphorylation and permeability in human pulmonary microvascular cell monolayers (62). In another study, Y27632 disrupted LT stimulated actin cable formation in human umbilical vein endothelial cells (45). These prior

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findings combined with the present ones suggest that rho-kinase has a different role in the changes in permeability measured in endothelial cell mono-layer systems and this *ex vivo* lung system. The findings also suggest that LF triggers non-rho-kinase associated mechanisms in this *ex vivo* whole organ system that contribute to the toxin's permeability effects (4). Although activation of VEGFR has also been associated with LTs permeability effects in the zebrafish, a VEGFR inhibitor administered at reportedly inhibitory concentrations did not alter LT's effects here(5, 6). Mechanisms underlying LTs permeability are likely not inflammatory in nature as well. Administration of LT *in vivo* had minimal effects on inflammatory mediator release in this Sprague-Dawley rat model (13) and actually inhibited this release in endotoxin challenged animals (10). Other mechanisms and pathways have been associated with LTs effects on permeability in *in vitro* and in other models including activation of apoptosis (25), inhibition of p38-MK-2 stimulated heat shock protein-27 (35), inhibition of DE-cadherin transport to adherens junctions (21), inhibition of angiopoietin-1 signaling through Tie-2 (18), and histamine activation (19, 52). Resource limitations prevented our investigating these additional pathways in the present studies.

In the present study, LT also produced increases in pulmonary artery pressure and permeability in Wistar and Brown Norway rats, two other strains sensitive to the toxin's lethal effects, but not in Lewis rats that are insensitive to these effects (42). These findings raise the possibility that LT's pulmonary effects here relate to some rat strain specific genes such as isoforms of the Nlrp1 gene. Polymorphisms in this gene in the rat have been linked to an association between specific rat strains and both macrophage sensitivity to lysis by LT and to the toxin's lethal effects. However, since non-rat species (e.g. mice, rabbits and canines) are also sensitive to the lethal effects of LT and LT increase lung permeability in a mouse study and

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466 across human endothelial cell monolayers, the present findings in the isolated rat lung appear
467 relevant to the pathogenic effects of LT in other non-rat species (30, 54).

468 Endotoxin [lipopolysaccharide (LPS)], a bacterial toxin commonly associated with the
469 development of sepsis associated lung injury and shock, has also been investigated in isolated
470 perfused rat lung models. In studies we reviewed, the effects of LPS on lung function in these
471 models appear to have varied based on the LPS dose and methodology employed. Two to three
472 hours of perfusion with LPS concentrations ≤ 50 ug/ml in perfusate with buffer alone produced
473 no effect on either pulmonary artery pressure or lung permeability (17, 58). Concentrations of
474 LPS of 200 to 300 ug/mL either did not alter pulmonary artery pressure but increased
475 permeability after 6h of perfusion with a perfusate including buffer and blood (9, 50) or
476 produced an immediate transient increase in the former without a change in the latter with whole
477 blood perfusion (8). Finally, a concentration of LPS of 400 ug/mL increased permeability but
478 not pulmonary artery pressure after 20min in lungs perfused with buffer alone (48). The
479 concentrations of LPS used in these models are comparable to ones producing lethality *in vivo* in
480 animals (5 to 20 mg/kg)(10), but are notably much higher than the concentration of LT
481 producing pulmonary effects or lethality in the lung model or *in vivo* (50ug/kg)(11-13). Also,
482 while LPS's pulmonary and lethal effects in both *ex vivo* lung models and *in vivo* have frequently
483 been associated with inflammatory mediator release (e.g. TNF, IL-1, lipid metabolites), lethal LT
484 doses in the rat may actually suppress this release (10, 11, 13).

485 Reductions in pulmonary artery and capillary pressures with ET both by itself and in
486 combination with LT in the present study are most likely related to this toxin's effect on
487 increasing intracellular cAMP levels. We have shown in an isolated aortic ring model and in *in*
488 *vivo* studies that ET causes potent systemic arterial vasodilation in association with increases in

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both tissue and circulating cAMP levels (11, 34, 53, 54). Adefovir, a nucleoside that selectively inhibits cAMP production by edema factor, blocks these vasodilatory effects of ET both in the aortic ring model and *in vivo* (34, 53). Since increases in cAMP stimulate pulmonary arterial relaxation, ET likely has similar effects on pulmonary and systemic vascular tone (43). While the vasodilatory effects of ET were less pronounced in the high compliance pulmonary vasculature, once tension was increased with LT in isolated lungs, the effects of ET were much more apparent. Uptake of EF was necessary for these vasodilatory effects of ET as PA-mAb reversed reductions in Ppa. Consistent with their effects on Ppa, LT increased and at the higher dose ET decreased Ppc. These opposing effects likely reflect the differing mechanisms potentially mediating these toxins' effects on Ppa. Edema toxin's effects on permeability in this lung model are less clear. Different from what its name would imply, ET did not increase Kf.c or W/D in any study. In fact, in each of three studies, compared to a PA control, ET was associated with decreases in Kf.c that were not significant in Study 1 and Study 8 with low and high dose ET but were significant in Study 9 with a high dose. However, PA-mAb did not alter ET's small decreases in Kf.c and ET had no measurable effect on increases in Kf.c with LT. Overall though, the present findings with ET are more consistent with the strengthening effects increased cellular cAMP can have on endothelial barrier function (47).

The present findings with LT and ET in this isolated lung model provide insights into how these toxins potentially alter lung function during *B. anthracis* infection. Increased permeability with LT exposure at either the alveolar endothelial or epithelial levels or both could produce direct alveolar protein and fluid accumulation, ventilation and perfusion mismatching, hypoxia and reduced lung compliance. Histopathology studies have demonstrated alveolar protein and fluid accumulation in some but not all models (13, 28, 39, 64). Permeability

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512 increases in the pleural visceral or parietal tissues, could also contribute to the recurrent pleural
513 effusions that characterize inhalational anthrax disease. Lethal toxin challenge has been shown to
514 produce pleural effusions in animals models (13, 39). When large clinically, these effusions
515 produce atelectasis and indirectly impair oxygenation and compliance. Finally, permeability
516 changes with LT within the mediastinal tissues could contribute to the marked mediastinal
517 edema observed in patients with inhalational disease and which in turn could alter mediastinal
518 lymphatic flow and aggravate pleural fluid accumulation. However, while histopathology study
519 in humans dying with inhalational anthrax demonstrate protein-rich fluid accumulation in all of
520 these lung tissues, to what extent these changes relate to LT versus other bacterial components or
521 some combination is unknown (20). Notably, the necrotizing-hemorrhagic mediastinitis and
522 disrupted lymphatic drainage seen in patients is thought to make an important contribution to the
523 recurrent pleural effusions (1, 24). Different from its permeability effects, LT's pulmonary
524 vasoconstrictor effects in this isolated lung model, while highly consistent and significant across
525 experiments, were relatively small and not likely sufficient alone to produce consequential
526 increases in pulmonary vascular resistance and secondary abnormalities in cardiac performance.
527 These constrictor effects could add to the cardiovascular effects of other bacterial components
528 such as cell wall constituents. Finally, ET's vasodilatory effects in this model, while potentially
529 countering LT's vasoconstrictor effects, could contribute to dysregulated pulmonary blood flow.
530 Importantly, ET associated vasodilation in the pulmonary vasculature could impair the protective
531 effects of hypoxic vasoconstriction in patients with anthrax associated pulmonary infection,
532 edema or pleural effusions.

533 This study has limitations. Changes were observed in the model over a relatively brief 4h
534 period and how long they would persist either after a single exposure to toxin or with repeated

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challenges is unknown. Also, it is unknown whether the changes noted in this *ex vivo* lung and toxin system will also occur in lungs exposed to toxin *in vivo*. Finally, *B. anthracis* produces other components that contribute to its pathogenesis and whether these components would synergize with or antagonize the pulmonary vascular effects of LT and ET is unknown.

The present findings support the possibility that LT contributes to the increased vascular permeability and extravasation of fluid and protein that characterizes *B. anthracis* infection. While other bacterial components, such as *B. anthracis* cell wall with its robust inflammatory properties, likely also participate in this process, our findings do not support a role for ET in this increased vascular permeability. However, ET may have pulmonary vasodilator effects which could counter protective adaptive mechanisms like hypoxic pulmonary vasoconstriction. These findings with LT and ET together add to others providing a basis for administration of agents that target both toxins during the development of shock and organ injury with *B. anthracis* infection.

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Disclosures

None of the authors have conflicts of interest to disclose.

Author Contributions

XC – study design, experimental conduct, data acquisition, data analysis, data interpretation, manuscript preparation, manuscript review; WX - experimental conduct, data acquisition, manuscript review; PN - experimental conduct, data acquisition, manuscript review; AWS - experimental conduct, data acquisition, manuscript review; RW - experimental conduct, data acquisition, manuscript review; BP- experimental conduct, data acquisition, manuscript review; YL - experimental conduct, data acquisition, manuscript review; MM – study design, data analysis and interpretation, manuscript review; SHL - study design, data analysis and interpretation, manuscript review; YF - experimental conduct, data acquisition, manuscript review; PQE - study design, experimental conduct, data acquisition, data analysis, data interpretation, manuscript preparation, manuscript review

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Figure Legends

Figure 1. This figure shows the time line for the procedures and measures performed and the challenges and interventions employed in the 11 studies described. See Table 1 for further details regarding each study including the number of lungs examined in each study group. PA – protective antigen; LT – lethal toxin; ET – edema toxin; NA – not applicable; TV – tidal volume; Ppa – pulmonary artery pressure; Ppc – pulmonary capillary pressure; Kf.c – pulmonary permeability coefficient; W/D – lung wet to dry weight ratio, FITC-albumin ,albumin–fluorescein isothiocyanate conjugate.

Figure 2. Panel A compares serial mean (±sem) changes in pulmonary artery pressure (Ppa) from baseline to 240 min for lungs challenged with lethal toxin (LT), edema toxin (ET) or LT+ET, versus those challenged with protective antigen alone (control, PA). The p-values in panel A are for the overall effect of either ET or LT+ET challenges compared to PA (designated chall) and for the change in the effect of each of these three challenges compared to PA over time (i.e. the time interaction and designated chall*time). The p-value for the overall effect of LT is not applicable (NA) since the time interaction was significant. This figure then compares for the same groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel B), initial pulmonary capillary pressure (Ppc) measures at 240 minutes (Panel C), and pulmonary permeability coefficients (Kf.c, Panel D) and subsequently measured wet-to-dry weight lung ratios (W/D, Panel E).

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799 Figure 3. Panel A compares serial mean (±sem) changes in pulmonary artery pressure (Ppa)
800 from baseline to 240 min for lungs challenged with a low dose of lethal toxin (LT 1ug/ml) versus
801 a low dose of PA (2ug/ml) and for those challenged with a high dose of LT (LT 5ug/ml) versus a
802 high dose of PA (control, PA 10 ug/ml). The p-values in panel A are for the change in the effects
803 over time of either LT 1ug/ml compared to PA 2ug/ml or for LT 5ug/ml compared PA 10 ug/ml
804 (i.e. the time interactions and designated chall*time). This figure then compares for the same
805 groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel B), initial
806 pulmonary capillary pressure (Ppc) measures at 240 minutes (Panel C), and pulmonary
807 permeability coefficients (Kf.c, Panel D) and subsequently measured wet-to-dry weight lung
808 ratios (W/D, Panel E). Panels B to E, also compare the effects of the low and high LT doses (p-
809 values designated with #). The data shown in this figure for the low LT and PA doses is the same
810 as the data shown in Figure 2. The concentrations of LT shown here represent the amounts of
811 lethal factor employed in the toxins, which is 50% of the concentration of PA included in the
812 toxin.
813
814 Figure 4. Panel A compares serial mean (±sem) changes in pulmonary artery pressure (Ppa) from
815 baseline to 240 min for lungs challenged with lethal toxin (LT) and perfused either under
816 constant pressure (LT+CP) or constant flow (LT+CF) versus lungs challenged with protective
817 antigen alone under constant flow (control, PA). Although PA lungs were perfused with
818 constant flow, pressure remained constant in these lungs. Therefore for this figure, PA lungs are
819 denoted as constant flow and pressure (PA+CF/CP). Panel B shows the same comparisons for
820 changes in perfusion rates from baseline to 240 min. The p-values in panel A are for the overall
821 effect of LT+CP compared to PA (designated chall) and for the change in the effect of LT+CP or

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LT+CF compared to PA over time (i.e. the time interaction and designated chall*time). The p-value for the overall effect of LT+CF is not applicable (NA) since the time interaction was significant. The p-value in Panel B is for the change in the effect of LT+CP versus PA over time and there is no comparison of PA and LT+CF because flow was constant for each. This figure then compares for the same groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel C), pulmonary permeability coefficients (Kf.c) measuremed at 240 minutes (Panel D), and subsequently measured wet-to-dry weight lung ratios (W/D) (Panel E).

Figure 5. Panel A compares serial mean (±sem) changes in pulmonary artery pressure (Ppa) from baseline to 240 min for lungs challenged with lethal toxin (LT) and treated with either nonspecific monoclonal antibody (LT+NS-mAb) or protective antigen directed mAb (PA-mAb) versus those challenged with protective antigen without treatment (control, PA). The p-values in panel A are for the change in the effects of LT+NS-mAb or LT+PA-mAb compared to PA over time (i.e. the challenge time interaction and designated chall*time) and for the change in the effect of LT+PA-mAb compared to LT+NS-mAb over time (i.e. the treatment time interaction and designated Rx*time). This figure then compares for the same groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel B), initial pulmonary capillary pressure (Ppc) measures at 240 minutes (Panel C), pulmonary permeability coefficients (Kf.c, Panel D) and subsequently measured wet-to-dry weight lung ratios (W/D, Panel E).

Figure 6. This figure compares serial mean (±sem) changes in pulmonary artery pressure (Ppa) from baseline to 240 min for lungs challenged with lethal toxin (LT) and treated with either the Rho-kinase inhibitors GSK269962 (Panel A) or Y27632 (Panel B) versus lungs challenged with

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LT and treated with diluent alone (controls). The p-values in panel A and B are for the changes in the effects of each treatment versus the diluent control over time. This figure then compares for the same groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel C), initial pulmonary capillary pressure (Ppc) measures at 240 minutes (Panel D), and pulmonary permeability coefficients (Kf.c, Panel E) and subsequently measured wet-to-dry weight lung ratios (W/D, Panel F).

Figure 7. Panel A compares serial mean (±sem) changes in pulmonary artery pressure (Ppa) from baseline to 240 min for lungs challenged with a low dose of lethal toxin (ET 1ug/ml) versus a low dose of PA (2ug/ml) or a high dose of ET (ET 5ug/ml) versus a high dose of PA (PA 10 ug/ml). The p-values in panel A are for the overall effect of either ET 1ug/ml versus PA 2ug/ml (designated chall) and for the change in the effects over time of either ET 1ug/ml compared to PA 2ug/ml over time or for ET 5ug/ml compared PA 10 ug/ml (i.e. the time interactions and designated chall*time). This figure then compares for the same groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel B), initial pulmonary capillary pressure (Ppc) measures at 240 minutes (Panel C), and pulmonary permeability coefficients (Kf.c, Panel D) and subsequently measured wet-to-dry weight lung ratios (W/D, Panel E). Panels B to E, also compare the effects of the low and high ET doses (p-values designated with #). The data shown in this figure for the low ET and PA doses is the same as the data shown in Figure 2. The concentrations of ET shown here represents the amounts of edema factor employed in the toxin, which is 50% of the concentration of PA included in the toxin.

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Figure 8. Panel A compares serial mean (±sem) changes in pulmonary artery pressure (Ppa) from baseline to 240 min for lungs challenged with lethal toxin (ET) and treated with either nonspecific monoclonal antibody (ET+NS-mAb) or protective antigen directed mAb (ET+PA-mAb) versus those challenged with protective antigen without treatment (control, PA). The p-values in panel A are for the overall effect of ET+NS-mAb versus PA and for the change in the effect of ET+NS-mAb or ET+PA-mAb compared to PA over time (i.e. the challenge time interaction and designated chall*time) and for the change in the effect of ET+PA-mAb compared to ET+NS-mAb over time (i.e. the treatment time interaction and designated Rx*time). This figure then compares for the same groups the mean (±sem) overall changes in Ppa from baseline to 240min (Panel B), initial pulmonary capillary pressure (Ppc) measures at 240 minutes (Panel C), pulmonary permeability coefficients (Kf.c, Panel D) and subsequently measured wet-to-dry weight lung ratios (W/D, Panel E).

Figure 9. This figure compares serial mean (±sem) lung tissue albumin–fluorescein isothiocyanate conjugate (FITC-albumin) concentration normalized by perfusate FITC-albumin concentration at 240 min in lungs challenged with lethal toxin (LT) (1 ug/ml) or protective antigen (PA)(2 ug/ml)(n=6 for all).

Figure 10. This figure compares serial mean (±sem) cAMP levels at 60, 120, 180 and 240 minutes from lungs challenged with low dose lethal toxin (LT 1ug/ml), high dose edema toxin (ET 5ug/ml) and LT+ET (both 1ug/ml)) versus lungs challenged with protective antigen (PA 5ug/ml, control) in Panel A and lungs challenged with ET 5ug/ml and treated with either

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nonspecific monoclonal antibody (NS-mAb) or PA directed mAb (PA-mAb) versus PA 10ug/ml (Panel B). The p-values in each panel are for the overall effect of toxin with or without treatment versus PA (designated chall) and for the change in the effects of toxin with or without treatment compared to PA over time (i.e. the time interaction and designated chall*time)

Figure 11. Panels A, B and C compare serial mean (±sem) changes in pulmonary artery pressure (Ppa) from baseline to 240 min for lungs isolated from Wistar, Brown Norway or Lewis rats respectively and challenged with lethal toxin (LT) versus those challenged with protective antigen alone (control, PA). The p-values in Panel A and B are for the change in the effects of LT compared to PA over time (i.e. the time interaction and designated chall*time) while the p-values in Panel C, are for the overall effect of LT and for the time interaction. Panels D, E and F compare the effect of LT versus PA on the mean (±sem) overall changes in Ppa from baseline to 240min, initial pulmonary capillary pressure (Ppc) measures at 240 minutes, and pulmonary permeability coefficients and subsequently measured wet-to-dry weight lung ratios in lungs from Wistar, Brown Norway and Lewis rats respectively.

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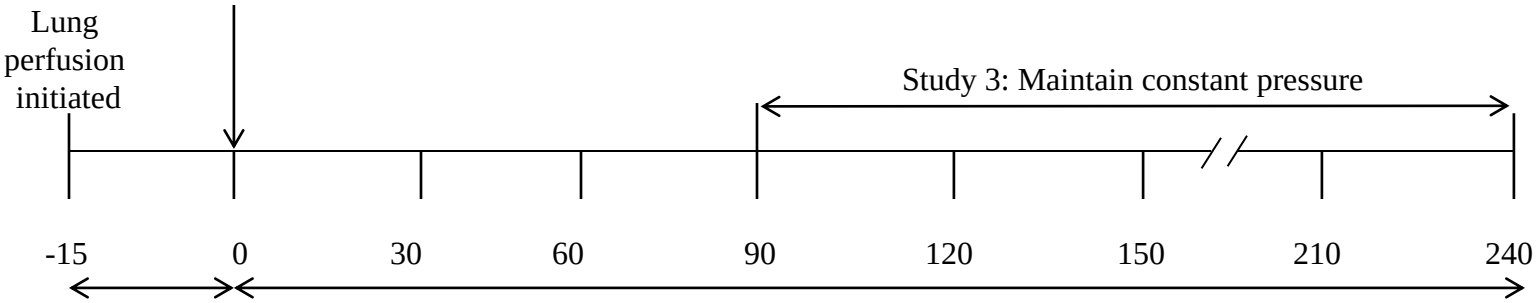
Figure 1.

Study	Challenge	Treatment/Rat Strain
1	PA, LT, ET or LT+ET	NA
2	LT or PA, High Dose	NA
3	LT or PA	Constant perfusion pressure or flow
4	LT or PA	PA-mAb or NS-mAb
5	LT	GSK269962 (Rho-kinase inhibitor)
6	LT	Y27632 (Rho-kinase inhibitor)
7	LT	ZM323881 (VEGFR inhibitor)
8	ET or PA, High Dose	NA
9	ET or PA, High Dose	PA-mAb or NS-mAb
10	LT or PA	Wistar, Brown Norway or Lewis rats



Equilibration with TV 8 ml/kg and flow 3 ml/100g: Exclude lungs with Ppa increase ≥ 1 cmH_2O and weight gain ≥ 1 g

Maintain constant flow (except Study 3), record Ppa, flow and lung weight every 15 min, collect perfusion samples every 30 min, and measure Ppc and Kf.c at 240 min followed by W/D.

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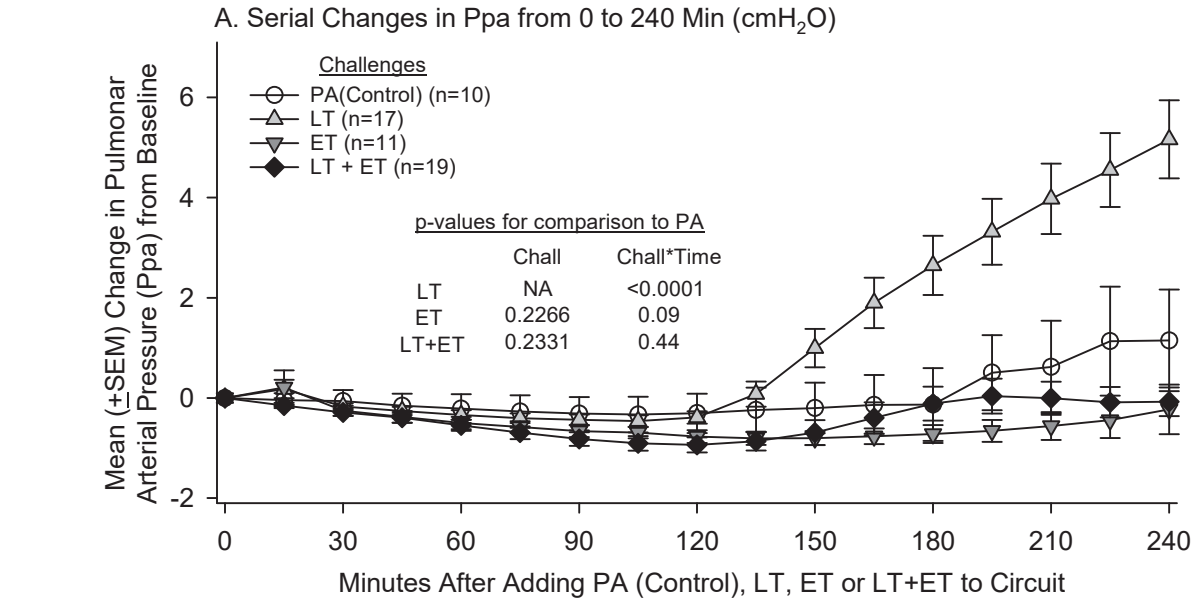
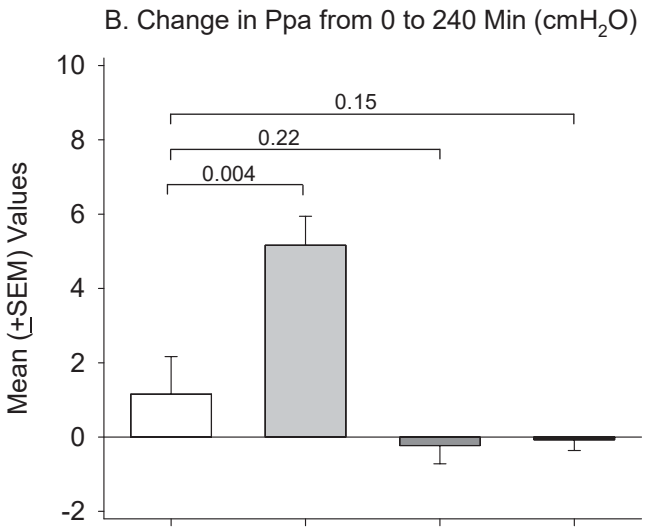
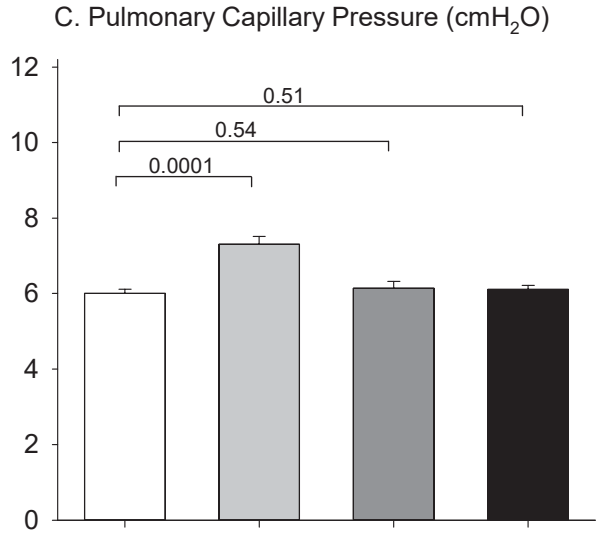
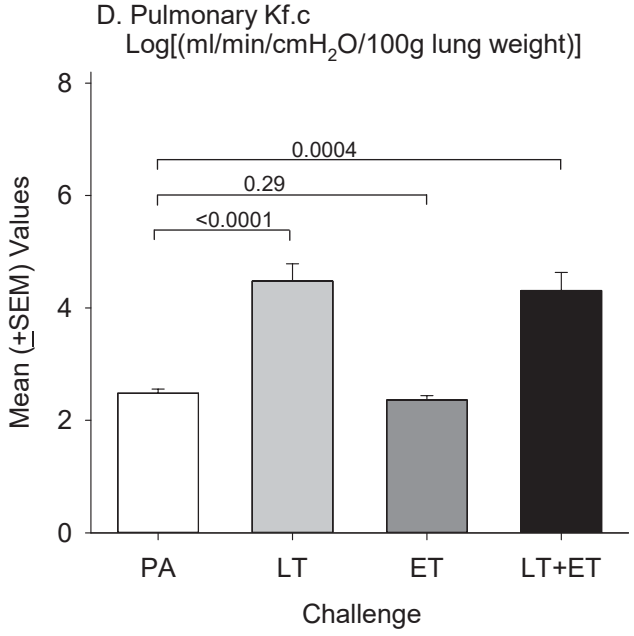
Fig 2









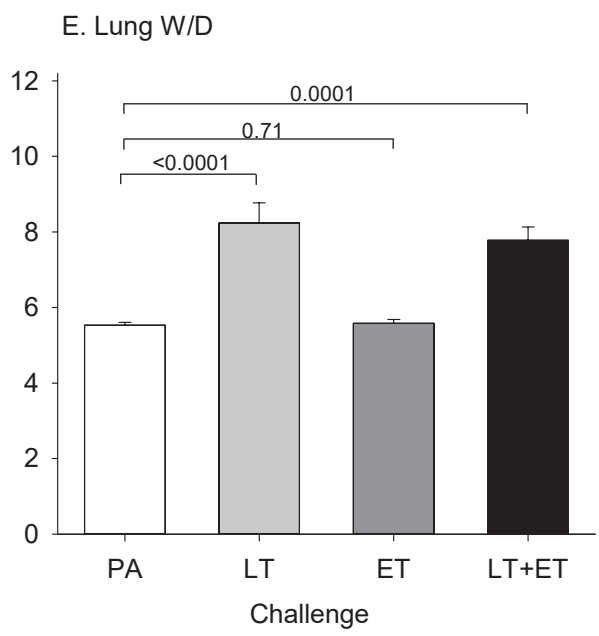


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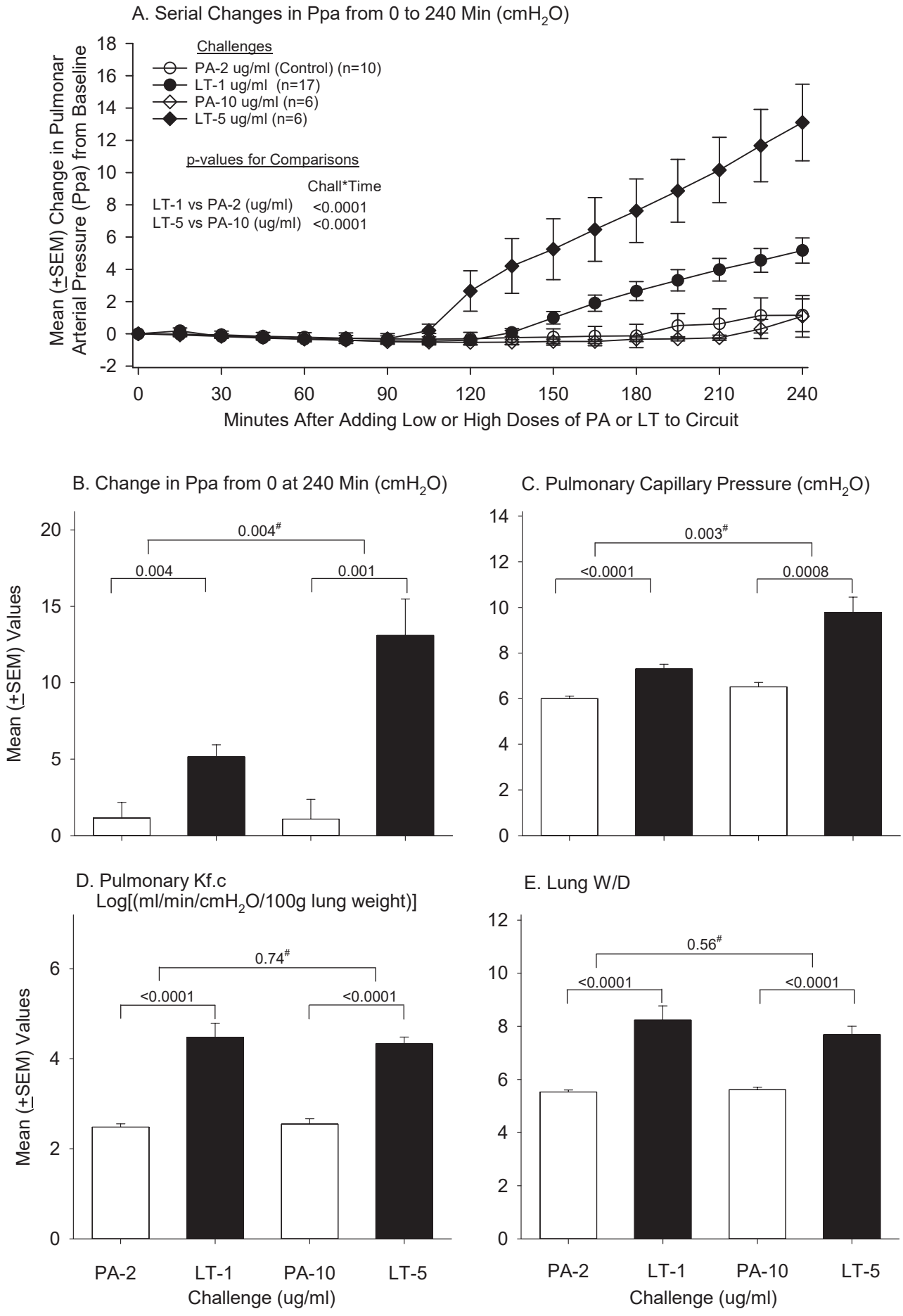
Fig 3



p values comparing the effect of LT 5 ug/ml vs LT1 ug/ml

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Fig 4

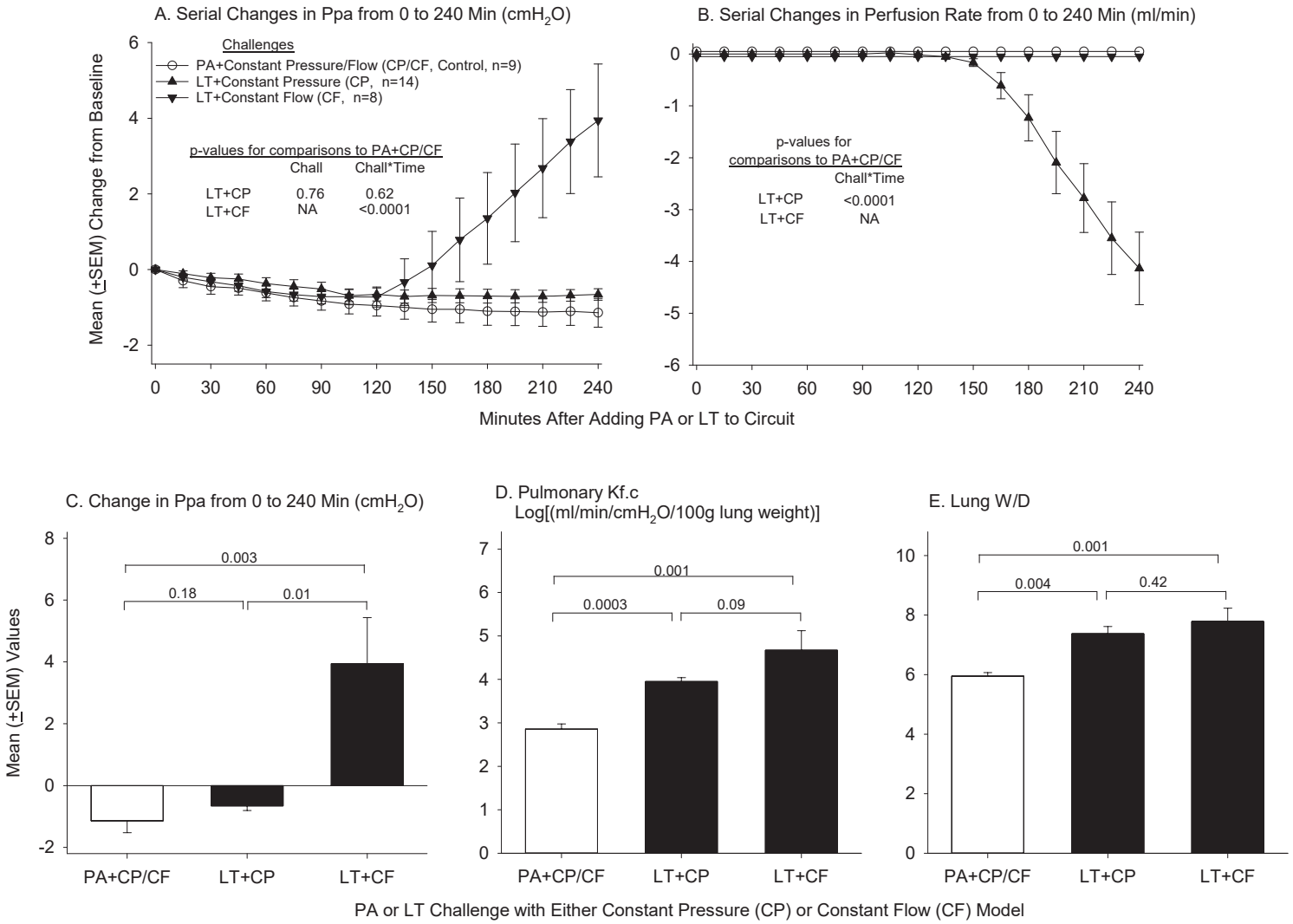

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Fig 5.

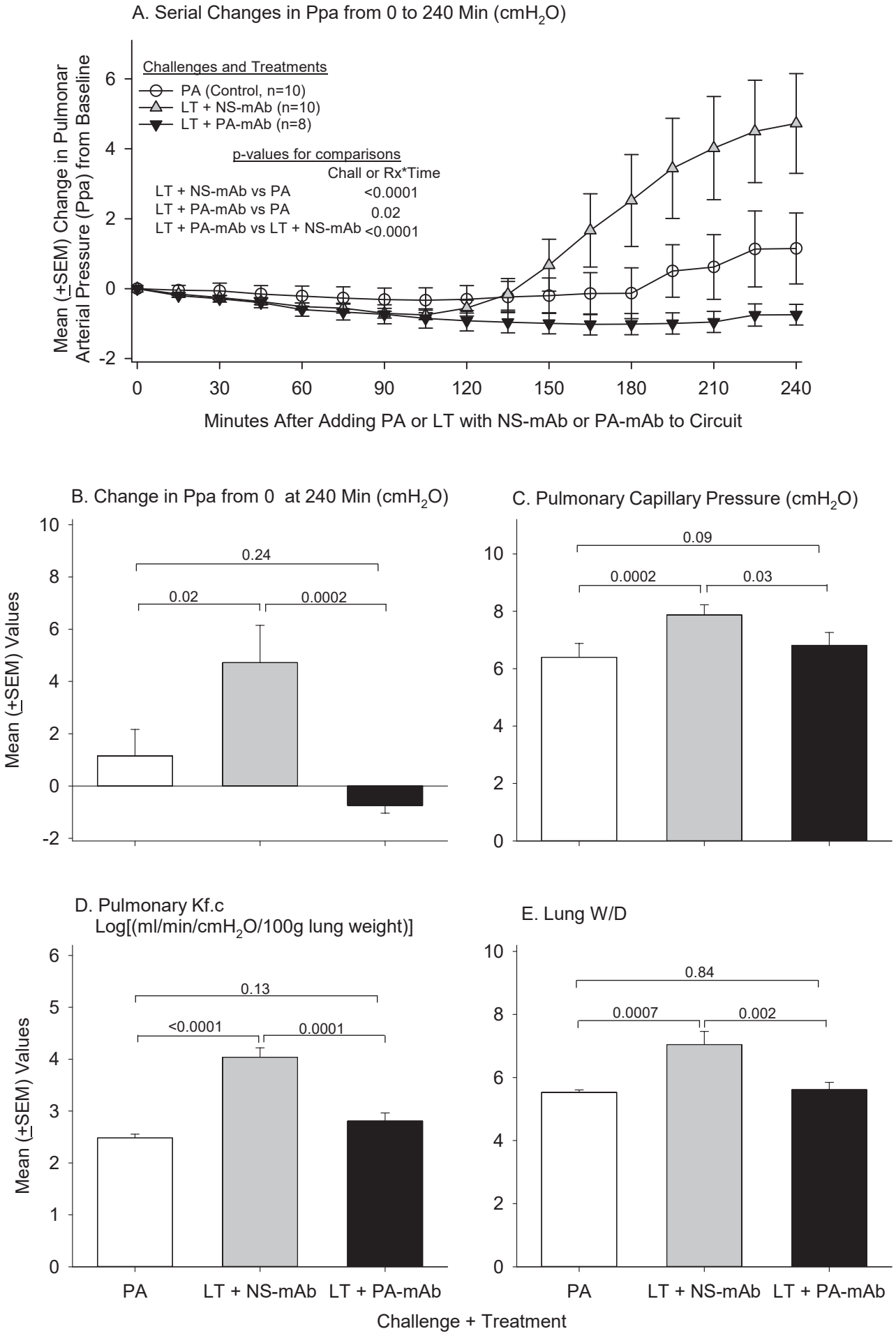

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Fig 6

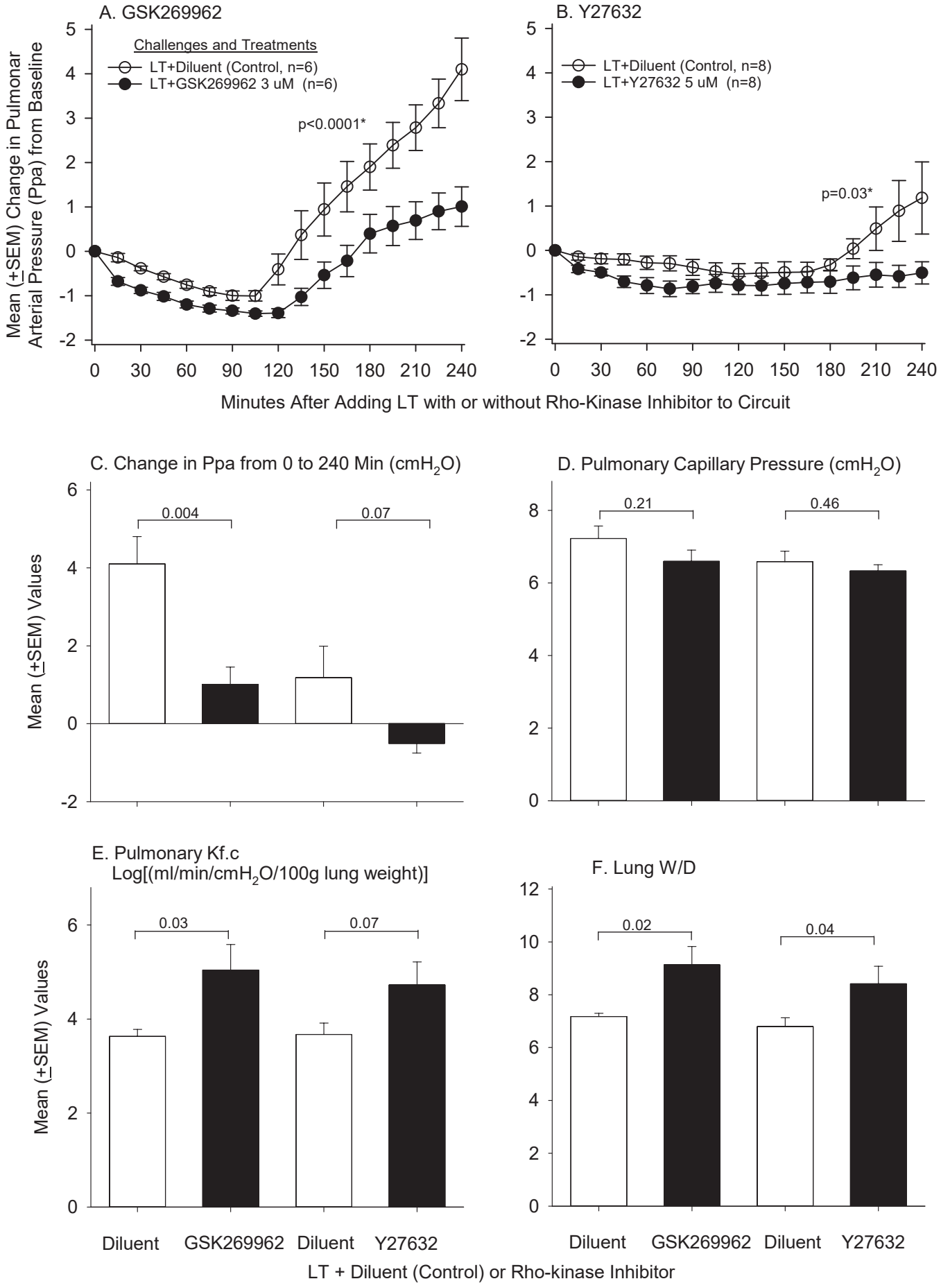

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Fig 7

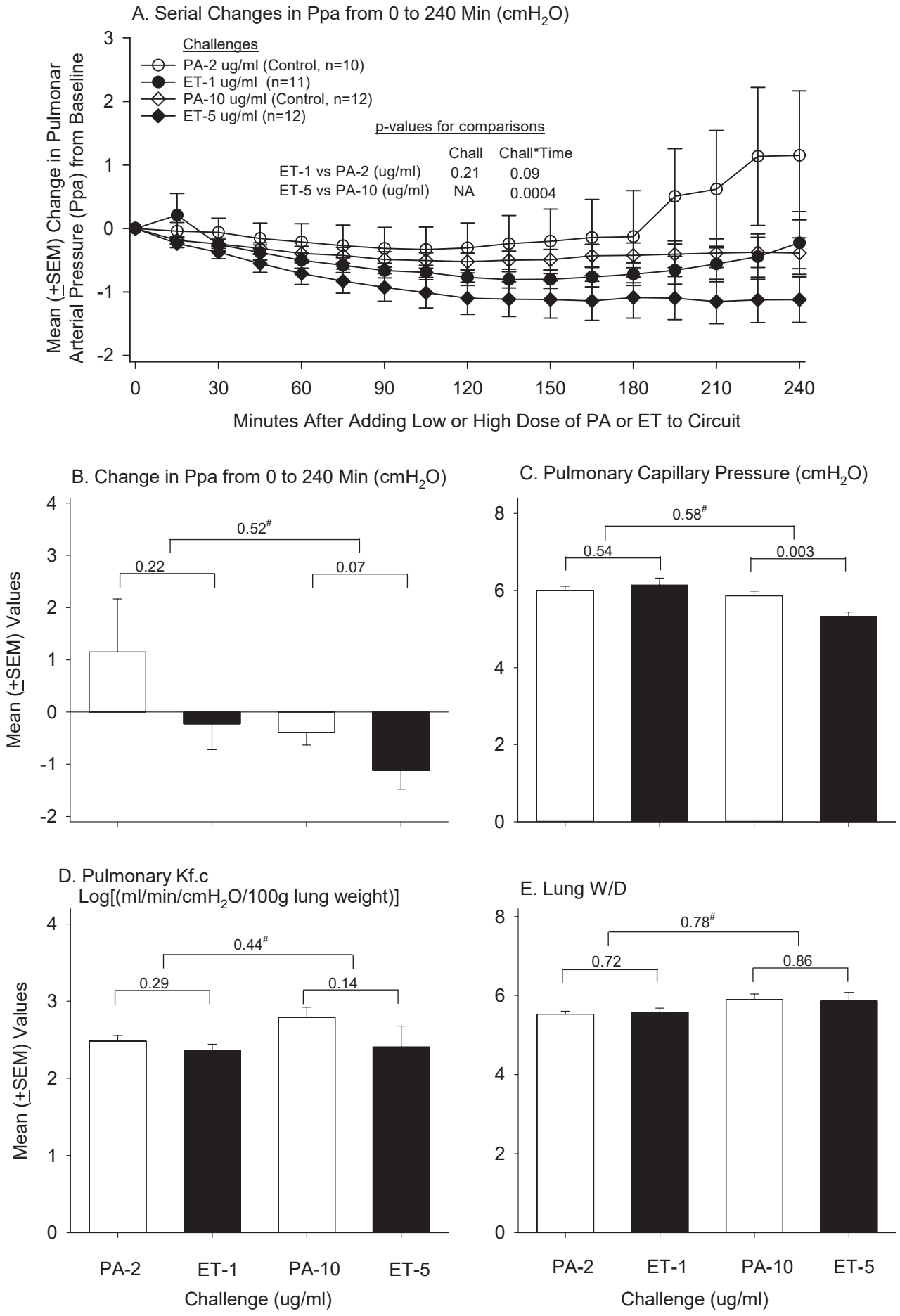

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Fig 8

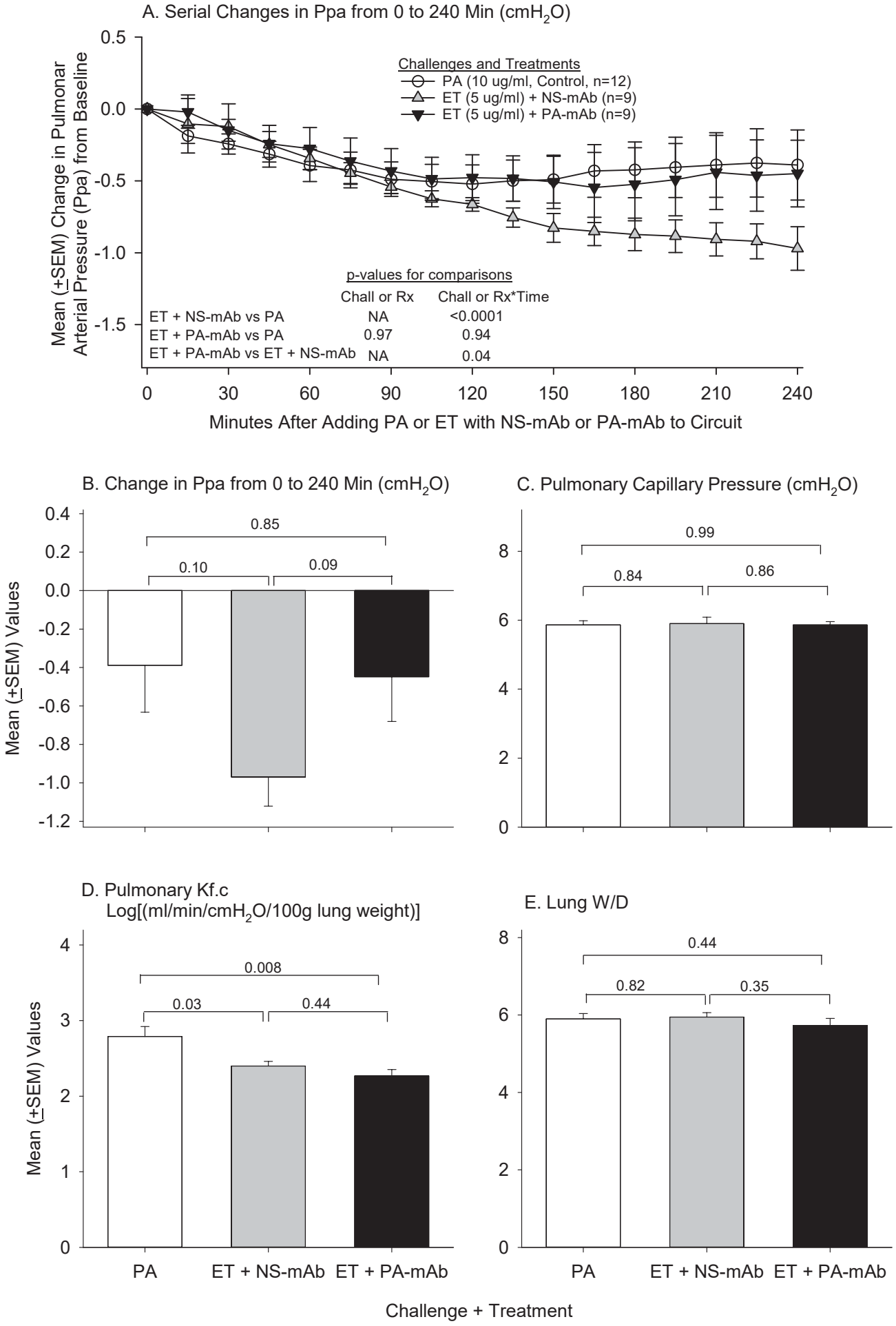

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Fig 9

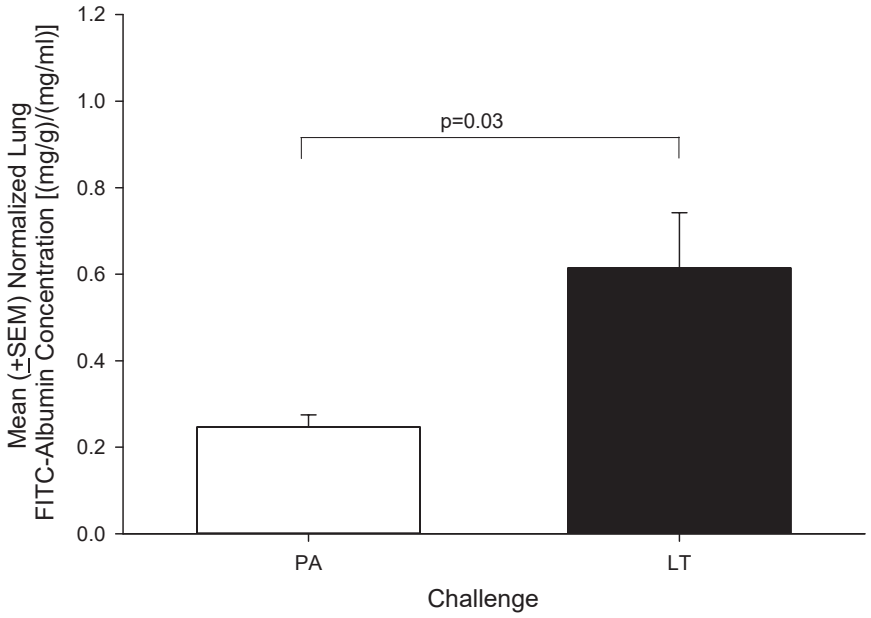

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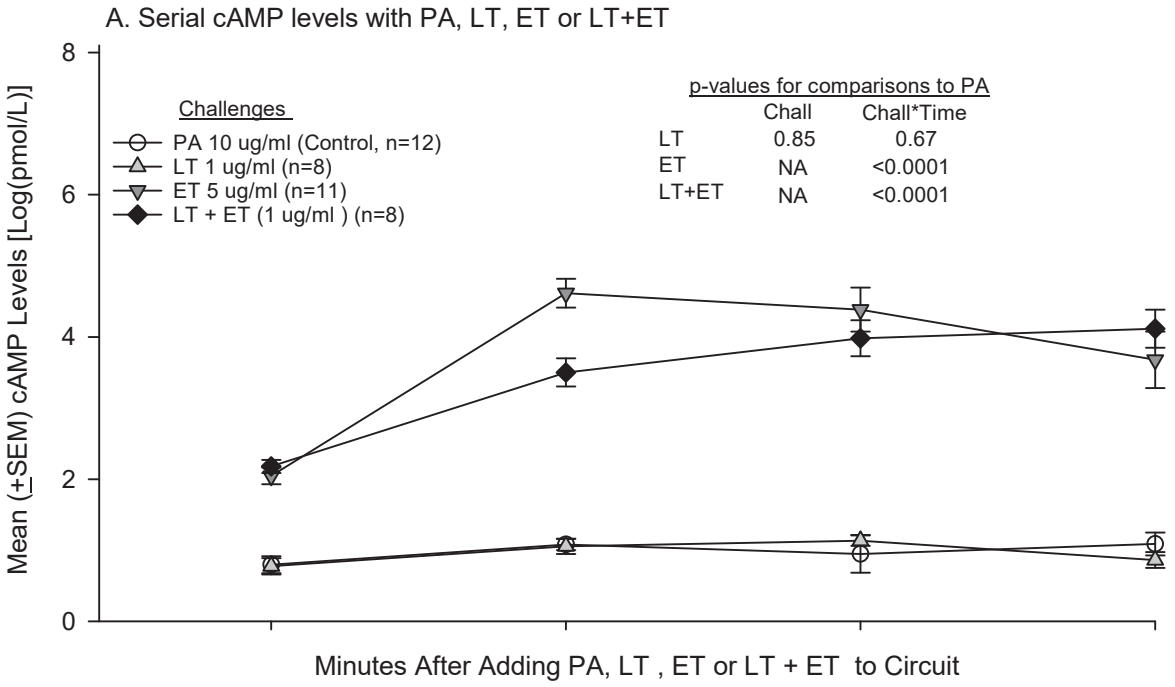
Fig 10



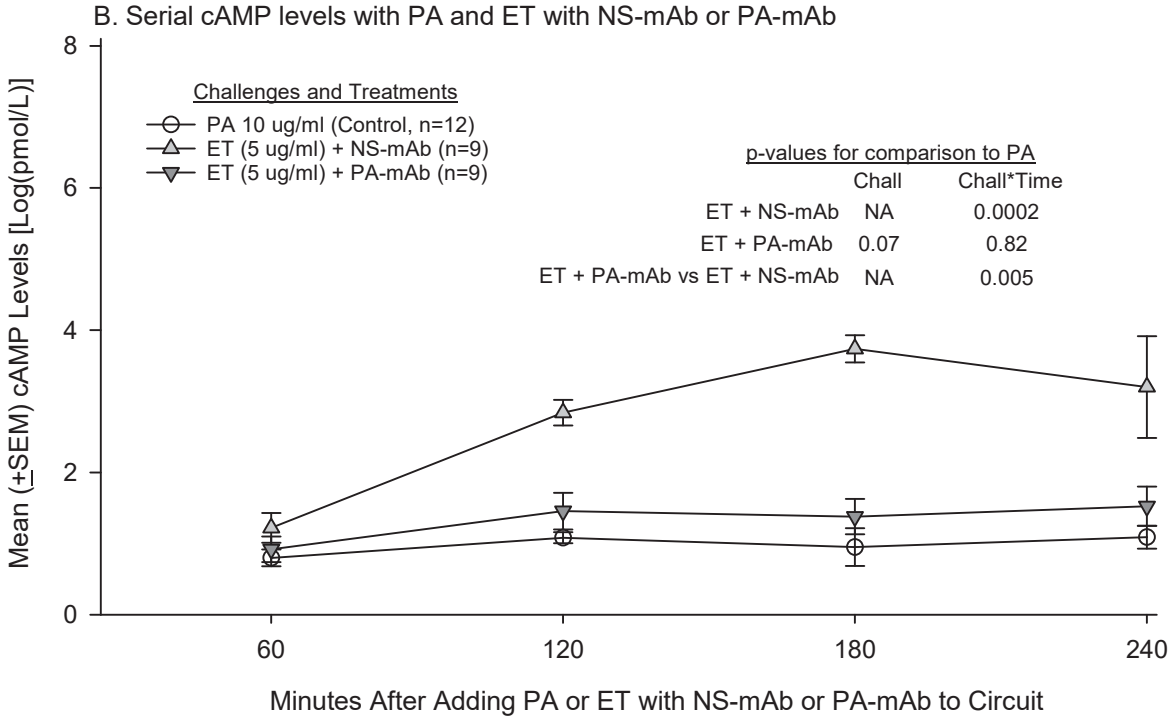


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Fig 11

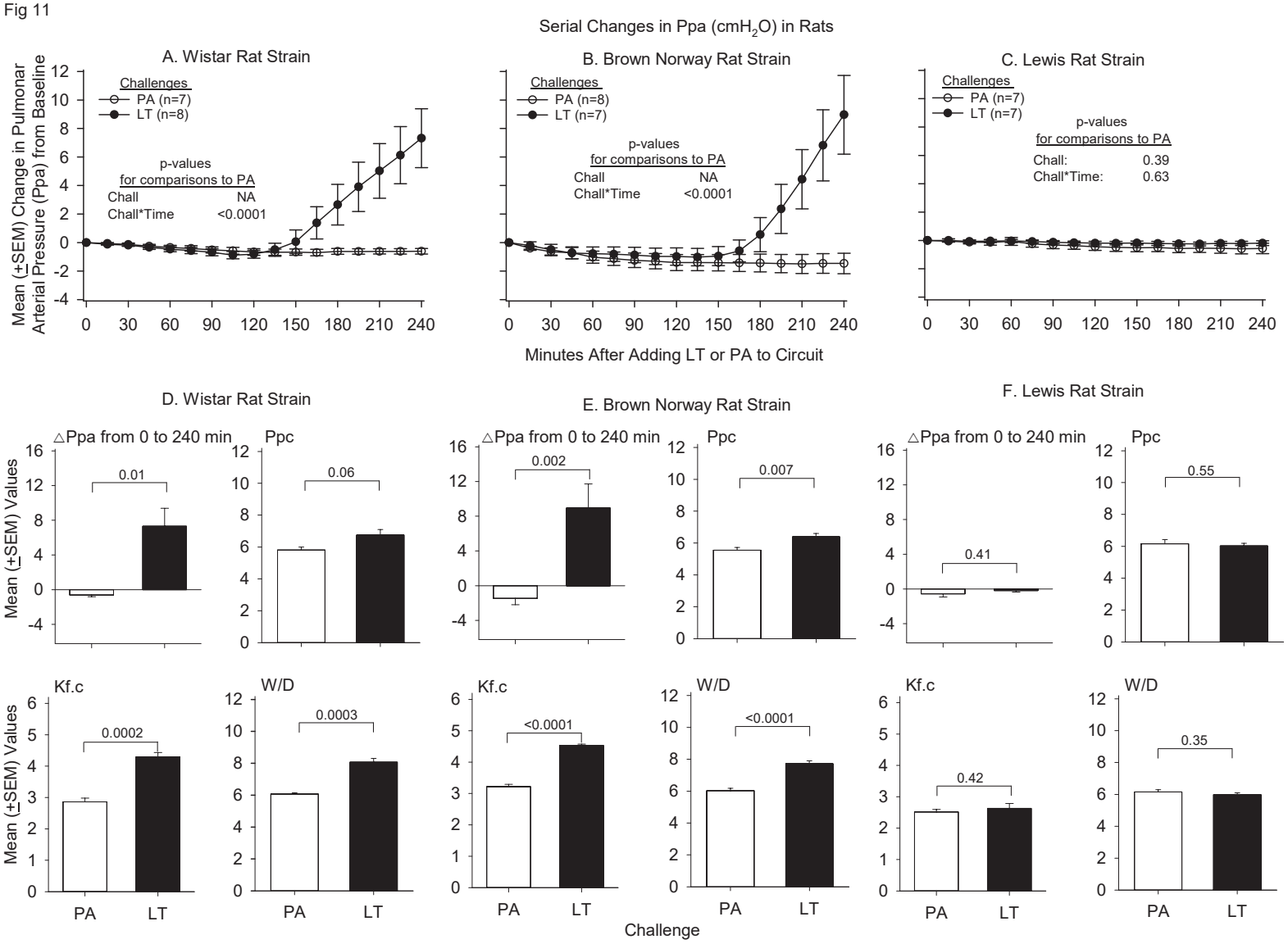

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Table 1. Summary of the Studies

Study	Perfusion	Challenge	Treatment/Strain	Number of Lungs
1: Effect of LT and ET alone or together	Constant Flow	PA 2 ug/ml	-	10
		LT 1 ug/ml	-	17
		ET 1 ug ml	-	11
		LT+ET 1 ug/ml	-	19
2: High Dose LT	Constant Flow	PA 10 ug/ml	-	6
		LT 5 ug/ml	-	6
3: Perfusion Pressure in LT Challenge	Constant Pressure	PA 2 ug/ml	-	9
	Constant Pressure	LT 1 ug/ml	-	14
	Constant Flow	LT 1 ug/ml	-	8
4: PA-mAb in LT	Constant Flow	PA 2 ug/ml	-	10
		LT 1 ug/ml	NS-mAb	10
		LT 1 ug/ml	PA-mAb 10×	8
5.GSK269962 in LT	Constant Flow	-	DMSO	3
		-	GSK269962 3 uM	3
		LT 1 ug/ml	DMSO	6
		LT 1 ug/ml	GSK269962 3 uM	6
6: Y-27632 in LT	Constant Flow	-	DMSO	3
		-	Y-27632 5 uM	3
		LT 1 ug/ml	DMSO	8
		LT 1 ug/ml	Y-27632 5 uM	8
7. ZM 323881 in LT	Constant Flow	LT 1 ug/ml	DMSO	7
		LT 1 ug/ml	Y-27632 5 uM	9
8. High Dose ET	Constant Flow	PA 10 ug/ml	-	12
		ET 5 ug/ml	-	12
9. PA-mAb in ET	Constant Flow	PA 10 ug/ml	-	12
		ET 5 ug/ml	NS-mAb	8
		ET 5 ug/ml	PA-mAb 10×	8
10. Effect of Rat Strain in LT	Constant Flow	PA 2 ug/ml	Wistar	7
		LT 1 ug/ml	Wistar	8
		PA 2 ug/ml	Brown Norway	8
		LT 1 ug/ml	Brown Norway	7
		PA 2 ug/ml	Lewis	7
		LT 1 ug/ml	Lewis	7

PA, Protective antigen; LT, lethal toxin; ET, edema toxin; PA-mAb, monoclonal antibody directed to PA;

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