

The lectin-like domain of tumor necrosis factor- α improves alveolar fluid balance in injured isolated rabbit lungs*

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Objective: Identification of mechanisms that preserve optimal alveolar fluid balance during pulmonary edema is of great clinical importance. This study was performed to determine whether the lectin-like domain of tumor necrosis factor- α (designated *TIP*) can improve fluid balance in experimental lung injury by affecting alveolocapillary permeability and/or fluid clearance.

Design: Prospective, randomized laboratory investigation.

Setting: University-affiliated laboratory.

Subjects: Adult male rabbits.

Interventions: *TIP*, a scrambled peptide (*scrTIP*), dibutyl cyclic adenosine monophosphate (*db-cAMP*), or saline was applied to isolated, ventilated, and buffer-perfused rabbit lungs by ultrasonic nebulization, after which hydrostatic edema or endo/exotoxin-induced lung injury was induced and edema formation was assessed. In studies evaluating the resolution of alveolar edema, 2.5 mL of excess fluid was deposited into the alveolar space of isolated lungs by nebulization in the absence or presence of *TIP*, *scrTIP*, *amiloride*, or *ouabain* or combinations thereof.

Measurements and Main Results: Microvascular permeability was largely increased during hydrostatic edema and endo/exotoxin-induced lung injury in saline-treated lungs, or lungs that received

scrTIP, as assessed by capillary filtration coefficient (K_{fc}) and fluorescein isothiocyanate-labeled albumin flux across the alveolocapillary barrier. In contrast, *TIP*- or *db-cAMP*-treated lungs exhibited significantly lower vascular permeability upon hydrostatic challenge. Similarly, extravascular fluid accumulation, as assessed by fluid retention, wet weight to dry weight ratio, and epithelial lining fluid volume measurements, was largely inhibited by *TIP* or *db-cAMP* pretreatment. Furthermore, *TIP* increased sodium-potassium adenosine triphosphatase (*Na,K-ATPase*) activity 1.6-fold by promoting *Na,K-ATPase* exocytosis to the alveolar epithelial cell surface and increased *amiloride*-sensitive sodium uptake, resulting in a 2.2-fold increase in active Na^+ transport, and hence improved clearance of excess fluid from the alveolar space.

Conclusions: Aerosolized *TIP* improved alveolar fluid balance by both reducing vascular permeability and enhancing the absorption of excess alveolar fluid in experimental lung injury. These data may suggest a role for *TIP* as a potential therapeutic agent in pulmonary edema. (*Crit Care Med* 2008; 36:1543–1550)

KEY WORDS: pulmonary edema; tumor necrosis factor- α ; alveolocapillary barrier; permeability; sodium transport; sodium-potassium adenosine triphosphatase

Alveolocapillary barrier function is essential to maintain alveolar fluid balance and adequate gas exchange. Perturbations to barrier integrity result in pulmonary edema, causing a life-threatening impairment of gas exchange. Formation of pulmonary edema is the hallmark of acute lung injury, which is characterized by diffuse epithelial and endothelial injury and thus increased permeability of the

alveolocapillary barrier (1). Alveolar edema is also a common complication of congestive heart failure that causes increased intravascular pressure in the lung (2). Alveolar fluid is cleared by the active transport of sodium across the alveolar epithelium. Sodium is actively pumped out of the alveolar epithelial cells into the interstitium by sodium-potassium adenosine triphosphatase (*Na,K-ATPase*), located on the basolateral membrane of

the epithelium, which in turn drives the uptake of sodium by *amiloride*-sensitive and -insensitive sodium channels, located on the apical membrane of the epithelial monolayer. This generates an osmotic gradient that drives the vectorial transport of water out of the alveolar space (3).

Identification of mechanisms that improve alveolar fluid balance by improving alveolocapillary barrier function and/or enhancing alveolar fluid resorption is of great clinical importance. Recently, the lectin-like domain of tumor necrosis factor- α (designated *TIP*), which corresponds to residues 99–115 of murine tumor necrosis factor (*TNF*)- α (4), has been recognized as an important regulator of alveolar fluid balance. *TIP* up-regulated alveolar fluid resorption in an *in situ* mouse lung and in *ex vivo* (5) and *in vivo* (6) rat lung models and increased membrane conductance in microvascular en-

*See also p. 1671.

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dothelial cells (7). However, no studies have assessed the impact of TIP on the barrier function of the lung endothelium and epithelium, which are key players in the formation of pulmonary edema. Moreover, the mechanism by which TIP leads to enhanced alveolar fluid clearance has not been fully elucidated. Previous studies on the fluid-clearing properties of TIP have been confined to healthy lungs, and the efficacy of TIP in injured lungs has not been investigated. In this study, we employed hydrostatic edema as well as an endo/exotoxin-induced lung injury model of permeability edema and assessed the effect of TIP on alveolocapillary barrier function and alveolar fluid resorption in isolated, ventilated, and buffer-perfused rabbit lungs. Our data suggest that TIP may improve alveolar fluid balance by both attenuating the formation of, and enhancing clearance of, alveolar edema.

MATERIALS AND METHODS

Peptides. The TNF-derived peptides were synthesized by Bachem (Bubendorf, Switzerland) as acetate salts: TIP, CGPKDTPEGAELK-PWYC; scrambled TIP (scrTIP), CGTKPWELCPDEKPAYC (4).

Lung Preparation. Animal experiments were approved by the Animal Ethics Authority (Regierungspräsidium) of Giessen, Germany. The isolation of rabbit lungs was described in detail previously (8–10).

Measurements of Endothelial Permeability, Extravascular Lung Water, and Alveolar Edema in Hydrostatic and Endo/Exotoxin-Induced Lung Injury. In our hydrostatic edema model, two transient step-elevations (10 cm H₂O, 8 mins) in pulmonary venous pressure (P_{PV}) were applied to assess endothelial permeability. The first transient step-elevation was applied after the lungs had been equilibrated for 30 mins and had reached a steady-state baseline P_{PV} of 2 cm H₂O. Pharmacologic agents (TIP, scrTIP, or dibutyl cyclic adenosine monophosphate [db-cAMP; Calbiochem, San Diego, CA]) were then applied by nebulization with an Aeroneb Pro nebulizer (Aerogen, Mountain View, CA) to ~1 mM in the epithelial lining fluid (ELF). Alternatively, lungs were sham-nebulized with physiologic saline. In all cases, ~0.7 mL of fluid was deposited into the lungs. In the groups with hydrostatic edema, the baseline P_{PV} was then permanently elevated to 10 cm H₂O, and after a further 45 mins, a second 8-min transient step-elevation in P_{PV} (of 10 cm H₂O) was applied, on top of the baseline P_{PV} of 10 cm H₂O. The experimental protocol is illustrated in Figure 1A. The capillary filtration coefficient (K_{f,c}), fluid retention, and vascular compliance were determined gravimetrically as described previously (8, 9, 11). The

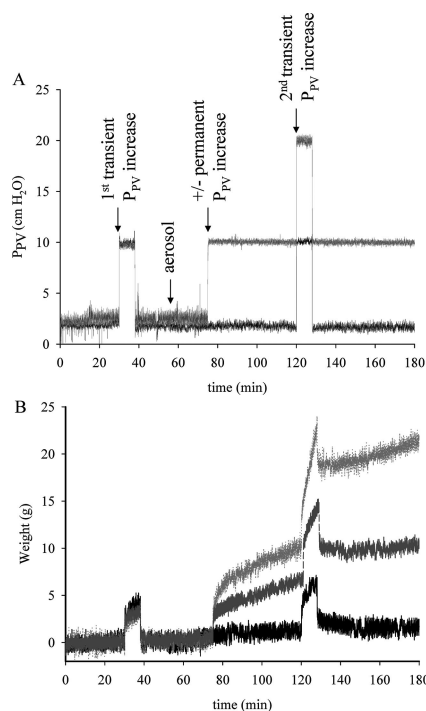


Figure 1. The lectin-like domain of tumor necrosis factor- α (TIP) peptide reduced the weight gain of isolated rabbit lungs in a model of hydrostatic edema. *A*, original, representative recordings of pulmonary venous pressure (P_{PV}). P_{PV} was maintained at 2 cm H₂O for the first 75 mins of the experiment, during which time pharmacologic agents or saline was deposited into the alveolar space of the lungs by aerosolization. In control experiments (*black*), the P_{PV} was maintained at 2 cm H₂O, whereas in experiments with hydrostatic edema, the P_{PV} was elevated to 10 cm H₂O for an additional 2 hrs in the absence (*red*) or presence (*blue*) of TIP (or scrambled peptide or dibutyl cyclic adenosine monophosphate, not illustrated). Two transient hydrostatic challenges were applied, the first of which was performed under baseline conditions, while a second challenge was applied 45 mins after the P_{PV} was elevated to 10 cm H₂O. *B*, original, representative recordings of lung weight in control lungs (*black*) and lungs with hydrostatic edema in the absence (*red*) or presence (*blue*) of TIP.

wet weight to dry weight ratio (W/D) and ELF volume (V_{ELF}) were determined as described previously (12).

As an alternative to K_{f,c}, a second, independent measurement of permeability was fluorescein isothiocyanate (FITC)-labeled albumin influx from the perfusate into the alveolar space. The FITC-albumin was added to the perfusate (to .16 mg/mL) after lungs had reached steady state, and FITC-albumin concentrations were determined from bronchoalveolar lavage fluids (200 μ L) in a Fusion microplate spectrofluorimeter (Packard Dreieich, Germany) at an emission wavelength of 480 nm and an excitation wavelength of 520 nm, as described previously (13).

The experimental protocol was identical to that described for the K_{f,c} measurements.

To assess the effect of TIP on fluid balance in permeability edema, we used an endo/exotoxin-induced lung injury model. This model has been demonstrated to impair endothelial integrity and to markedly increase both water and albumin permeability in isolated rabbit lungs (14) and *in vitro* (15). In this model of lung injury, four transient step-elevations (10 cm H₂O, 8 mins) in P_{PV} were applied to assess endothelial permeability. The first of these transient step-elevations was applied after the lungs had reached steady-state conditions (30 mins), after which pharmacologic agents (TIP, scrTIP, db-cAMP, or saline) were applied by nebulization. Fifteen minutes after the nebulization step, lungs were perfused for 180 mins in the absence or presence of *Salmonella enterica* serovar Abortusequi lipopolysaccharide (endotoxin, 7.5 ng/mL; Sigma, Taufkirchen, Germany) followed by bolus injection of *Staphylococcus aureus* α -toxin (exotoxin, .75 ng/mL; Sigma; Taufkirchen, Germany) or vehicle into the pulmonary artery. Transient step-elevations in P_{PV} were again performed 30, 60, and 90 mins after exotoxin administration to measure K_{f,c}, fluid retention, and vascular compliance. V_{ELF} was determined at the conclusion of each experiment.

Measurement of Alveolar Fluid Clearance. Active transepithelial sodium transport and passive paracellular permeability to small solutes were assessed by monitoring ²²Na and [³H]mannitol clearance from the alveolar space essentially as described previously (10, 12, 13). Lungs were allowed to establish steady-state baseline conditions for 30 mins, after which the organs were perfused in the absence or presence of the irreversible Na,K-ATPase inhibitor ouabain (10⁻⁵ M). Physiologic saline (1.5 mL) was deposited into the lung by nebulization. In some cases, TIP or amiloride, or combinations thereof (n = 6 of each condition), were simultaneously applied, with a deposition of ~1 mM TIP (or scrTIP) and ~10 μ M amiloride into the ELF. After a 60-min re-equilibration period, radioactive tracers were applied by nebulization of a mixture of ²²NaCl (1 μ Ci/mL) and [³H]mannitol (6 μ Ci/mL), over 10 mins, with an Optineb ultrasonic nebulizer (Nebu-Tec, Elsenfeld, Germany) with a deposition of ~1.2 μ Ci ²²Na⁺ and ~7.2 μ Ci [³H]mannitol. Elimination of ²²Na⁺ from the lung was monitored on-line by γ -detectors (Target System Electronic, Solingen, Germany) for 70 mins post-nebulization. Transit of the [³H]mannitol tracer was followed by discontinuous perfusate sampling (at 5, 10, 15, 20, 30, 50, and 70 mins postnebulization). Tracer-clearance curves were referenced to a 100% starting point at the end of nebulization. The V_{ELF} was determined at the end of each experiment.

Ouabain-Sensitive ⁸⁶Rb⁺ Uptake and Amiloride-Sensitive ²²Na⁺ Uptake Assays. Alveolar type II (ATII) cells were isolated from

male Sprague-Dawley rats (12, 16, 17). Approximately 3 million cells were plated in each well of six-well plates, and studies were performed on 90% to 95% confluent monolayers on day 2 after isolation. Ouabain-sensitive uptake of the K^+ -mimic $^{86}Rb^+$ into primary rat ATII cells was used to estimate potassium transport mediated by Na,K-ATPase (18), while uptake of amiloride-sensitive $^{22}Na^+$ by ATII cells was measured to assess Na^+ channel activity (19, 20). In the $^{86}Rb^+$ uptake studies, cells were incubated in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum in the absence or presence of ouabain (1.67 mM) or with TIP or scrTIP (1 mM) for 1 hr at 37°C. Medium was aspirated and replaced with identical fresh medium containing 1 $\mu Ci/mL$ $^{86}Rb^+$, and plates were incubated for a further 5 mins at 37°C, with gentle (100 rpm) rotation. Rubidium uptake was terminated by aspiration of the medium and addition of ice-cold 10 mM Tris-Cl, 150 mM MgCl₂, pH 7.4. Wells were washed extensively with 10 mM Tris-Cl, 150 mM MgCl₂ (3 \times 5 mL), and plates were air-dried overnight. Cells were solubilized in .2% (mass/vol) sodium dodecyl sulfate, and $^{86}Rb^+$ was quantified by liquid scintillation counting. Protein was quantified with the BioRad D_C Protein Assay Reagent (BioRad, Hercules, CA), adapted for samples containing detergent. In the $^{22}Na^+$ uptake studies, cells were incubated in Dulbecco modified Eagle medium in the presence of ouabain (1.67 mM) and in the absence or presence of amiloride (100 μM) for 30 mins and in the absence or presence of TIP (1 mM) for an additional 30 mins at 37°C. Medium was replaced with fresh medium containing 2 $\mu Ci/mL$ $^{22}Na^+$, and plates were incubated for 8 mins. Termination of $^{22}Na^+$ uptake was performed as described previously, and $^{22}Na^+$ in cell extracts was quantified in a 1480 Wallac Wizard 3-inch γ -counter (PerkinElmer, Rodgau-Jügesheim, Germany).

Preparation of Cell Lysates and Cell-Surface Labeling. Monolayers of ATII cells, plated in 60-mm tissue culture dishes, were treated exactly as described previously for ouabain-sensitive rubidium-uptake assay. Cell lysates were prepared in 50 mM Tris-Cl, 150 mM NaCl, 1% (vol/vol) NP-40, and 1% (mass/vol) sodium deoxycholate, pH 8, supplemented with Complete protease inhibitor cocktail (Roche, Mannheim, Germany). Biotinylation of cell-surface proteins was undertaken as described previously (12, 21). The Na,K-ATPase was detected with mouse monoclonal immunoglobulin G_{1 κ} anti-Na,K-ATPase α_1 -subunit (clone C464.6; Upstate, Waltham, MA).

Statistical Analysis. Numerical values represent the mean \pm sd. Comparisons between groups were made using a one-way analysis of variance with *post hoc* Dunnett or Tukey test. We considered $p < .05$ to be significant.

RESULTS

TIP Decreases Microvascular Permeability and Attenuates Alveolar Edema Formation in a Hydrostatic Edema Model. To investigate the potential effects of TIP on the formation of pulmonary edema, we first employed a model of hydrostatic edema in isolated, ventilated, and buffer-perfused rabbit lungs. The experimental protocol is illustrated in Figure 1A. Under baseline conditions (P_{PV} 2 cm H₂O), the pulmonary arterial pressure (P_{PA}) remained stable under all experimental conditions (data not shown). During the first transient P_{PV} increase, a parallel increase in P_{PA} (from 10.04 ± 1.12 to $14.63 \pm .51$ cm H₂O in saline-treated controls) was observed, which immediately returned to baseline levels after the conclusion of the hydrostatic challenge. When the P_{PV} was permanently elevated to 10 cm H₂O, a similar increase in P_{PA} (from $10.13 \pm .86$ to 15.17 ± 1.24 cm H₂O in saline-treated controls) was observed, while during the second, transient hydrostatic challenge (resulting in a P_{PV} of 20 cm H₂O), the P_{PA} increased from 10.29 ± 1.10 to 22.58 ± 2.09 cm H₂O in the saline-treated group. The increase in P_{PA} after the transient and permanent P_{PV} elevations was similar in all experimental groups and was not influenced by TIP, scrTIP, or db-cAMP treatment (data not shown).

In control lungs, organ weight was markedly increased after elevation of baseline hydrostatic pressure. In contrast, lungs pretreated with TIP before hydrostatic edema formation exhibited a significantly lower weight gain compared with lungs pretreated with saline. Original lung weight recordings are illustrated in Figure 1B. Similarly, microvascular permeability (assessed by K_{fc}) and alveolocapillary permeability (assessed by FITC-albumin flux) were significantly increased after the transient P_{PV} elevation (Fig. 2). In contrast, lungs that had been treated with TIP exhibited a significantly smaller magnitude of permeability increase compared with lungs maintained under control conditions or lungs treated with scrTIP (Fig. 2A). Similar to the effect of TIP, pretreatment of the lungs with db-cAMP, an agent that has been proposed to decrease microvascular permeability (and increase fluid resorption) (22, 23), also reduced magnitude of the increase in permeability induced by the elevated hydrostatic pressures (Fig. 2).

In line with these findings, in the hydrostatic edema model, fluid retention

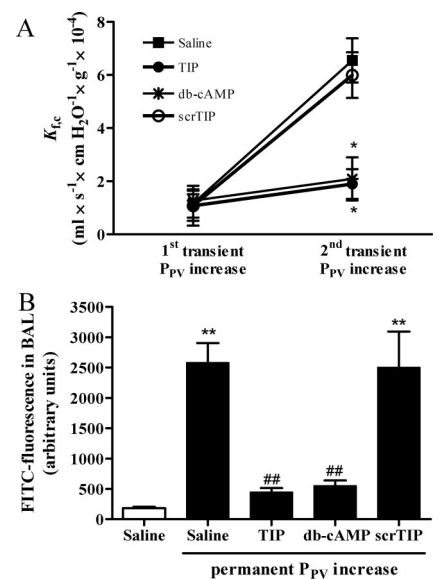


Figure 2. The lectin-like domain of tumor necrosis factor- α (TIP) peptide reduced alveolocapillary permeability in a model of hydrostatic edema. A, endothelial permeability was assessed from capillary filtration coefficients (K_{fc}) determined at baseline conditions (first challenge) and 45 mins after the pulmonary venous pressure (P_{PV}) was permanently elevated to 10 cm H₂O (second challenge) in the presence of TIP, dibutyl cyclic adenosine monophosphate (db-cAMP), or a scrambled peptide (scrTIP) or in saline-treated lungs. * $p < .05$ compared with saline-treated group after permanent P_{PV} elevation. B, in identical experiments, alveolocapillary permeability was assessed from fluorescein isothiocyanate (FITC)-albumin transit from the vascular to the alveolar space. In all cases, data represent the mean \pm SD (n = 6, per group). ** $p < .01$ compared with saline-treated group without permanent P_{PV} elevation; ## $p < .01$ compared with saline-treated group after permanent P_{PV} elevation. BAL, bronchoalveolar lavage.

(assessed by increases in lung weight after transient hydrostatic challenge) increased from $1.02 \pm .88$ mL to 21.69 ± 6.28 mL in saline-treated lungs (Fig. 3A). Similar results were obtained in lungs that were treated with scrTIP. In contrast, lungs that were treated with TIP or db-cAMP before the onset of the hydrostatic edema formation exhibited significantly lower fluid retention compared with control lungs or lungs treated with scrTIP (Fig. 3A). Moreover, extravascular lung water, as assessed by W/D ratios, was markedly increased in the hydrostatic edema model in saline- or scrTIP-treated lungs and was significantly reduced by both TIP and db-cAMP (Fig. 3B).

Epithelial lining fluid volume was measured at the end of each experiment to assess the amount of extravascular

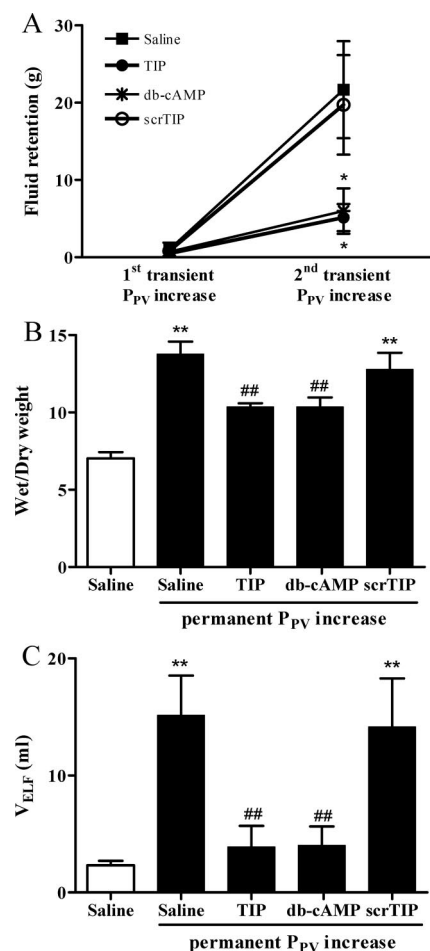


Figure 3. The lectin-like domain of tumor necrosis factor- α (*TIP*) peptide reduced extravascular water in a model of hydrostatic edema. *A*, fluid retention was determined as the increase in lung weight that remained after the transient hydrostatic challenges in the presence of *TIP*, dibutyl cyclic adenosine monophosphate (*db-cAMP*), or a scrambled peptide (*scrTIP*) or in saline-treated lungs. * $p < .05$ compared with saline-treated group after permanent pulmonary venous pressure (P_{PV}) elevation. *B*, extravascular lung water was assessed by the wet weight to dry weight ratio. *C*, at the conclusion of each experiment, alveolar fluid accumulation was assessed by measurements of epithelial lining fluid volume (V_{ELF}). In all cases, data represent the mean \pm SD ($n = 6$ per group). In *B* and *C*, ** $p < .01$ compared with the saline-treated group without permanent P_{PV} elevation; ## $p < .01$ compared with the saline-treated group after permanent P_{PV} elevation.

fluid that was retained in the alveolar space after the hydrostatic challenge (Fig. 3C). In sham-treated control lungs, hydrostatic injury resulted in a V_{ELF} of 15.15 ± 3.39 mL at the conclusion of the experiment. However, lungs treated with *TIP* or *db-cAMP* exhibited a significantly lower V_{ELF} (3.89 ± 1.80 and 4.03 ± 1.61

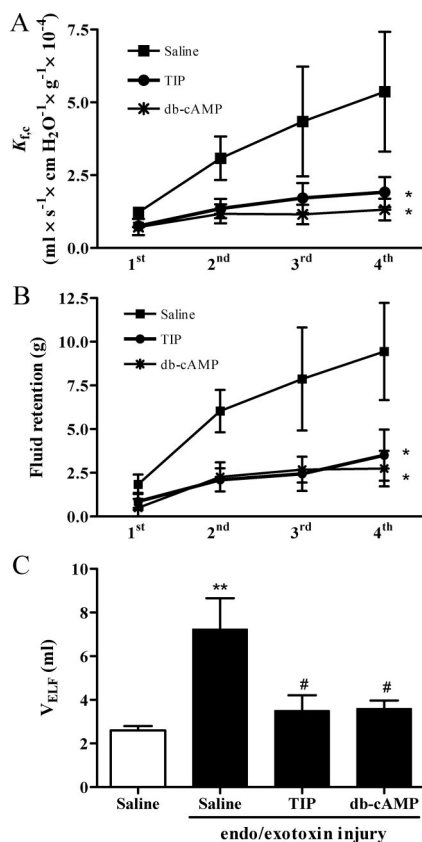


Figure 4. The lectin-like domain of tumor necrosis factor- α (*TIP*) peptide reduced endothelial permeability and extravascular lung water in a model of permeability edema. *A*, endothelial permeability was assessed from capillary filtration coefficients (K_{fc}) determined at baseline conditions (first challenge) and 30, 60, and 90 mins after endo/exotoxin-induced injury (second, third, and fourth challenges, respectively) in the presence of dibutyl cyclic adenosine monophosphate (*db-cAMP*) or *TIP* or in saline-treated lungs. *B*, fluid retention was determined as the increase in lung weight that remained after the transient hydrostatic challenges in the presence of *db-cAMP*, or *TIP* or in saline-treated lungs. In *A* and *B*, * $p < .05$ compared with saline-treated group after endo/exotoxin-induced injury. *C*, at the conclusion of each experiment, alveolar fluid accumulation was assessed by measurements of epithelial lining fluid volume (V_{ELF}). In all cases, data represent the mean \pm SD ($n = 6$, per group). In *C*, ** $p < .01$ compared with saline-treated group without lung injury; # $p < .05$ compared with saline-treated group after lung injury.

mL, respectively), whereas *scrTIP* had no effect on V_{ELF} (Fig. 3C).

To further investigate the potential effects of *TIP* on the formation of permeability edema, we employed a model of endo/exotoxin-induced lung injury in the isolated rabbit lungs. Capillary filtration and fluid retention during the transient hydrostatic challenges were significantly

increased 30, 60, and 90 mins following exotoxin treatment after an initial 3-hr priming with endotoxin (Fig. 4, *A* and *B*). In contrast, lungs treated with *TIP* or *db-cAMP* exhibited significantly lower K_{fc} and fluid retention (Fig. 4, *A* and *B*). While the endo/exotoxin-injured lungs exhibited an approximately 2.8-fold increase in V_{ELF} , lungs that had been pretreated with *TIP* exhibited a V_{ELF} increase of only $\sim 65\%$ compared with noninjured lungs (Fig. 4C). Similar results were obtained when lungs were treated with *db-cAMP* (Fig. 4C), while P_{PA} remained unchanged under all experimental conditions (data not shown).

TIP Increases Active Transepithelial Sodium Transport and Alveolar Fluid Clearance in Intact Lungs. Active transport of sodium and passive paracellular movement of small solutes were assessed by measurements of ouabain and amiloride-sensitive and -insensitive fluxes of $^{22}\text{Na}^+$ and [^3H]mannitol from the alveolar space to the vascular compartment. Original clearance curves depicting $^{22}\text{Na}^+$ transit from the alveolar to the vascular compartments in intact rabbit lungs are illustrated in Figure 5A. The methodology, which permits distinction between active and passive transport processes, has been described in detail previously (13). Under control conditions, $^{22}\text{Na}^+$ clearance is mediated by both active and passive transport processes. In contrast, in lungs treated with ouabain and amiloride (potent and specific inhibitors of Na,K-ATPase and the epithelial sodium channel [ENaC], respectively), $^{22}\text{Na}^+$ transit was almost exclusively passive (10, 24). Administration of *TIP* to the alveolar space by ultrasonic nebulization markedly increased the clearance of $^{22}\text{Na}^+$ from the alveolar space, as $60.87\% \pm 3.65\%$ of $^{22}\text{Na}^+$ deposited in the alveolar space was cleared over the course of the experiment (Fig. 5A) compared with control conditions or lungs treated with a *scrTIP*, which exhibited $48.46\% \pm 2.16\%$ and $48.21\% \pm 1.14\%$ clearance of $^{22}\text{Na}^+$, respectively. Pretreatment with ouabain and/or amiloride before the application of *TIP* significantly reduced the *TIP*-induced increase in $^{22}\text{Na}^+$ transit (Fig. 5A). Passive paracellular movement of small solutes, as assessed by [^3H]mannitol clearance from the alveolar space, was unaffected under all experimental conditions (Fig. 5C). Thus, the increase we observed in the $^{22}\text{Na}^+$ clearance after *TIP* treatment was due to enhanced active sodium transport. This active fraction of

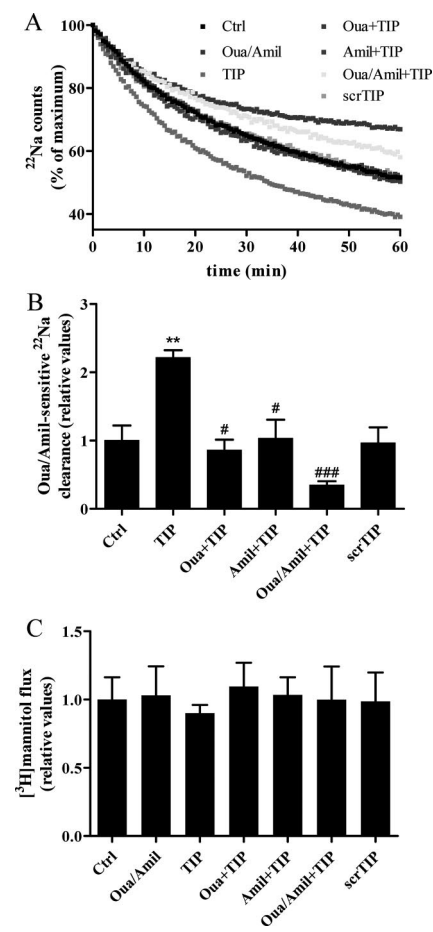


Figure 5. The lectin-like domain of tumor necrosis factor- α (*TIP*) peptide increased active trans-epithelial Na^+ transport in intact lungs. *A*, after deposition of radioactive tracers ($^{22}\text{NaCl}$ and ^3H]mannitol) into the lung by nebulization, ^{22}Na clearance from the lung was continuously monitored for 60 mins. Before application of tracers, 1.5 mL of excess liquid (saline) was deposited into the alveolar space by nebulization. In some cases, *TIP* or amiloride (*Amil*), or combinations thereof, were simultaneously applied, with a deposition of ~ 1 mM *TIP* (or scrambled peptide, *scrTIP*) and ~ 10 μM *Amil* in the epithelial lining fluid in the absence or presence of ouabain (*Oua*, 10^{-5} M) in the vascular space. For the purposes of clarity, standard errors have been omitted; however, they are incorporated into the analyses of these data in *B*. *B*, active ^{22}Na transport was quantified from data in *A*. Values are relative to the ouabain- and amiloride-sensitive active ^{22}Na transport in sham-treated lungs. *C*, passive ^3H]mannitol flux was monitored by liquid scintillation counting of perfusate samples taken at discrete time intervals after nebulization of the ^3H]mannitol tracer to the lungs. Values are relative to the passive ^3H]mannitol flux in sham-treated lungs. In *B* and *C*, bars represent the mean \pm SD ($n = 6$ for all groups); $^{**}p < .01$ compared with controls (*Ctrl*); $^{\#}p < .05$ and $^{###}p < .001$ compared with *TIP*-treated lungs.

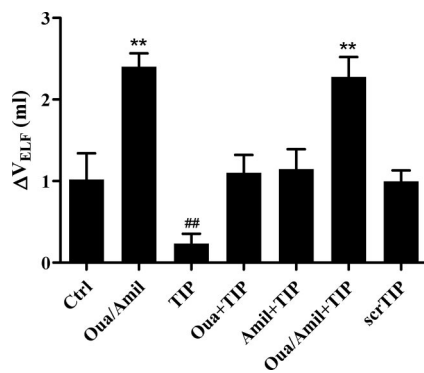


Figure 6. The lectin-like domain of tumor necrosis factor- α (*TIP*) peptide increased alveolar fluid clearance in intact lungs. During the two nebulization steps, approximately 2.5 mL of fluid was deposited into the alveolar space. Data indicate the increase in epithelial lining fluid volume (V_{ELF}) at the conclusion of the experiments in the presence of various treatments when compared with nonnebulized controls (*Ctrl*). Bars represent the mean \pm SD ($n = 6$ for all groups); $^{**}p < .01$ compared with controls; $^{\#}p < .01$ compared with control lungs or lungs that had been pretreated with ouabain (*Oua*) and amiloride (*Amil*) before *TIP* administration. *scrTIP*, scrambled peptide.

trans-epithelial $^{22}\text{Na}^+$ transit is illustrated in Figure 5*B*. Administration of *TIP* increased active sodium transport by approximately 2.2-fold and 2.3-fold when compared with sham-treated controls or lungs treated with *scrTIP*, respectively. This change in active Na^+ transport resulted in an increase in alveolar fluid clearance (assessed by measurements of V_{ELF}) in lungs to which aerosolized *TIP* was delivered, compared with all other groups (Fig. 6). During the two nebulization steps, approximately 2.5 mL of fluid was deposited into the alveolar space. Under control conditions, lungs cleared approximately 60% of the liquid deposited into the alveolar space over the course of the experiment, leading to an excess V_{ELF} of $1.02 \pm .32$ mL at the conclusion of the experiment. Lungs that received *TIP* by nebulization cleared significantly more fluid from the alveolar space and exhibited a final excess V_{ELF} of $.23 \pm .12$ mL. In contrast, lungs that were pretreated with ouabain and amiloride were unable to clear the liquid deposited in the alveolar compartment, resulting in $2.41 \pm .17$ mL of fluid retained in the alveolar space. Similar results were obtained in the presence of *TIP* in lungs that were pretreated with ouabain and amiloride, while *scrTIP* had no effect on lung liquid clearance compared with sham-treated controls. Taken together, these data suggest that

TIP enhanced both ouabain- and amiloride-sensitive sodium transport across the alveolar epithelium, thus augmenting lung liquid clearance.

TIP Increases *Na,K-ATPase* Activity and Amiloride-Sensitive Na^+ Uptake in *ATII* Cells. To investigate the role of *Na,K-ATPase* in the *TIP*-induced activation of sodium transport, *ATII* cells were treated with *TIP* (or *scrTIP*), and the activity of *Na,K-ATPase* was measured by ouabain-sensitive $^{86}\text{Rb}^+$ uptake. Application of *TIP* (1 mM, 1 hr) caused a significant, 1.6-fold increase in *Na,K-ATPase* activity, while application of *scrTIP* did not change *Na,K-ATPase* activity when compared with sham-treated control lungs (Fig. 7*A*). To determine whether this increase in the activity of *Na,K-ATPase* was due to an increase in *Na,K-ATPase* abundance at the plasma membrane, we performed cell-surface biotinylation studies. Administration of *TIP* resulted in a $1.82 \pm .32$ -fold increase in *Na,K-ATPase* abundance at the plasma membrane of *ATII* cells, while pretreatment with *scrTIP* did not alter *Na,K-ATPase* cell-surface density compared with controls (Fig. 7*B*). In contrast, the total cellular pools of *Na,K-ATPase* were unchanged under all experimental conditions, suggesting that the increase in *Na,K-ATPase* activity and abundance at the cell surface was due to *Na,K-ATPase* exocytosis from intracellular compartments to the plasma membrane. To study the role of amiloride-sensitive Na^+ channels in the *TIP*-induced enhancement of sodium transport, uptake of $^{22}\text{Na}^+$ by *ATII* cells was measured in the absence or presence of *TIP*. Application of *TIP* increased $^{22}\text{Na}^+$ uptake by approximately 87%, which was significantly reduced in cells that had been pretreated with amiloride (Fig. 7*C*).

DISCUSSION

Mechanisms that drive pulmonary edema resolution by enhancing alveolar-capillary barrier function or alveolar fluid resorption are of great clinical importance. Both *TNF- α* and its lectin-like domain located at the distal tip of the *TNF- α* polypeptide (hence called the *TIP domain*) have emerged as potentially important regulators of alveolar fluid balance. Although *TNF- α* down-regulates αENaC gene expression and hence fluid transport by *ATII* cells *in vitro* (25, 26), alveolar fluid clearance was potentiated in a *TNF- α* -dependent manner in both a

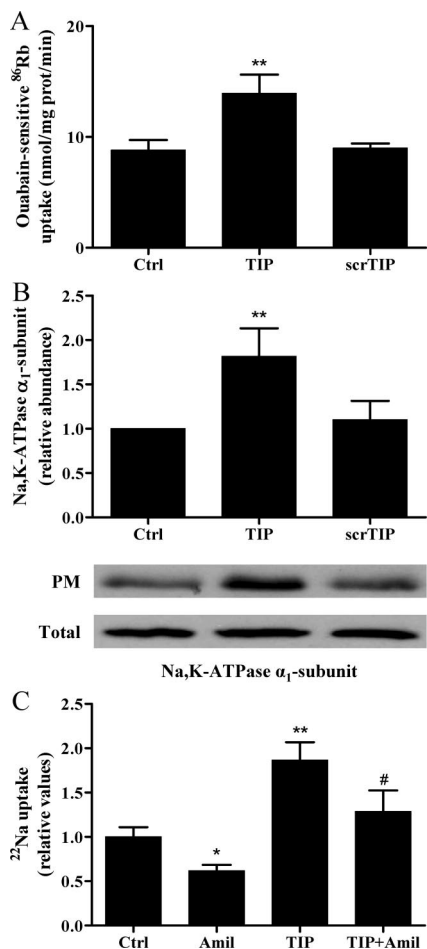


Figure 7. The lectin-like domain of tumor necrosis factor- α (*TIP*) peptide increased sodium-potassium adenosine triphosphatase (*Na,K-ATPase*) activity and Na^+ uptake in alveolar type II (*A2II*) cells. *A*, ouabain-sensitive uptake of $^{86}\text{Rb}^+$ by *A2II* cells was assessed in the absence (*Ctrl*) or presence of *TIP* or scrambled peptide (*scrTIP*, 1 mM, 1 hr). Each bar represents the mean \pm SD ($n = 4$ for each experimental condition). $**p < .01$. *B*, 90% to 95% confluent monolayers of *A2II* cells were sham-treated (*Ctrl*) or treated with *TIP* or *scrTIP* (1 mM, 1 hr), after which cell-surface proteins were biotinylated and cell lysates were prepared. Biotinylated proteins in 100 μg of cell lysates were extracted with streptavidin-agarose beads, and both pulled-down proteins and total cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed for *Na,K-ATPase* α_1 -subunit. Representative immunoblots of the *Na,K-ATPase* α_1 -subunit in the plasma membrane (*PM*) as well as in the total cell lysate (*Total*) are shown. Bars represent the relative density of the *Na,K-ATPase* α_1 -subunit protein in the plasma membrane compared with controls. Values are given as mean \pm SD ($n = 4$ for each experimental condition). $**p < .01$. *C*, uptake of $^{22}\text{Na}^+$ by *A2II* cells was assessed in the absence (*Ctrl*) of ouabain and the absence or presence of *TIP* or amiloride (*Amil*). Each bar represents the mean \pm SD ($n = 4$ for each experimental condition). $*p < .05$ and $**p < .01$ compared with controls, and $\#p < .05$ when compared with *TIP*-treated cells.

rodent pneumonia model (27) and intestinal ischemia-reperfusion injury (28). These opposing effects of $\text{TNF-}\alpha$ on alveolar fluid dynamics have been attributed to two different domains with opposing functions within the $\text{TNF-}\alpha$ polypeptide (6). One of these domains, the *TIP* domain, enhanced alveolar fluid clearance in ventilated rats (29). Recently, Elia et al. (5) demonstrated that $\text{TNF-}\alpha$ promoted fluid resorption in wild-type mice and in mice deficient in both $\text{TNF-}\alpha$ receptors, indicating that the effect of *TIP* on alveolar fluid clearance was independent of both $\text{TNF-}\alpha$ receptors. Intratracheal application of a synthetic peptide based on the *TIP* sequence to an isolated, ventilated, autologous blood-perfused rat lung model caused a significant reduction in lung water and improvement in lung compliance compared with lungs treated with a scrambled peptide of the same length (5). However, no study has explored the impact of *TIP* in alveolocapillary barrier integrity or the exact mechanism by which sodium transport is enhanced by *TIP*.

To address these questions, we first employed a hydrostatic edema model in isolated, ventilated, and buffer-perfused rabbit lungs, in which we elevated P_{PV} for 2 hrs and assessed endothelial barrier function and formation of pulmonary edema. Pulmonary capillary pressure is an important determinant of fluid flux across the pulmonary capillary wall, as increases in hydrostatic pressure in the lung vasculature result in accumulation of extravascular lung liquid (30, 31), due to increased vascular permeability in response to the hydrostatic pressure (32–34). We observed that a prolonged increase of P_{PV} from 2 to 10 mm Hg resulted in a massive extravasation of fluid in rabbit lungs, a significant amount of which accumulated in the alveolar space. The application of *TIP* largely reduced this liquid filtration and alveolar edema accumulation as assessed by measurements of fluid retention, W/D , and V_{ELF} . This protective effect of *TIP* on edema formation was not due to a modification of the microvascular pressure by *TIP*, since the increase in P_{PA} after P_{PV} elevation was not reduced by *TIP* treatment and was similar under all experimental conditions. Rather, pretreatment of lungs with *TIP* significantly reduced the hydrostatic pressure-induced increase in endothelial permeability. Similarly, administration of the cAMP analog db-cAMP prevented edema formation by

reducing microvascular permeability during elevated hydrostatic pressures. In fact, strategies that increase or mimic cAMP have been credited with a protective role in endothelial barrier integrity (35–38). In particular, it was recently proposed that the β_2 -adrenergic agonist salmeterol reduces vascular leakage in a model of acid-induced lung injury by decreasing endothelial permeability (23). Furthermore, it has been reported that elevation of intracellular cAMP, via activation of protein kinase A and consequent stabilization of the actin cytoskeleton and microtubule network, promotes endothelial barrier function (38, 39). Thus, our data indicate that *TIP*, similar to db-cAMP, prevents alveolar edema formation in a model of hydrostatic edema by enhancing microvascular barrier function. Since the positive effects of *TIP* on alveolar fluid balance were almost identical to the effects of cAMP stimulation, one might postulate that the clinical use of *TIP* may not be greater than cAMP enhancing agents. Further studies are warranted to address this important question.

To confirm our findings that *TIP* reduces capillary permeability, we also employed a model of endo/exotoxin-induced permeability edema and assessed capillary filtration as well as extravascular lung liquid. Indeed, similar to the effects of *TIP* in the hydrostatic model, *TIP* also significantly reduced capillary permeability and thus edema formation after lungs were injured with endo/exotoxin, a model that has been shown to markedly increase microvascular permeability in isolated rabbit lungs (14). The low concentrations (.75 ng/mL) were optimized such that they increased vascular permeability without affecting vascular pressures, which has been documented at higher doses (10–30 ng/mL) (14).

In another set of experiments, we studied the effect of *TIP* on alveolar edema clearance in a model of alveolar fluid challenge. In these studies we deposited a significant amount of fluid into the alveolar space of isolated rabbit lungs and monitored Na^+ transport as well as resorption of liquid from the alveolar compartment. The primary driving force of alveolar fluid resorption across the epithelial monolayer is the active transport of Na^+ (3, 40), which is undertaken primarily by ENaC and *Na,K-ATPase* (40–43), while chloride and potassium channels also influence fluid clearance (44,

45). During this process, Na,K-ATPase in the basolateral membrane pumps Na⁺ out of the epithelium, creating an osmotic gradient that leads to the entry of Na⁺ through apically located amiloride-sensitive and -insensitive Na⁺ channels. This vectorial transport of Na⁺ drives the passive movement of water from the alveolar to the interstitial space (40). It has been well established that alveolar fluid resorption is largely inhibited by amiloride, an inhibitor of ENaC, as well as by the potent and irreversible Na,K-ATPase inhibitor, ouabain (40). Furthermore, up-regulation of ENaC and Na,K-ATPase enhances active Na⁺ transport and thus increases the ability of the lungs to clear alveolar edema (46–48). In our experiments, TIP increased transepithelial Na⁺ transport, leading to a marked increase in alveolar fluid clearance. In contrast, in lungs that were pretreated with ouabain and/or amiloride before TIP administration, TIP failed to increase epithelial transport of Na⁺ and the lungs were unable to clear the excess fluid deposited into the alveolar space, suggesting that the positive effect of TIP on alveolar fluid clearance was mediated by up-regulation of ENaC and/or Na,K-ATPase. In fact, it has already been reported that the lectin-like domain of TNF- α can enhance amiloride-sensitive alveolar fluid resorption, suggesting that ENaC is probably involved in this process (5). In our studies with isolated rabbit lungs and AII cells, we were able to confirm these findings, as a significant portion of transepithelial ²²Na⁺ clearance, as well as ²²Na⁺ uptake by AII cells, was amiloride sensitive.

The activity of Na,K-ATPase can be regulated transcriptionally and posttranslationally (49). However, the short time frame of our experiments precludes transcriptional regulation as a major contributor. In contrast, rapid, posttranslational regulation of Na,K-ATPase function includes mechanisms that either alter activity of cell surface-localized Na,K-ATPase or result in trafficking of preexisting Na,K-ATPase molecules between the plasma membrane and intracellular compartments, thereby modulating the number of Na,K-ATPase molecules that are located on the cell surface (50). Under physiologic conditions, Na,K-ATPase is not saturated by the intracellular Na⁺ concentration ([Na⁺]_i), and an increase in [Na⁺]_i alone can lead to activation of Na,K-ATPase (51). Given that TIP enhances ENaC activity, leading to enhanced entry of Na⁺

into the alveolar epithelium, it is possible that the increase of Na,K-ATPase activity after application of TIP is secondary to the increase in ENaC activity. However, it is well established that an increase in [Na⁺]_i results in enhanced activity of Na,K-ATPase without changing its cell surface abundance (50). In contrast, in our experiments, application of TIP resulted in a significant increase in the number of Na,K-ATPase molecules on the cell surface of alveolar epithelial cells, suggesting that the enhanced Na⁺ pump activity after application of TIP was probably not secondary to increased [Na⁺]_i but rather was mediated by trafficking of Na,K-ATPase from intracellular compartments to the cell surface. Thus, it is likely that the mechanism by which TIP augments the resolution of alveolar edema involves trafficking of Na,K-ATPase. Indeed, it is well known that mechanisms resulting in increased Na,K-ATPase abundance in the plasma membrane lead to enhanced transport of Na⁺ and thus increased alveolar fluid clearance (41, 48, 49). For example, it has been reported that short-term stimulation of β_2 -adrenergic receptors by isoproterenol resulted in Na,K-ATPase exocytosis from intracellular pools to the plasma membrane of alveolar epithelial cells and a consequent increase in Na,K-ATPase activity (52), and this increase in the activity of Na,K-ATPase led to enhanced fluid resorption from the alveolar space of isolated rat lungs (53). Similarly, dopamine increases fluid clearance by up-regulating Na,K-ATPase activity through its translocation to the cell surface in the alveolar epithelium (54–56). Taken together, our data indicate that TIP activates transepithelial Na⁺ transport and thus alveolar fluid clearance by up-regulating both amiloride-sensitive Na⁺ channels and Na,K-ATPase in the epithelium.

CONCLUSIONS

This experimental study suggests that TIP may regulate both the formation and the clearance of excess alveolar fluid and thus might represent a therapeutic agent in pulmonary edema. Further studies are warranted to identify the intracellular signaling mechanisms by which TIP regulates alveolar fluid balance.

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