Transient Receptor Potential Vanilloid 4 and Serum Glucocorticoid–regulated Kinase 1 Are Critical Mediators of Lung Injury in Overventilated Mice *In Vivo*

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ABSTRACT

Background: Mechanical ventilation can cause lung endothelial barrier failure and inflammation cumulating in ventilator-induced lung injury. Yet, underlying mechanotransduction mechanisms remain unclear. Here, the authors tested the hypothesis that activation of the mechanosensitive Ca^{2+} channel transient receptor potential vanilloid (TRPV4) by serum glucocorticoid–regulated kinase (SGK) 1 may drive the development of ventilator-induced lung injury.

Methods: Mice (total n = 54) were ventilated for 2 h with low (7 ml/kg) or high (20 ml/kg) tidal volumes and assessed for signs of ventilator-induced lung injury. Isolated-perfused lungs were inflated with continuous positive airway pressures of 5 or 15 cm H_2O (n = 7 each), and endothelial calcium concentration was quantified by real-time imaging.

Results: Genetic deficiency or pharmacologic inhibition of TRPV4 or SGK1 protected mice from overventilation-induced vascular leakage (reduction in alveolar protein concentration from 0.84 ± 0.18 [mean ± SD] to 0.46 ± 0.16 mg/ml by TRPV4 antagonization), reduced lung inflammation (macrophage inflammatory protein 2 levels of 193 ± 163 in *Trpv4*$^{-/-}$ *vs.* 544 ± 358 pmol/ml in wild-type mice), and attenuated endothelial calcium responses to lung overdistension. Functional coupling of TRPV4 and SGK1 in lung endothelial mechanotransduction was confirmed by proximity ligation assay demonstrating enhanced TRPV4 phosphorylation at serine 824 at 18% as compared to 5% cyclic stretch, which was prevented by SGK1 inhibition.

Conclusions: Lung overventilation promotes endothelial calcium influx and barrier failure through a mechanism that involves activation of TRPV4, presumably due to phosphorylation at its serine 824 residue by SGK1. TRPV4 and SGK1 may present promising new targets for prevention or treatment of ventilator-induced lung injury. **(Anesthesiology 2017; 126:300-11)**

MECHANICAL ventilation is the only life-saving treatment in acute respiratory distress syndrome (ARDS); yet, long-term mechanical ventilation may also trigger critical adverse effects in that it promotes vascular barrier dysfunction, edema formation, local inflammation, and release of proinflammatory cytokines.[1,2] This phenomenon, known as ventilator-induced lung injury (VILI), was initially considered to result from physical rupture of lung tissue due to excessive volumes and pressures during mechanical ventilation.[2,3] Recently, however, VILI has become recognized as being primarily the result of active mechanotransduction at the alveolocapillary barrier. Yet, the distinct pathways of mechanotransduction that initiate the inflammatory response and alveolocapillary barrier failure in VILI still remain largely unclear.

The Ca^{2+} permeable and mechanosensitive transient receptor potential vanilloid 4 (TRPV4) cation channel presents a particularly attractive candidate in this context, as it has been implicated in stretch-induced endothelial barrier failure in isolated lungs.[4–7] TRPV4 plays a critical role in the transduction of mechanical forces and gets activated by

What We Already Know about This Topic

- Ventilator-induced lung injury appears to be primarily the result of active mechanotransduction initiating the inflammatory response and alveolocapillary barrier failure

What This Article Tells Us That Is New

- By using animal genetic models and pharmacologic approaches, the authors found attenuated ventilator-induced lung injury (VILI) by the inhibition of transient receptor potential vanilloid (TRPV4) and involvement of serum glucocorticoid–regulated kinase (SGK) 1 in the TRPV4 Ca^{2+}-mediated VILI through molecular interaction and phosphorylation of TRPV4 at serine 824
- The results of this study suggest TRPV4 and SGK1 as potential targets for treatment of VILI

Submitted for publication February 19, 2016. Accepted for publication October 19, 2016. From the Institute of Physiology (L.M., L.E., U.W., W.M.K.) and Department of Nephrology, Medizinische Klinik (M.v.d.G.), Charité—Universitaetsmedizin Berlin, Berlin, Germany; Departments of Medicine, Neurology, and Neurobiology, Duke University, Durham, North Carolina (W.L.); Keenan Research Centre for Biomedical Science, St. Michael's Hospital, Toronto, Ontario, Canada (W.M.K.); Departments of Surgery and Physiology, University of Toronto, Toronto, Ontario, Canada (W.M.K.); and German Heart Institute, Berlin, Germany (W.M.K.).

 Anesthesiology 2017; 126:300-11

<zdoi;10.1097/ALN.0000000000001443>

cell swelling[8–13] and surface expansion.[14] In endothelial cells, TRPV4 is activated by uniaxial, circumferential stretch or shear stress and mediates Ca^{2+} influx into the endothelium, which in turn increases endothelial permeability.[4–6,15,16] In parallel, TRPV4 activation can trigger inflammatory pathways in both parenchymal and immune cells.[17–20]

Regulation of TRPV4 activity and channel open probability depends on extracellular and intracellular Ca^{2+} concentrations, as well as posttranslational modifications, like nitrosylation[21] and phosphorylation.[22,23] The best characterized phosphosites of TRPV4 are Tyr110,[24] Tyr253,[5] and serine 824 (Ser824),[14] which are regulated by various kinases including protein kinases A and C.[22,23] Recently, the serum glucocorticoid–regulated kinase (SGK) 1 was identified as a novel TRPV4 regulating kinase, which amplifies the subsequent TRPV4 response to appropriate stimuli.[14] Notably, SGK1 is similarly involved in the regulation of cell volume[25,26] and cell fluid transport,[27] *i.e.*, mechanosensation pathways that closely mirror those regulating TRPV4.

Based on their demonstrated roles in mechanosensation and/or transduction, we tested for the role of TRPV4 and its possible regulation by SGK1 in the context of VILI. Here, we demonstrate a key role for TRPV4 in stretch-induced mechanotransduction in lung endothelial cells *in situ* and lung edema formation and inflammation *in vivo*. Furthermore, we identified SGK1 as a novel upstream regulator of TRPV4 in VILI in that SGK1 inhibition attenuated TRPV4-mediated endothelial Ca^{2+} entry and lung vascular leak and inflammation *in vivo* and prevented stretch-induced Ser824 phosphorylation of TRPV4 *in vitro*. These findings reveal SGK1 and TRPV4 as important signaling components of the endothelial mechanotransduction pathway and hence as putative targets for the prevention of VILI.

Materials and Methods

Animals

Experiments were performed as prospective, randomized, and controlled studies in male TRPV4-deficient (*Trpv4*$^{-/-}$)[8] and corresponding C57BL/6J wild-type (WT) mice with body weights (bw) of 25 to 30 g. Procedures were approved by the animal care and use committee of the local government authorities (Landesamt für Gesundheit und Soziales, Berlin, Germany) and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Washington, D.C., 7th edition, 1996). Sample size was based on previous experience[19] and targeted for group sizes of n = 8 to 9 animals, with deviations being attributable to inclusion of pilot experiments or to missing data points due to technical problems with sample collection or analysis.

In Vivo *Model of VILI*

WT or *Trpv4*$^{-/-}$ mice were anesthetized (ketamine 100 mg/kg, xylazine 20 mg/kg bw), tracheotomized, and polyethylene catheters were surgically introduced into the right carotid artery and the left jugular vein. Animals were randomly assigned to either ventilation (MidiVent; Harvard Apparatus, USA) with low tidal volume (LV_T) of 7 ml/kg bw and a respiratory rate of 150 min^{-1} or injurious ventilation with high tidal volume (HV_T) of 20 ml/kg and a respiratory rate of 60 min^{-1} at an inspiratory fraction of oxygen of 1.0 and a positive end-expiratory pressure of 2 cm H_2O for 2 h. Respiratory rates were adjusted to maintain $Paco_2$ levels within 35 to 45 mmHg for each group. For pharmacologic interventions, the TRPV4 antagonist HC-067047 (20 nM; Sigma Aldrich, Germany) or the SGK1 inhibitor GSK650394 (100 nM; Tocris Bioscience, United Kingdom) were continuously infused *via* the jugular vein at a rate of 400 µl/h, while control groups received 400 µl/h saline. A 1-mg bolus of human serum albumin (HSA; Baxter, Germany) was given intravenously 60 min before the end of the experiment for subsequent analysis of pulmonary hyperpermeability to proteins greater than or equal to 60 kd.[28] Unventilated controls were subjected to the same procedures without connection to the ventilator.

After 2 h with or without mechanical ventilation, animals were euthanized, lungs were excised, and *post hoc* analyses were performed in a blinded fashion. Lung edema was estimated as wet/dry lung weight ratio from the apical lobe of the right lung. The right middle lobe was fixed in 4% formalin and paraffin embedded for histologic sectioning; slides were stained by hematoxylin and eosin, and lung injury was assessed by a semiquantitative score.[29] The lower lobe and the accessory lobe of the right lung were frozen in liquid nitrogen, stored at –80°C, and utilized for *post hoc* protein analyses. The left lung was lavaged with ice-cold phosphate-buffered saline (4 × 150 µl), and protein concentration in the bronchoalveolar lavage fluid (BALF) was quantified by Bradford assay (Bio-Rad, USA). The concentration of HSA in BALF and plasma was measured by ELISA (Bethyl, USA), and macromolecular extravasation was calculated as ratio of HSA concentrations in BALF over plasma ×1,000. Interleukin-1β, interleukin-10, macrophage inflammatory protein 2 (MIP-2; CXCL2), keratinocyte-derived chemokine (KC; CXCL1), regulated on activation, normal T cell expressed and secreted (RANTES; CCL5), and monocyte chemotactic protein 1 (MCP-1; CCL2) were quantified in BALF by multiplex assay (Bio-Rad).

Real-time Imaging of Endothelial Ca^{2+} Concentration ($[Ca^{2+}]_i$) in Intact Lungs

Lungs from WT or *Trpv4*$^{-/-}$ mice were isolated, ventilated, and continuously perfused with Hanks balanced salt solution containing 20% fetal bovine serum at a rate of 1 ml/min.[30] For imaging of endothelial $[Ca^{2+}]_i$, Fura-2AM (5 µM; Promokine, Germany), which deesterifies intracellularly to the Ca^{2+}-sensitive dye Fura-2, was infused for 20 min.[31] Lungs were positioned under an upright fluorescence microscope, and Fura-2 fluorescence in endothelial cells of subpleural

lung venular capillaries of 15 to 30 μm in diameter was excited at 340, 360, and 380 nm by monochromatic illumination (Polychrome IV; T.I.L.L. Photonics, Germany), collected through an approchromat objective (UAPO 40× W2/340; Olympus, Germany) and dichroic and emission filters (FT 425 and BP 505–530; Zeiss, Germany) by a CCD camera (Sensicam; PCO, Germany), and subjected to digital image analysis (TILLvisION 4.01; T.I.L.L. Photonics). Images were obtained at baseline with lung inflation by constant positive airway pressure (CPAP) of 5 cm H_2O and subsequently more than 15 min after an acute CPAP increase to 15 cm H_2O. Endothelial $[Ca^{2+}]_i$ responses were determined as changes in the 340/380 ratio and are expressed relative to the individual baseline.

Proximity Ligation Assay

Primary human pulmonary microvascular endothelial cells (HPMVECs; PromoCell, Germany) were seeded at 8 × 105 cells per well onto ProNectin-coated BioFlex plates (Flexcell International, USA), mounted onto a FX-4000T FLEXCELL Tension Plus system (Flexcell International) and exposed to cyclic stretch of either 5% at 2.5 Hz (mimicking LV_T ventilation) or 18% elongation at 1.0 Hz (mimicking HV_T) for 2 h as described.[32] After 2 h of cyclic stretch cells were fixed and phosphorylation of TRPV4 at Ser824 was assessed by proximity ligation assay using Duolink (Sigma-Aldrich) according to manufacturer's instructions. In brief, cells were incubated with goat anti-TRPV4 (1:50; Santa Cruz Biotechnology Inc., Germany) and rabbit anti-pSer824 (1:100; Cell Signaling Technology, USA) as primary antibodies. After enzymatic ligation and rolling circle amplification of short DNA strands attached to the species-specific secondary antibodies, proximity between the two binding sites for the primary antibodies of less than 40 nm becomes detectable by confocal microscopy (Nikon A1Rsi+; Nikon GmbH, Germany) as red puncta of 5 to 100 pixels in size, which mark individual sites of TRPV4 Ser824 phosphorylation. Number of TRPV4 Ser824 phosphorylation sites per cell was quantified by FIJI software (ImageJ, National Institutes of Health, USA).

Coimmunoprecipitation and Immunoblotting

For coimmunoprecipitation analyses, lung tissue was homogenized in lysis buffer (137 mM NaCl, 20 mM Tris, 2 mM EDTA, 1 mM PMSF, 10% glycerol, 1% Nonidet-40, and protease and phosphatase inhibitor cocktail), and protein concentration was determined by Bradford assay. Five hundred micrograms of total protein was precleared with 20-μl magnetic beads (Cell Signaling) incubated with a TRPV4 antibody[33] (1:5,000; kindly provided by Dr. S. Heller, Stanford University, USA), and antibody-bound protein was captured with 30-μl magnetic beads at 4°C. Captured proteins were separated in a sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. Membranes were blocked and incubated with anti-TRPV4,[33] anti-pSer824 (phosphomotif RXRXXS*/T*; Cell Signaling Technology), anti-SGK (Santa Cruz Biotechnology Inc.), and horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology Inc.). Immunoblots were visualized by enhanced chemoluminescence (GE Healthcare, Germany), and band intensities were quantified by LI-COR Image Studio (LI-COR Biotechnology, Germany).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Prism 6.0; GraphPad Software Inc., USA). All data are presented as means ± SDs. Different treatment groups were compared by Mann–Whitney U test, one-way analysis of variance followed by Dunnett *post hoc* test, or two-way analysis of variance followed by Šidák *post hoc* test as appropriate. Correlation was tested by calculation of the Pearson product–moment coefficient, and linear regression analysis was performed. Statistical significance was assumed at $^*P < 0.05$.

Results

TRPV4 Deficiency or Inhibition Prevents Experimental VILI In Vivo

To address the functional role of TRPV4 *in vivo*, we first probed for the effects of genetically encoded TRPV4 deficiency or pharmacologic inhibition of TRPV4 channel activity in mice during mechanical ventilation with injurious tidal volumes of 20 ml/kg bw. As compared to animals ventilated with LV_T or unventilated controls, animals subjected to HV_T ventilation showed increased lung wet/dry weight ratio (fig. 1A), indicating the formation of lung edema. Impairment of alveolocapillary barrier function was further evidenced by a marked increase in total protein concentration in the BALF (fig. 1B) and the extravasation of high molecular weight HSA from plasma into the alveolar compartment (fig. 1C). TRPV4 inhibition by the specific antagonist HC-067047 prevented the HV_T-induced increases in lung wet/dry weight ratio (5.89 ± 0.55 *vs.* 5.12 ± 0.19 with HC-067047) and reduced BALF protein concentration (from 0.84 ± 0.18 to 0.46 ± 0.16 mg/ml) and HSA extravasation (from 29.00 ± 14.10 to 7.98 ± 3.85 U), in line with endothelial barrier stabilization. A similar protective effect was evident in *Trpv4*$^{-/-}$ mice.

Evaluation of lung parenchymal damage from histologic sections revealed thickening of alveolar septa and infiltration of inflammatory cells in HV_T lungs, resulting in a marked increase in the histologic lung injury score as compared to LV_T lungs or unventilated controls (fig. 1, D and E). Analogous to the protective effects on vascular hyperpermeability, HC-067047-treated and TRPV4-deficient HV_T groups showed less histologic evidence of lung injury.

To probe for the role of TRPV4 in the release of inflammatory and modulatory cytokines, we assessed BALF for levels of interleukin-1β and interleukin-10 and the chemokines MCP-1, RANTES, MIP-2, and KC, which mediate

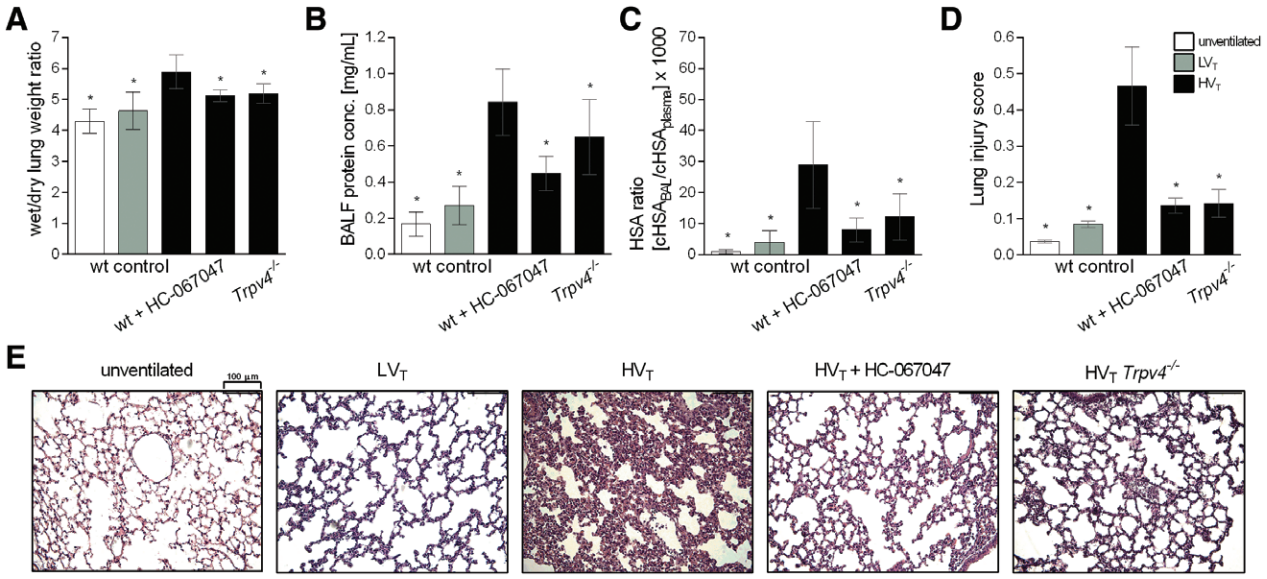

Fig. 1. Pharmacologic transient receptor potential vanilloid 4 (TRPV4) inhibition or genetic deficiency attenuate formation of lung edema, protein extravasation, and histologic characteristics of lung injury in a 2-h murine model of ventilator-induced lung injury. (*A*) Wet/dry lung weight ratio (n = 5/8/11/12/11 for unventilated wild-type [wt] control mice, mice ventilated with either low tidal volume [LV_T] of 7 ml/kg body weight or high tidal volume [HV_T] of 20 ml/kg body weight, HV_T ventilated mice treated with the TRPV4 antagonist HC-067047 [20 nM], or HV_T ventilated *Trpv4*$^{-/-}$ mice), (*B*) protein concentration in bronchoalveolar lavage fluid (BALF) (n = 5/8/8/9/9), (*C*) human serum albumin (HSA) BALF/plasma concentration ratio as measure of protein hyperpermeability (n = 5/8/8/9/9), and (*D*) semiquantitative analysis of histologic signs of lung injury (n = 5/8/8/8/8). (*E*) Representative histologic images show hematoxylin and eosin–stained lung sections, *scale bar*: 100 µm; *$P < 0.05$ *versus* HV_T wt control.

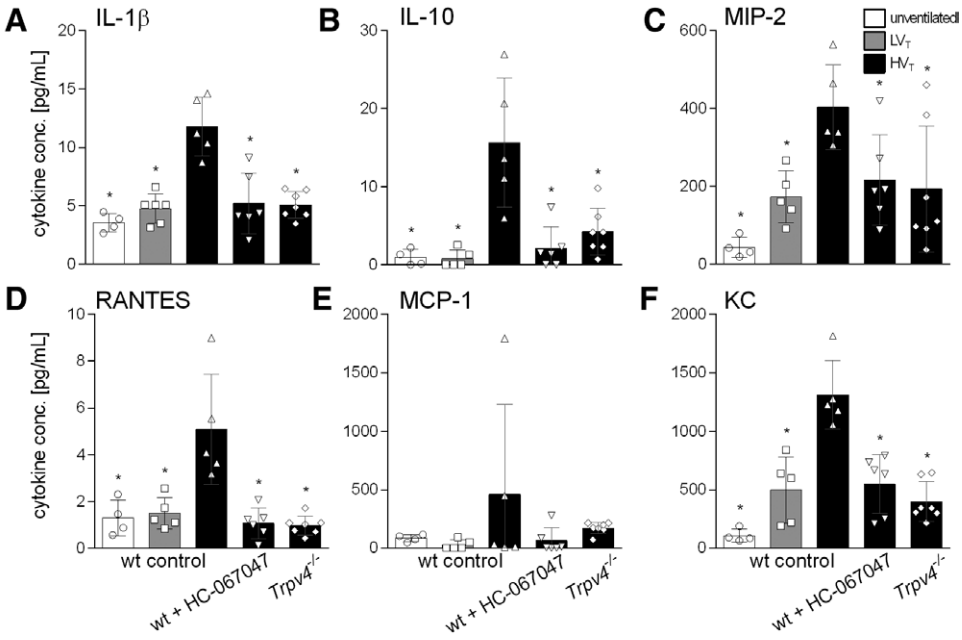

Fig. 2. Pharmacologic transient receptor potential vanilloid 4 (TRPV4) inhibition or genetic deficiency attenuate increased cytokine concentrations in bronchoalveolar lavage fluid (BALF) in a 2-h murine model of ventilator-induced lung injury. (*A*) interleukin-1β (IL-1β), (*B*) IL-10, (*C*) macrophage inflammatory protein 2 (MIP-2), (*D*) regulated on activation, normal T cell expressed and secreted (RANTES), (*E*) monocyte chemotactic protein-1 (MCP-1), and (*F*) keratinocyte-derived chemokine (KC) were determined in BALF of unventilated wild-type (wt) control mice (n = 4), mice ventilated with either low tidal volume (LV_T) of 7 ml/kg body weight (n = 5) or high tidal volume (HV_T) of 20 ml/kg body weight (n = 5), HV_T ventilated mice treated with the TRPV4 antagonist HC-067047 (20 nM; n = 6), or HV_T ventilated *Trpv4*$^{-/-}$ mice (n = 7); *$P < 0.05$ *versus* HV_T wt control.

leukocyte recruitment to the inflammatory site (fig. 2, A–F). With the exception of MCP-1, which showed a high variability between experiments in the HV_T group, all tested cytokines were found to be significantly increased in HV_T as compared to LV_T lungs or lungs from unventilated mice. TRPV4 inhibition or deficiency again attenuated this response, as exemplified by a reduction in MIP-2 levels from 544 ± 358 pmol/ml in WT mice to 193 ± 163 pmol/ml in *Trpv4*$^{-/-}$ mice. Taken together, these data identify TRPV4 as a critical mediator of the characteristic hallmarks of VILI, namely, vascular hyperpermeability and edema formation, inflammatory cell infiltration, and the release of inflammatory cytokines.

SGK1 Inhibition Prevents Experimental VILI In Vivo

Recently, SGK1 has been identified as novel regulator of TRPV4.[14] Like TRPV4, SGK1 is involved in osmoregulation and cell volume homeostasis,[25,26,34] suggesting that SGK1 is also regulated by mechanical cues. We therefore hypothesized that SGK1 may similarly be involved in VILI and tested the effects of the pharmacologic SGK1 antagonist GSK650394 in our murine model. Analogous to TRPV4 antagonization, inhibition of SGK1 attenuated the formation of lung edema and pulmonary vascular hyperpermeability in HV_T lungs in that it reduced the wet/dry lung weight ratio (fig. 3A), the accumulation of protein in the BALF (fig. 3B), and the extravasation of HSA (fig. 3C) as compared to untreated HV_T mice. Similarly, SGK1 inhibition attenuated histologic signs of lung injury in representative hematoxylin-eosin sections and a semiquantitative score (fig. 3, D and E) and reduced cytokine levels of interleukin-1β, interleukin-10, MIP-2, RANTES, and KC in BALF (fig. 4, A–F). Inhibition of SGK1 thus phenocopied the effects of TRPV4 antagonization in experimental VILI, indicating a potential functional link between both molecules in lung mechanotransduction. Consistent with this view, the relative mitigating effects of both TRPV4 and SGK1 inhibition on the individual readout parameters of lung injury in overventilated mice showed a strong linear correlation (fig. 5).

Both, TRPV4 and SGK1, Mediate the Endothelial $[Ca^{2+}]_i$ *Response to Ventilation-induced Mechanical Stretch*

In previous work, we and others showed that Ca^{2+} influx *via* TRPV4 is a critical regulator of lung endothelial permeability and inflammatory cell signaling.[6,19,35] We therefore aimed next to consolidate the notion that both TRPV4 and SGK1 are critical for mechanotransduction and the induction of endothelial $[Ca^{2+}]_i$ signaling in mechanically ventilated lungs. To this end, we applied real-time imaging in the isolated, buffer-perfused mouse lung to quantify the endothelial $[Ca^{2+}]_i$ response to an increase in CPAP from 5 to 15 cm H_2O (fig. 6A), which corresponds to the mean peak airway pressures recorded *in vivo* at baseline LV_T (5.4 ± 0.5 cm H_2O) or HV_T (14.8 ± 2.3 cm H_2O). In control lungs, acute CPAP elevation caused a rapid increase in endothelial $[Ca^{2+}]_i$, evident as rise in the 340/380 Fura-2

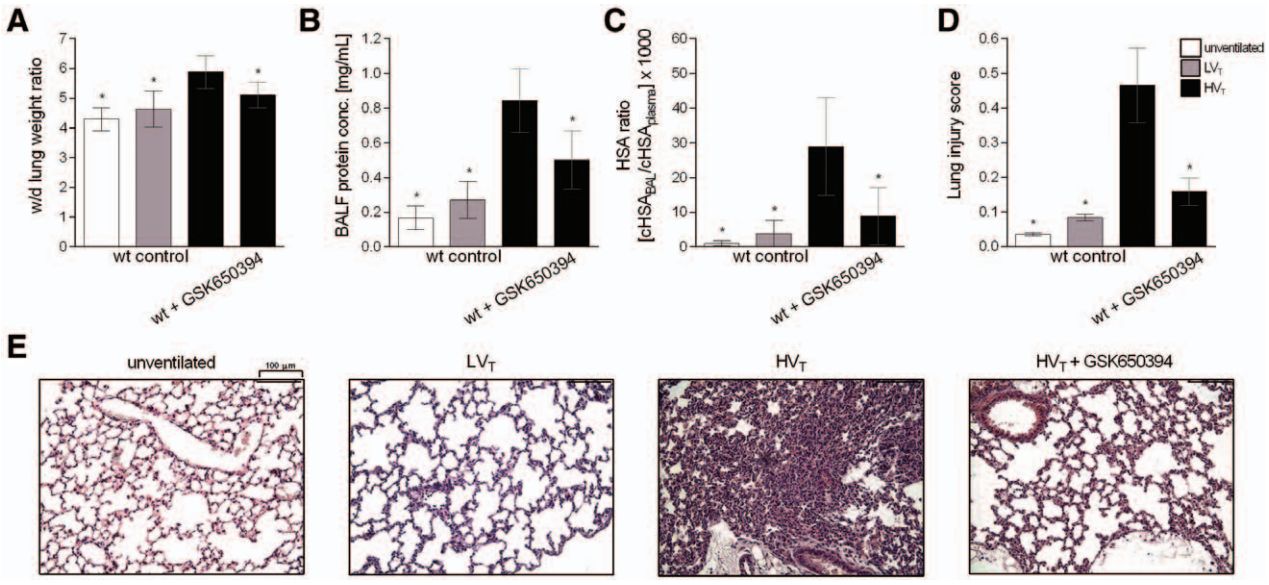

Fig. 3. Pharmacologic serum glucocorticoid–regulated kinase 1 (SGK1) inhibition attenuates formation of lung edema, protein extravasation, and histologic characteristics of lung injury in a 2-h murine model of ventilator-induced lung injury. (*A*) Wet/dry lung weight ratio (n = 5/8/11/12 for unventilated wild-type [wt] control mice, mice ventilated with either low tidal volume [LV_T] of 7 ml/kg body weight or high tidal volume [HV_T] of 20 ml/kg body weight, or HV_T ventilated mice treated with the SGK1 inhibitor GSK650394 [100 nM]), (*B*) protein concentration in bronchoalveolar lavage fluid (BALF; n = 5/8/8/8), (*C*) human serum albumin (HSA) BALF/plasma concentration ratio as measure of protein hyperpermeability (n = 5/8/8/8), and (*D*) semiquantitative analysis of histologic signs of lung injury (n = 5/8/8/8). (*E*) Representative histologic images show hematoxylin and eosin–stained lung sections, *scale bar*: 100 μm; *$P < 0.05$ *versus* HV_T wt control.

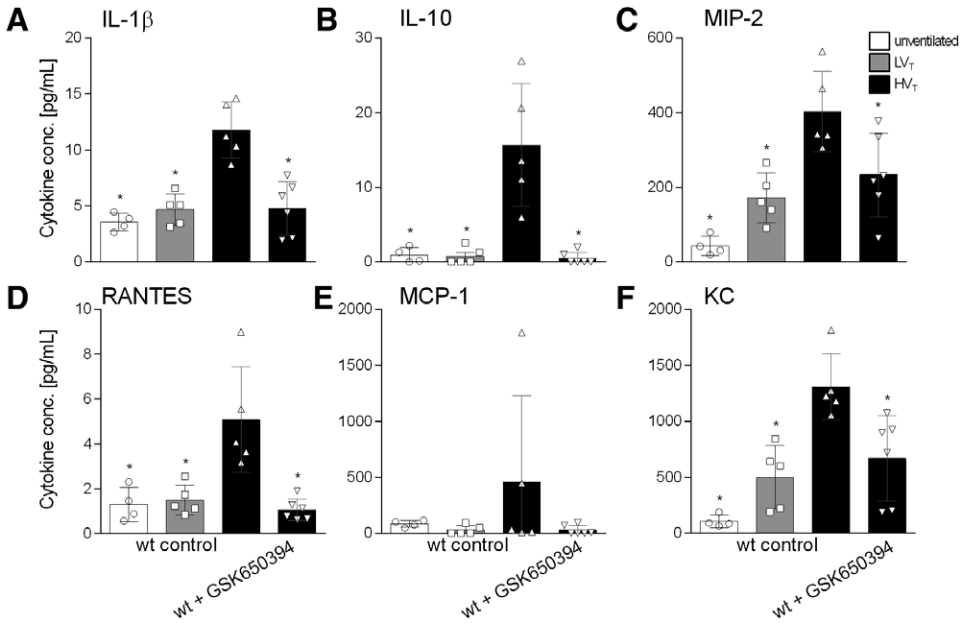

Fig. 4. Pharmacologic serum glucocorticoid–regulated kinase 1 (SGK1) inhibition attenuates increased cytokine concentrations in bronchoalveolar lavage fluid (BALF) in a 2-h murine model of ventilator-induced lung injury. (*A*) interleukin-1β (IL-1β), (*B*) IL-10, (*C*) macrophage inflammatory protein 2 (MIP-2), (*D*) regulated on activation, normal T cell expressed and secreted (RANTES), (*E*) monocyte chemotactic protein-1 (MCP-1), and (*F*) keratinocyte-derived chemokine (KC) were determined in BALF of unventilated wild-type (wt) control mice (n = 4), mice ventilated with either low tidal volume (LV_T) of 7 ml/kg body weight (n = 5) or high tidal volume (HV_T) of 20 ml/kg body weight (n = 5), or HV_T ventilated mice treated with the SGK1 inhibitor GSK650394 (100 nM; n = 6); *$P < 0.05$ *versus* HV_T wt control.

fluorescence ratio, that was sustained for more than 15 min (fig. 6B). In line with the well-documented role of TRPV4 as mechanoregulated Ca^{2+} channel, this $[Ca^{2+}]_i$ increase was attenuated in lungs where TRPV4 was either deficient or pharmacologically inhibited, respectively. A similar inhibition was also observed in lungs perfused with the SGK1 inhibitor GSK650394, suggesting a regulatory role of SGK1 in TRPV4-mediated Ca^{2+} entry (fig. 6, C and D).

SGK1 Phosphorylates TRPV4 at Ser824 in Stretched HPMVECs and in Overventilated Lungs

Recently, SGK1 has been found to phosphorylate TRPV4 at its serine residue 824, thereby enhancing channel activity and thus TRPV4-mediated Ca^{2+} influx.[14] To corroborate the notion that SGK1 may act upstream of TRPV4 in VILI, we next tested whether SGK1 may regulate TRPV4 by Ser824 phosphorylation under supraphysiologic stretch *in vitro*. To this end, HPMVECs were subjected to either 5 or 18% stretch, and TRPV4 phosphorylation at Ser824 was quantified by PLA technology. In line with an SGK1-mediated activation of TRPV4, application of 18% biaxial stretch increased TRPV4 phosphorylation at Ser824, yet this effect was attenuated by approximately 60% by the SGK1 inhibitor GSK650394 (fig. 7, A and B). Coimmunoprecipitation analyses from lung lysate indicate a similar mechanism of TRPV4 activation in overventilated lungs *in vivo*, in as much as HV_T increased TRPV4 Ser824 phosphorylation as compared to unventilated or LV_T lungs, and this effect was again reduced by approximately 50% by SGK1 inhibition (fig. 7C). In line with a mechanosensitive signaling axis *via* SGK1 and TRPV4, coimmunoprecipitations further revealed an increased SGK1-TRPV4 interaction in HV_T as compared to LV_T lungs (fig. 7D).

Discussion

Here, we report a new signaling axis *via* SGK1 and TRPV4 that mediates critical hallmarks of VILI. Both pharmacologic inhibition and genetic deficiency of TRPV4 attenuated endothelial hyperpermeability, lung edema formation, and inflammatory cytokine release in overventilated mice and normalized at large the histologic appearance of the lung microstructure. Pharmacologic inhibition of SGK1 replicated these effects, indicating that TRPV4 and SGK1 may act through the same signaling pathway. Indeed, both TRPV4 and SGK1 have been implicated in cellular mechanotransduction,[8,25,27,36] and SGK1 can activate TRPV4 *via* phosphorylation of its Ser824 residue.[14] Consequentially, SGK1 inhibition blocked the TRPV4-inhibitor–sensitive endothelial $[Ca^{2+}]_i$ response to an acute increase in inflation pressure in isolated-perfused mouse lungs, and attenuated TRPV4 phosphorylation at Ser824 in endothelial cells exposed to 18% cyclic stretch *in vitro*, and in lungs of overventilated mice *in vivo*. These findings indicate a new signaling axis in overventilated lungs in that SGK1 activates TRPV4 *via* phosphorylation of its Ser824 residue and thus identify TRPV4 and SGK1 as putative targets for the prevention of VILI (fig. 8).

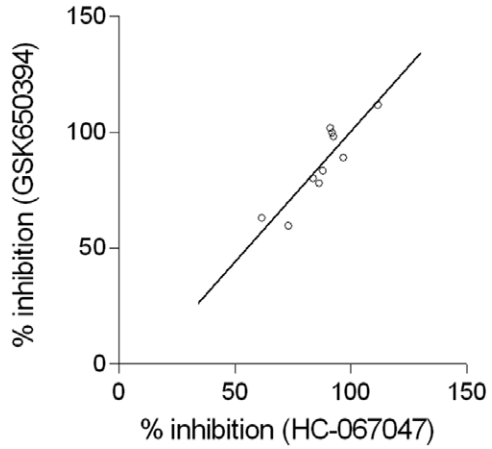

Fig. 5. Correlation between the mitigating effects of transient receptor potential vanilloid 4 (TRPV4) *versus* serum glucocorticoid–regulated kinase 1 (SGK1) inhibition on characteristics of ventilator-induced lung injury. Scatter plot and linear regression analysis show the relative inhibition induced by the TRPV4 inhibitor HC-067047 *versus* the relative inhibition of the same parameter by the SGK1 inhibitor GSK650394. Relative inhibition was calculated as (high tidal volume [HV_T] inhibitor – HV_T control)/(low tidal volume [LV_T] control – HV_T control) [in %] for mean values of the wet/dry lung weight ratio, protein concentration in bronchoalveolar lavage fluid (BALF), human serum albumin BALF/plasma concentration ratio, histologic lung injury score, and BALF concentrations of IL-1β, IL-10, macrophage inflammatory protein 2 (MIP-2), regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), and keratinocyte-derived chemokine (KC). Each individual parameter is represented by one data point. $R^2 = 0.803$; $P = 0.0004$.

TRPV4 is a widely expressed cation channel that has recently become implicated in numerous physiologic and pathophysiologic processes including nociception,[37–39] regulation of vascular tone,[40,41] or thermoregulation.[42] In the lung, TRPV4 is expressed in various cell types with direct or indirect involvement in VILI, including microvascular endothelial cells, alveolar and bronchial epithelial cells, vascular smooth muscle cells, alveolar macrophages, and neutrophils.[43] Of specific interest for the current study was the role of TRPV4 in lung microvascular endothelial cells, where it had been shown to mediate endothelial barrier failure and lung edema formation upon pharmacologic activation.[7,44,45] Studies by us[6] and others[35,46] revealed that lung endothelial TRPV4 is rapidly activated when lung microvessels undergo circumferential mechanical stretch in response to increased vascular pressures, resulting in a Ca^{2+}-dependent increase in endothelial permeability and formation of hydrostatic lung edema. Based on its role in endothelial mechanotransduction,[16] we speculated for a similar relevance of TRPV4 in pathologies when endothelial cells undergo longitudinal rather than circumferential stretch, as is the case in mechanically (over)ventilated lungs.[47] In a seminal study in isolated lungs, Hamanaka *et al.*[5] demonstrated that vascular hyperpermeability in VILI is associated with Ca^{2+} entry *via* TRPV4. However, the role of TRPV4 in overventilated lungs *in vivo* has so far not been addressed. Using both a genomic and a pharmacologic approach to minimize off target effects, we demonstrate here a critical role for TRPV4 in a murine model of overventilation-induced lung vascular hyperpermeability and edema formation.

Of relevance, TRPV4 inhibition or deficiency not only prevented vascular hyperpermeability in overventilated lungs, but also attenuated the release of proinflammatory cytokines and histologic signs of lung injury and inflammatory cell infiltration. This is consistent with the recent work by us and others demonstrating that TRPV4 antagonization not only prevents endothelial leak, but also attenuates inflammation in experimental models of acute lung injury induced by intratracheal hydrochloric acid instillation or chlorine gas inhalation.[4,19] Although TRPV4 is expressed in circulating or resident immune cells such as macrophages[48] or neutrophils,[19] experiments in bone marrow chimeric mice revealed that the proinflammatory role of TRPV4 in experimental lung injury was largely attributable to its role in lung parenchymal cells.[19]

We therefore focused our subsequent studies on the lung microvascular endothelium and show here that (1) mechanical stretch results in TRPV4 phosphorylation in HPMVECs *in vitro* and (2) increased lung inflation causes an increase in endothelial $[Ca^{2+}]_i$ that can be blocked by deficiency or inhibition of TRPV4. These findings underline the role and regulation of TRPV4 in the endothelial $[Ca^{2+}]_i$ response to mechanical stretch; however, they do notably not preclude contributions of TRPV4-dependent mechanisms from other cell types. Of particular relevance for VILI, a TRPV4-dependent contribution of alveolar macrophages has been demonstrated in that intratracheal instillation of *Trpv4*$^{+/+}$ macrophages into the lungs of *Trpv4*$^{-/-}$ mice restored the characteristic increase in lung filtration coefficient in response to high positive inspiratory pressure that is seen in lungs of *Trpv4*$^{+/+}$ mice, but absent in lungs of *Trpv4*$^{-/-}$.[48] Macrophages not only respond to TPRV4 activation by increased production of reactive oxygen and nitrogen species, spreading and lamellipodia formation,[48] but also express SGK1, which again regulates macrophage migration and recruitment.[49] It is thus tempting to speculate that the actual mechanism of TRPV4 activation in macrophages in VILI may thus, similar to endothelial cells, again involve SGK1; yet, this notion still awaits experimental testing.

Despite the well-documented role of TRPV4 in mechanotransduction, there is considerable controversy whether TRPV4 functions as direct mechanosensor,[15] or alternatively as an essential signaling hub downstream of an initial mechanosensing machinery.[50] Recently, SGK1 was identified as a novel regulator of TRPV4, facilitating its transition from an inactive to an active form.[14] Here, we show for the first time a functional role for SGK1 in pulmonary endothelial cells in that inhibition of SGK1 could effectively reduce the endothelial $[Ca^{2+}]_i$ response to lung

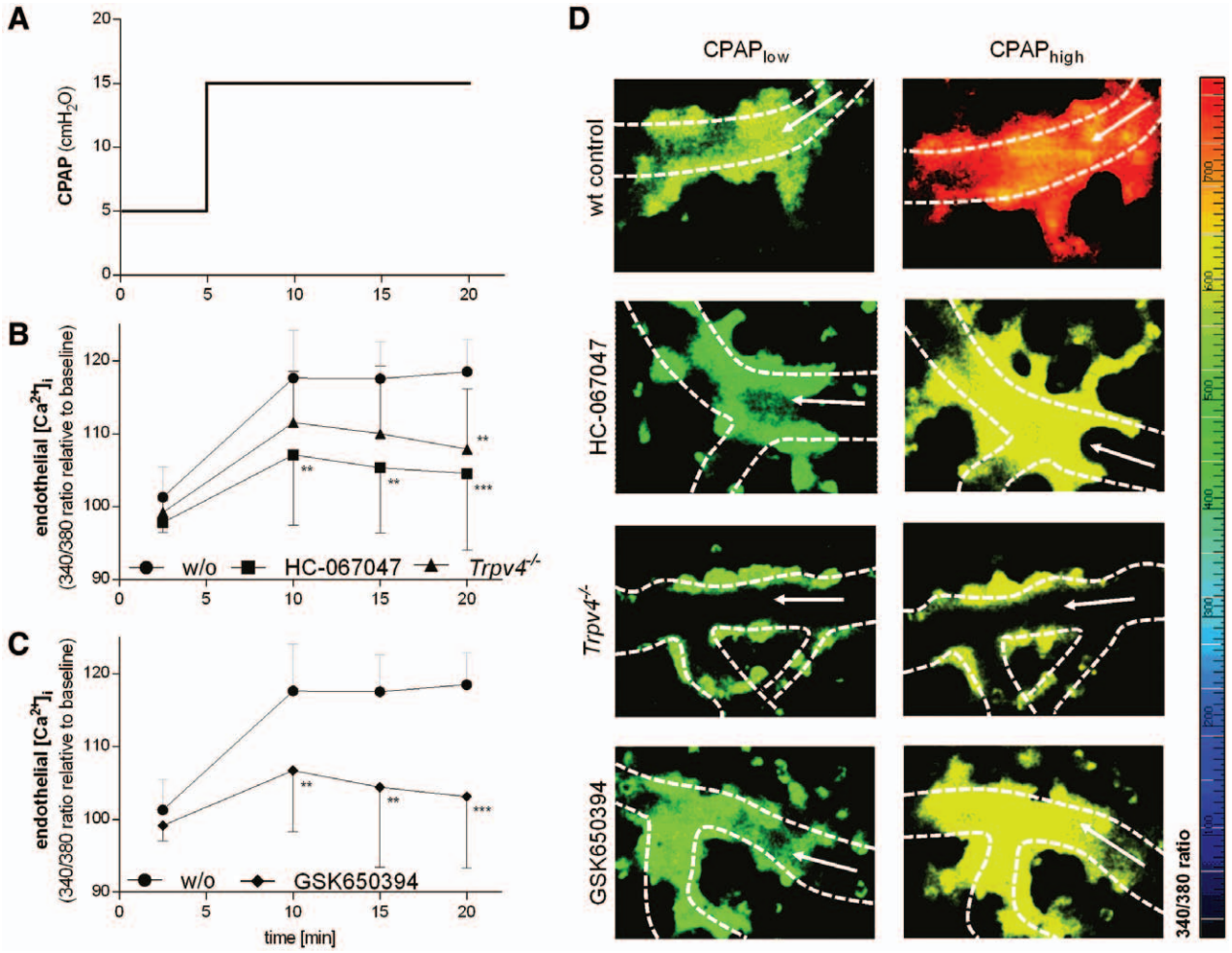

Fig. 6. Transient receptor potential vanilloid 4 (TRPV4) and serum glucocorticoid–regulated kinase 1 (SGK1) mediate endothelial Ca^{2+} influx in response to ventilation-induced mechanical stretch in isolated perfused mouse lungs. (*A*) Experimental protocol. Line and scatter plots show endothelial Ca^{2+} concentration ($[Ca^{2+}]_i$) (as 340/380 ratio relative to the individual baseline) at low (5 cm H_2O) and high (15 cm H_2O) continuous positive airway pressure (CPAP) in the absence (w/o) or presence of (*B*) the TRPV4 inhibitor HC-067047 (20 nM), or in *Trpv4*$^{-/-}$ lungs, or (*C*) in the presence of the SGK1 inhibitor GSK650394 (100 nM); (*D*) representative images of endothelial $[Ca^{2+}]_i$ (color coded for 340/380 ratio) in microvessels of the isolated perfused mouse lung; *white arrows* indicate the direction of blood flow, *dotted lines* the vessel margins; n = 9/7/7/7 for w/o, HC-067047, *Trpv4*$^{-/-}$, and GSK650394; **P* < 0.05 *versus* w/o at the identical timepoints.

overinflation. The analogy of this finding to the effects of TRPV4 inhibition or deficiency is consistent with the common involvement of both proteins in cellular mechanotransduction pathways underlying cell volume regulation.[13] In murine VILI, SGK1 inhibition recapitulated the protective effects of TRPV4 inhibition both qualitatively and quantitatively, suggesting a coinvolvement of SGK1 and TRPV4 in lung mechanotransduction in response to overventilation. The open probability of TRPV4 is regulated by phosphorylation of its C-terminal cytoplasmic domain by various kinases including protein kinase A and protein kinase C.[22,23,51–53] When the channel is closed, N and C terminus stick together due to their electrical charge. Upon phosphorylation and binding of calcium-calmodulin (CaM) to the calmodulin-binding domain (CaMBD), the C terminus gets detached due to the resulting change in electrical charge, and the channel opens. SGK1 phosphorylates TRPV4 at Ser824 within the CaMBD, thereby facilitating the binding of CaM to CaMBD and the subsequent opening and activation of TRPV4.[23,54] In keeping with a similar regulation of TRPV4 by SGK1 in VILI, we detected protein–protein interaction between SGK1 and TRPV4 in intact lungs, which was enhanced by overventilation. Furthermore, biaxial cyclic stretch of HPMVECs by 18% elongation mimicking mechanical overventilation[32] increased phosphorylation of the Ser824 residue of TRPV4, the classic target of SGK1,[14] which in turn was attenuated by approximately 60% by the SGK1 inhibitor GSK650394. A comparable extent of inhibition of TRPV4 phosphorylation at Ser824 by GSK650394 was detectable by coimmunoprecipitation in overventilated lungs. Taken together, these findings establish a novel signaling

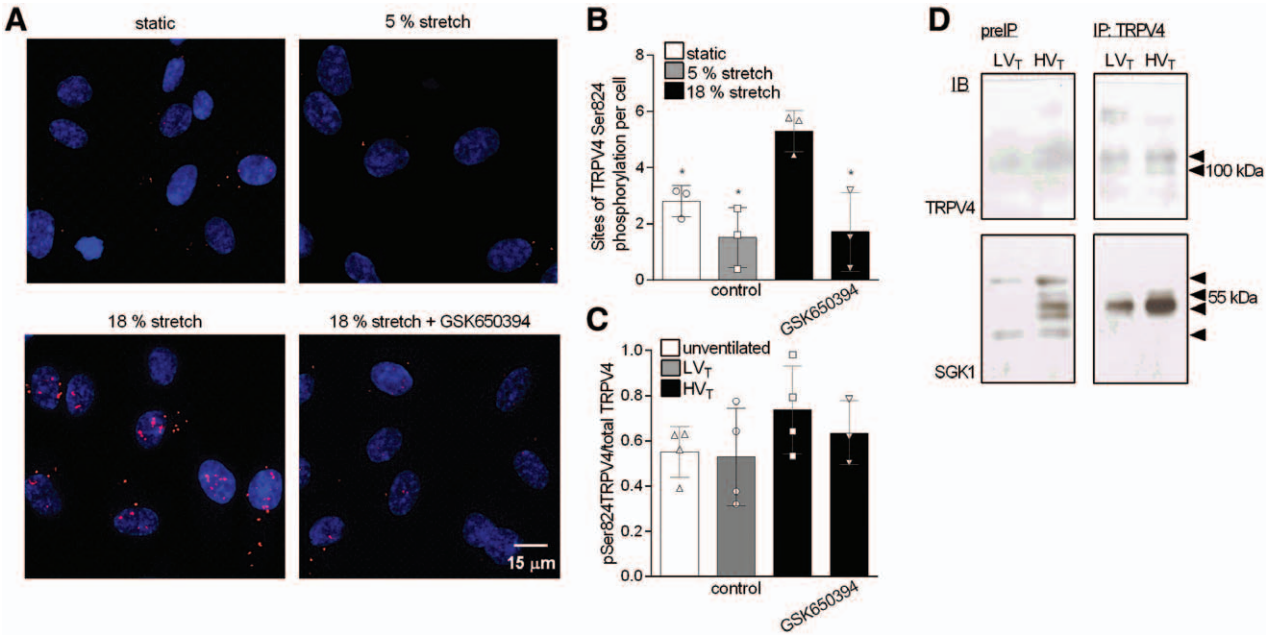

Fig. 7. Stretch-induced phosphorylation of transient receptor potential vanilloid 4 (TRPV4) at serine 824 (Ser824) is mediated by serum glucocorticoid–regulated kinase 1 (SGK1). Representative confocal microscopic images (*A*) and quantitative bar graph (*B*) show sites of TRPV4 Ser824 phosphorylation, visualized by proximity ligation assay as red puncta, in human pulmonary microvascular endothelial cells exposed to static conditions or cyclic stretch of either 5 or 18% elongation in the absence or presence of the SGK1 inhibitor GSK650394 (100 nM) for 2 h. DNA was counterstained with 4′,6-diamidino-2-phenylindole (*blue*). *Scale bar* represents 15 μm; n = 3 independent replicates with five image sections averaged per slide, **P* < 0.05 *versus* 18% stretch control. (*C*) Wild-type mice were left unventilated or subjected to either low tidal volume (LV_T) of 7 ml/kg body weight or high tidal volume (HV_T) of 20 ml/kg body weight for 2 h in the absence or presence of the SGK1 inhibitor GSK650394 (100 nM). Whole-lung lysates were collected, immunoprecipitated for TRPV4, and immunoblotted for total TRPV4 and the phosphomotif pSer824. Densitometric quantification shows the expression of the pSer824 motif relative to total TRPV4; n = 3/4/4/3 for unventilated control, LV_T control, HV_T control, and HV_T + GSK650394. (*D*) Wild-type mice were subjected to either LV_T or HV_T, and whole-lung lysates were collected. Immunoprecipitation was performed using an anti-TRPV4 antibody. Whole-lung lysates were collected, immunoprecipitated for TRPV4, and immunoblotted (IB) for TRPV4 or SGK1 with 500-μg total protein for immunoprecipitates (IP), or 30 μg for preimmunoprecipitates (pre-IP), respectively. Replicated in n = 3 experiments each.

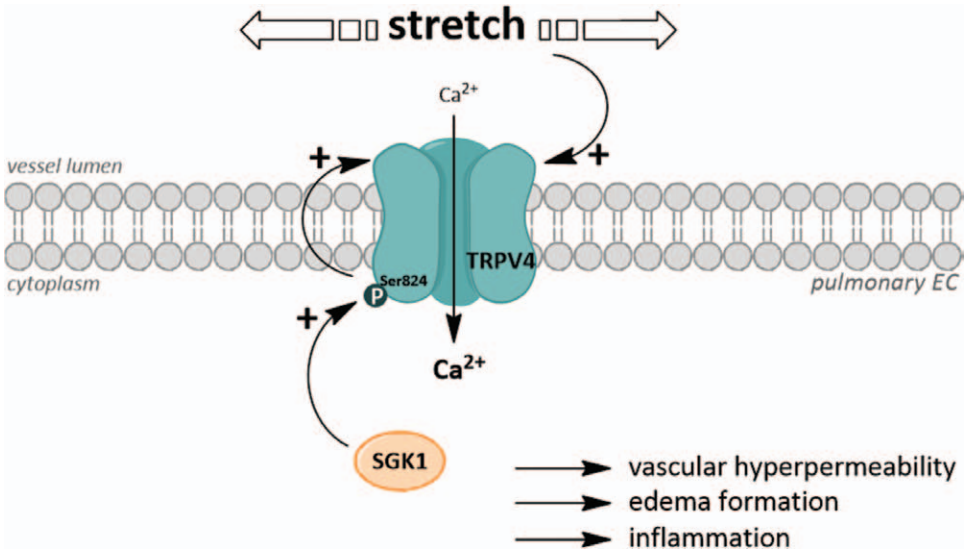

Fig. 8. Proposed signaling pathway for the effects of mechanical overventilation in lung endothelial cells. Mechanical stretch activates serum glucocorticoid–regulated kinase 1 (SGK1), which binds to and activates transient receptor potential vanilloid 4 (TRPV4), at least in part *via* phosphorylation of its serine 824 (Ser824). The resulting conformational change of the TRPV4 channel allows for Ca^{2+} influx into the endothelium, which in turn triggers vascular hyperpermeability and edema formation and exacerbates inflammation, thus contributing to the characteristic hallmarks of ventilator-induced lung injury.

axis in overventilated lungs in that SGK1 activates TRPV4-mediated endothelial Ca^{2+} influx and subsequent vascular barrier failure and proinflammatory signaling *via* SGK1-dependent Ser824 phosphorylation of TRPV4.

The Acute Respiratory Distress Syndrome Network trial has revolutionized our understanding of the detrimental effects of mechanical ventilation and resulted in a universal change of medical practice with the introduction of protective lung ventilation.[55] VILI, however, remains an imminent problem in critical care. Although adherence to the current guidelines will preclude ventilation at tidal volumes of 20 ml/kg bw, our findings from the current murine model of overventilation bear important clinical and translational potential. Even though global tidal volumes in ventilated patients rarely surpass 10 ml/kg bw in 2016, regional tidal volumes will frequently exceed values of 20 ml/kg bw and accordingly cause significant lung overdistension in ventilated patients with heterogeneous lung diseases such as ARDS.[56] As injured areas with alveolar collapse or fluid extravasation will only receive a small fraction of the total tidal volume, the majority of tidal volume will be delivered to noninjured areas with relatively normal compliance. Since only the latter present the actual ventilated lung tissue, the lungs of ARDS patients are in fact "functionally small," a notion described as "baby lung."[57] Recently, this concept was elegantly confirmed in 42 ARDS patients in that the volume increase during a recruitment maneuver was significantly lower than the corresponding predicted inspiratory capacity.[58] The measured volume increase was in fact almost exactly one third of the predicted inspiratory capacity, indicating that two thirds of the total lung volume did not participate in mechanical ventilation. Accordingly, the most commonly applied ventilation strategy of 7 ml/kg bw[59] will in fact result in an effective tidal volume in the ventilated lung areas of these ARDS patients of approximately 20 ml/kg, identical to the overventilation strategy applied in the current study.

In principle, all patients receiving ventilator support may potentially benefit from strategies that minimize VILI.[60] As a result, a series of approaches have aimed to alleviate the biomechanical stresses posed by mechanical ventilation on the lung, including lower tidal volumes, high positive end-expiratory pressure and recruitment maneuvers, high-frequency oscillatory ventilation, prone positioning, neuromuscular blocking agents, or neurally adjusted ventilatory assist.[60–62] Apart from these biomechanical strategies, pharmacologic interventions, *e.g.*, statins have shown promise in attenuating VILI in preclinical animal models,[28,63] however, failed to show a detectable benefit in clinical ARDS trials.[64,65] Other therapeutic strategies, such as the tyrosine kinase inhibitor imatinib, effectively attenuate lipopolysaccharide-induced lung injury, yet surprisingly exacerbated VILI in preclinical studies.[66] Hence, VILI continues to pose a large unmet clinical need in critical care medicine. The emerging recognition of TRPV4 as a promising drug target in respiratory diseases[67] and the impending initiation of the first clinical trials to reduce lung edema by TRPV4 channel blockers (NCT02497937) may provide for an intriguing new strategy to prevent or attenuate VILI in patients prone to experience high levels of biomechanical stress during mechanical ventilation. In this context, our study identifies TRPV4 as a promising pharmacologic therapeutic target in VILI and reveals SGK1 as important regulating kinase in the mechanical activation of TRPV4 and the ensuing regulation of endothelial barrier function and inflammatory signaling in overventilated lungs.

Acknowledgments

The authors thank the Advanced Medical Bioimaging Core Facility (AMBIO) of Charité—Universitätsmedizin Berlin (Berlin, Germany) for the support in acquisition of the imaging data.

Research Support

Supported in part by grant No. KU 1218/4-2 from the Deutsche Forschungsgemeinschaft (Berlin, Germany).

Competing Interests

The authors declare no competing interests.

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