The Role of MMP2 and MMP9 in TRPV4-Induced Lung Injury

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Author Contributions: PCV contributed to experimental design, completed all of the work described in the manuscript including data analysis, prepared manuscript; PR and MIT contributed to experimental design, assisted with data interpretation, revised manuscript.

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Ca\(^{2+}\) entry through TRPV4 (transient receptor potential vanilloid 4) results in swelling, blebbing and detachment of the epithelium and capillary endothelium in the intact lung. Subsequently, increased permeability of the septal barrier and alveolar flooding ensue. In this study, we tested the hypothesis that TRPV4 activation provides a Ca\(^{2+}\) source necessary for proteolytic disruption of cell-cell or cell-matrix adhesion by matrix metalloproteinases (MMPs) MMP2 and MMP9, thus increasing septal barrier permeability. In our study, C57BL/6 or TRPV4\(^{-/-}\) mouse lungs were perfused with varying doses of the TRPV4 agonist GSK1016790A (Sigma) and then prepared for Western Blot. Lung injury, assessed by increases in lung wet-to-dry weight ratios and total protein levels in the bronchoalveolar lavage fluid, was increased in a dose-dependent fashion in TRPV4\(^{+/+}\) but not TRPV4\(^{-/-}\) lungs. In concert with lung injury, we detected increased active MMP2 and MMP9 isoforms, suggesting that TRPV4 can provide the Ca\(^{2+}\) source necessary for increased MMP2/9 activation. Further, tissue inhibitor of metalloproteinases (TIMP) 2 levels in the TRPV4-injured lungs were decreased, suggesting that TRPV4 activation increases the availability of these active MMPs. We then determined whether MMP2 and MMP9 mediate TRPV4-induced lung injury. Pharmacological blockade (SB-3CT, 1\(\mu\)M, Sigma) of MMP2 and MMP9 resulted in protection against TRPV4-induced lung injury. We conclude that TRPV4 activation and the subsequent Ca\(^{2+}\) transient initiates a rapid cascade of events leading to release and activation of the gelatinase MMPs, which then contribute to lung injury.

Keywords: TRPV4, MMP2, MMP9, lung permeability, lung injury
INTRODUCTION

Activation of TRPV4, whether via pharmacological tools, mechanical ventilation or pulmonary venous hypertension (18, 22), increases lung endothelial permeability in a Ca$^{2+}$ entry-dependent fashion. We have previously shown that pharmacologic activation of TRPV4 promotes disruption of the alveolar septal barrier, characterized by cell swelling, blebbing, and detachment of the alveolar septal epithelium and capillary endothelium in the intact lung. As a result, permeability of the septal barrier is increased and alveolar flooding results (2). These structural alterations are similar to those seen in the alveolar septal wall of humans with acute respiratory distress syndrome (ARDS) (53), but distinct from inter-endothelial gap formation observed in extra-alveolar vessels following Ca$^{2+}$ entry evoked by store-depletion (6, 24, 31, 32). Notably, the downstream effectors through which TRPV4 activation increases lung permeability have not yet been identified. Understanding these processes may then provide insight into the development of ARDS.

We propose that TRPV4 activation may provide a Ca$^{2+}$ source necessary for increased MMP2/9 activity in the lung based on several lines of evidence. Mechanical stress, which activates TRPV4 in alveolar macrophages and lung endothelial cells (17, 45), can induce MMP2 and MMP9 release from these cells (19, 36). Active MMP2 and MMP9 degrade components of the alveolar basement membrane (10, 52), non-matrix components such as integrins (16, 47), and intercellular targets such as E-cadherin (29, 43). Indeed, active MMP2 and MMP9 alter barrier integrity in cultured lung epithelial and microvascular endothelial cells (38, 49). Furthermore, increased MMP2 and MMP9 in bronchoalveolar lavage fluid (BALF) have been noted in patients with ARDS (8, 28, 46). In several experimental models, MMP2 and MMP9 have been implicated in the development of lung injury (7, 11, 14), while their inhibition attenuated injury
(5, 14, 42, 54). Of relevance, a broad-spectrum MMP inhibitor attenuated lung injury caused by high volume ventilation (14). While ventilator-induced lung injury (VILI) does require TRPV4 activation (18), the specific link between the TRPV4-mediated Ca\(^{2+}\) signal, MMP2/9 activation, and acute lung injury has not been documented. Thus, this study was designed to test the hypothesis that TRPV4 activation leads to increased MMP2 and/or 9 expression in the intact lung, which then mediate TRPV4-induced lung injury. However, MMP2 and MMP9 activities are influenced by local concentrations of their preferred endogenous inhibitors, TIMP2 and TIMP1, respectively (35, 57), and further TRPV4 activation may not only impact expression of the gelatinase MMPs. Thus, our study design incorporated assessment of expression patterns for these TIMPs, along with that of MMP8 and MMP14, which have been variously implicated in lung injury.

**MATERIALS AND METHODS**

**Animals**

Wild type C57BL/6 mice (also noted as TRPV4\(^{+/+}\)) were purchased from Charles River Laboratory. TRPV4\(^{-/-}\) mice (C57BL/6 background strain) were bred in our animal facility. All animal studies were completed under protocols approved by our Institutional Animal Care and Use Committee, conforming to the NIH Guide for the Care and Use of Laboratory Animals.

**In Situ Lung Injury Model**

Mice of either gender (29.0±7.2 g body weight, mean±SD) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), containing heparin (100 U), then intubated and ventilated (~90 breaths/min, positive end-expiratory pressure ~3 cmH\(_2\)O). The anterior chest wall was removed to expose the lungs. Cannulas were inserted into the pulmonary artery and left ventricle...
and secured, then lungs were perfused in situ at constant flow (2 mL/min) without recirculation using 4% bovine albumin in Earle’s buffer containing 2 mM Ca^{2+} (37 °C). In each case, lungs were perfused for 15 min prior to addition of either vehicle (DMSO) or the specific TRPV4 agonist GSK1016790A (3-3000 nM, Sigma) for 30 min. This dose response series allowed us to determine an effective agonist concentration required to elicit TRPV4-mediated lung injury using this in situ perfusion model. To confirm a definitive role for TRPV4, lungs in TRPV4^{-/-} mice were perfused with vehicle or selected doses of GSK1016790A. At the end of the experiment, lung tissue aliquots were harvested and weighed, before and after drying at 65 °C to measure the lung wet-to-dry weight ratio (W/D). Separate aliquots of lung tissue were snap-frozen in liquid nitrogen then stored (-80 °C) for later Western blotting (see below). To assess vascular albumin leak, one bronchus was clamped (allowing this lung to be harvested for W/D or Western blotting), and the contralateral lung lavaged twice with 0.3 mL buffered saline. The BALF was then stored (-80 °C) until analysis for total protein, using the BCA protein assay (Pierce™).

To determine whether MMP2 and/or MMP9 contribute to TRPV4-mediated acute lung injury, we used the specific MMP2/9 inhibitor SB-3CT (Sigma). In wild-type mice, after the initial 15 min flush of the lung vasculature, vehicle or 1 µM SB-3CT was added to the perfusate (Earle’s buffer with 4% albumin). After 30 min pretreatment, lungs were challenged with vehicle or 150 nM GSK1016790A (the EC_{50} for an increase in lung W/D) for an additional 30 min. Lung tissue and BALF were collected at that time. After the equilibration period, 150 nM GSK1016790A was added to the perfusate. After 30 min, lung tissue for W/D and BALF were collected.

**Western Blot Analysis**
On analysis day, tissues were thawed on ice, weighed, and then sonicated in Hunter’s lysis buffer containing protease and phosphatase inhibitors (10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mM PMSF, and 1 mg/mL sodium orthovanadate). Lysates were shaken at 4 °C for 1.5 hr prior to centifugation at 14000 rpm (4 °C) for 15 min. The supernatant was obtained for subsequent analysis. Total protein concentration in each sample was measured using the BCA Protein Assay Kit (Pierce™). Either 7.5% or 12% SDS-polyacrylamide gels were loaded with 40 µg total protein from each lysate supernatant to analyze MMP2/ MMP9 and TIMP1/ TIMP2 expression, respectively. Following transfer to nitrocellulose membranes (Amersham), blots were probed for proteins with use of the following antibodies: anti-mouse MMP2 (Abcam); anti-mouse MMP9 (Abcam); anti-mouse MMP8 (Abcam); anti-mouse MMP14 (Millipore); anti-mouse TIMP1 (R&D Systems); anti-mouse TIMP2 (Abcam); and anti-mouse β-actin (Sigma). HRP-conjugated secondary antibodies (anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG; Cell Signaling) were detected by chemiluminescence (Super Signal West Femto and West Pico Substrate Kits by Pierce™). Band intensity from Western blot analyses was assessed with Un-Scan-IT software, referenced to that of β-actin. In our hands, Western blotting for MMP2/ MMP9 in lung tissue yielded much more consistent outcomes than gelatin zymography. The latter does not necessarily yield a picture of net gelatinase activity in tissue due to artifacts induced by activation of zymogens during tissue extraction, apparent activity of partially refolded but uncleaved zymogens, and/or dissociation of inhibitor TIMPs (48).

Statistical Analyses

Data were expressed as mean ± SEM. One-way ANOVA was used to identify significance (p< 0.05). When significant, specific differences between groups were identified by Dunnett’s or Tukey’s multiple comparison post hoc tests, noted in figure legends where applicable. One-way
ANOVA assumes similar variances in groups being compared. Therefore, in data sets with marked heterogeneity in variance, a log transformation of the data was performed prior to statistical analysis with one-way ANOVA and Tukey’s multiple comparison test (1).

RESULTS

We developed an in situ perfused mouse lung model that allows discrete measurement of alveolar septal barrier permeability and edema by measuring the lung W/D and the total protein recovered in the BALF, respectively. TRPV4 activation with the specific agonist GSK1016790A significantly increased the BALF total protein and the lung W/D in a dose-dependent manner in wild type mouse lungs, with an EC₅₀ of ~250 and ~150 nM, respectively (Figure 1A and B). These responses were specifically due to Ca²⁺ entry via TRPV4, as neither the BALF total protein nor the lung W/D increased in lungs of TRPV4⁻/⁻ mice (Figure 1C).

To determine whether TRPV4 activation by GSK1016790A increased active MMP2 and MMP9 protein levels in the lung, we used Western blotting in lung tissue lysates to compare expression of these MMPs. Compared to vehicle controls, treatment with the doses of GSK1016790A ≥ 150 nM increased the protein expression of the active isoform of MMP2 (64 kD) in TRPV4⁺/⁺, but not TRPV4⁻/⁻ lungs (Figure 2). When all active MMP9 isoforms were assessed collectively, we identified a significant increase in expression at the 1000 nM dose of GSK1016790A in wild type, but not TRPV4⁻/⁻ lungs. In contrast to TRPV4-induced upregulation of active MMP2 and MMP9 expression, TRPV4 activation with GSK1016790A downregulated expression of active MMP14 but did not impact expression of active MMP8 (Figure 3). These data suggest that TRPV4-induced lung injury is associated with increased availability of active MMP2 and MMP9 in mouse lung.
We used the same mouse lung tissue lysates used to assess MMP2 and MMP9 expression to probe for the expression of TIMP2 (28 kD) and TIMP1 (21 kD), the major endogenous inhibitors of active MMP2 and MMP9 (Figure 4). TIMP2, but not TIMP1, protein levels were decreased in TRPV4+/+ lungs at GSK1016790A doses that induced injury. Importantly, no changes in TIMP2 expression were noted in lungs from TRPV4−/− mice. Given the increased levels of active MMP2 and MMP9 isoforms in wild type lungs, along with the decreased TIMP2 levels at the higher GSK1016790A doses, it is likely that TRPV4 activation creates an environment that favors proteolytic activity by these MMPs in lung tissue.

Once establishing that TRPV4-induced lung injury correlated with increased protein levels of active MMP2 and MMP9, as well as decreased expression of TIMP2, we sought to determine whether there was a cause-effect relationship between TRPV4-induced increases in MMP2/9 and TRPV4-induced lung injury. We found that pretreatment of TRPV4+/+ lungs with SB-3CT (1 µM), an inhibitor of both MMP2 and MMP9, significantly attenuated the TRPV4-induced increases in BALF total protein and lung W/D compared to outcomes in lungs treated with GSK1016790A (150 nM) alone (Figure 5). While the BALF total protein in the lungs treated with SB-3CT and GSK1016790A was no different than that in the vehicle control group, the lung W/D was increased. Note that in wild-type lungs, the EC50 for GSK1016790A-induced increases in BALF total protein was higher than that required for increases in lung W/D (Figures 1A and 1B). Thus, protection afforded by SB-3CT in lungs challenged with 150 nM GSK1016790A may be more evident in BALF than in W/D.

**DISCUSSION**

We have previously reported that activation of the mechanosensitive cation channel
TRPV4 increases lung permeability in settings of high vascular or airway pressure (17, 18, 22).

In addition, we have found that direct activation of TRPV4 with 4αPDD or 14,15-EET induces lung injury similar to that seen in ARDS, with structural defects in the alveolar septal barrier and alveolar flooding (2). The current study, utilizing the specific TRPV4 agonist GSK1016790A, confirms TRPV4-induced increases in lung W/D accompanied by appearance of protein in bronchoalveolar lavage fluid. More importantly, we provide the first evidence linking the downstream effectors MMP2 and MMP9 to TRPV4-induced increases in lung permeability and edema. In concert with lung injury, TRPV4 activation in the lung led to increased expression of active MMP2 and MMP9 isoforms along with decreased expression of the endogenous MMP inhibitor TIMP2. Concomitant pharmacologic blockade of MMP2 and MMP9 prevented the permeability response to TRPV4 activation.

The gelatinase MMPs, MMP2 and MMP9, have been previously implicated in lung injury. Increases in MMP2 and MMP9 in the bronchoalveolar lavage fluid (BALF) or tracheal aspirates have been noted in patients with ARDS (8, 26, 28, 46). Early resolution of clinical ARDS is associated with decreased BALF levels of MMP2 and MMP9 (28). MMP2 and/or MMP9 have also been implicated in the development of experimental acute lung injury. LPS treatment or cardiopulmonary bypass leads to the appearance of these gelatinases in BALF (7, 11), while ischemia-reperfusion and VILI upregulate MMP2 and 9 expression in lung tissue (14, 40).

While this collective evidence is suggestive, specific causal links between increased expression of MMP2 and 9 and lung injury are complicated by several factors. First, concomitant increments in both MMPs are not always observed. For example, in a rat model of pancreatitis-induced lung injury, MMP9 increased in lung tissue while MMP2 remained unchanged (23).

Further, depending upon the model, MMPs other than the gelatinases can be upregulated as well,
including MMP1, MMP3, MMP7, MMP8, and MMP13 and MMP14 (13, 14, 19, 26, 33). Thus, although broad-spectrum MMP inhibitors such as Prinomastat or Batimastat have been shown to attenuate injury, interpretation of outcomes is complicated by the non-specificity of these drugs (14, 23).

Hamanaka and colleagues previously demonstrated that activation of TRPV4 initiates VILI (17, 18). Since inhibition of MMPs with Prinomastat attenuates lung injury caused by high volume ventilation (14), we considered a potential link between TRPV4 activation, MMPs and ARDS. We now show that in concert with lung injury, TRPV4 activation in the lung leads to increased expression of active MMP2 and MMP9 isoforms along with decreased TIMP2 expression. Since TIMP2 binding to either of the gelatinase MMPs limits their activity (37, 51), the decrease in TIMP2 at the higher doses of GSK1016790A could favor increased availability of active MMP2 and MMP9 isoforms in the setting of TRPV4-induced lung injury. This TRPV4-mediated effect is not due to non-specific upregulation of MMPs, as MMP14 levels fell and MMP8 levels remained unchanged. Further, the same treatment with GSK1016790A in lungs from TRPV4−/− mice had no impact on MMP2 or MMP9 expression. Moreover, we can specifically implicate MMP2 and 9, since concomitant inhibition of both gelatinases with SB-3CT prevented the lung injury. To our knowledge, this is the first report of functional downstream effectors for TRPV4 activation that promote increases in lung permeability and edema. SB-3CT is a selective mechanism-based competitive inhibitor of the gelatinases, MMP2 and MMP9 (Kₜₛ of 14 and 600 nM, respectively). It binds in the zinc pocket of the gelatinase MMPs, in much the same manner as the TIMPs. Unlike Prinomastat and Batimastat, SB-3CT has little impact on activity of MMPs other than MMP2 and 9 (3, 4). Specifically, Kₜₛ range from 15-206 μM for MMP3, MMP7 and MMP1 (4). In fact, SB-3CT in rat heart had no impact
on expression or activity of MMPs 1-3, 7, 8, 12, 13 or MT1-MMP (9). SB-3CT does inhibit
TNF-α converting enzyme (a member of the ADAM17 family of disintegrins). However,
compared to the action of SB-3CT on gelatinases, it acts on TNF-α converting enzyme via a
non-competitive, low binding affinity mechanism, albeit with a Kᵢ of 4 μM (41).

This study does not specifically address mechanism(s) by which active MMPs might
contribute to acute TRPV4-induced lung injury. However, the literature provides potential clues.
MMP activity in aggregate contributes to the balance between matrix deposition and degradation
in vivo (10, 52). Specifically, active MMP2 and MMP9 degrade numerous fibrillar collagens
including collagen IV and laminin, key structural components of the alveolar basement
membrane in vivo (10). Notably, the MMP2/MMP9-mediated degradation of collagen IV and
laminin is blocked by SB-3CT (9). Further, active MMP2 and MMP9 degrade non-matrix
components such as integrins (16, 47) and intercellular targets such as E-cadherin (29, 43).
Dysfunction of integrin-matrix tethering and/or cell-cell tethering would certainly contribute to
increased barrier permeability. Thus, TRPV4-mediated increases in the availability of active
MMP2 and MMP9 in the alveolar septal compartment could initiate cellular detachment and/or
development of inter-cellular gaps. Indeed, active MMP2 and MMP9 alter barrier integrity in
cultured lung epithelial and microvascular endothelial cells (38, 49). Similarly, TRPV4
activation is known to elicit endothelial cell detachment in vitro and detachment/disruption of the
lung septal endothelial barrier in vivo (2, 50, 56).

Mechanisms underlying the TRPV4-mediated activation of MMP2 and MMP9 are potentially
multifaceted. MMP14 has been implicated in MMP2 activation, a process that requires TIMP2-
mediated tethering of proMMP2 to active MMP14. However this may not always be the case.
We also found increased expression of active MMP2 in lung after TRPV4 activation as well as
decreased expression of both TIMP2 and active MMP14. These outcomes raise the question of how TRPV4 activation leads to increased expression of active MMP2. Several possibilities can be considered. First, if the molar balance of TIMP2, MMP14 and pro-MMP2 within a local microdomain in the lung remains optimal, MMP2 processing may still proceed, albeit yielding smaller quantities of active enzyme (37). Given the marked increase in active MMP2 after TRPV4 activation, this scenario seems unlikely to contribute substantially. Alternatively, MMP14-independent processing of pro-MMP2 due to involvement of other membrane-tethered MMPs has been reported. Indeed all of the MT-MMPs except MT4-MMP (MMP17) can do so, and several are expressed in monocytes/macrophages as well as other leukocytes (30, 51).

However, we did not investigate the impact of TRPV4 activation on these MMPs, nor are we aware of studies assessing expression of MT-MMPs specifically in lung tissue.

In addition to any role for MT-MMPs, other mechanisms of MMP activation are potentially relevant. Specifically, other secreted proteases have been implicated in MMP activation, such as tryptase and chymase released from tissue mast cells (16, 37). While mast cells have been reported to express TRPV4, this mechanism seems unlikely to explain outcomes in our study as tissue mast cell density in C57BL/6 mouse lung is quite low (15, 25). Alveolar macrophages are more likely to play a direct role in increased MMP2 and MMP2 activity. Alveolar macrophages express TRPV4, are activated by TRPV4 agonists, and release oxidants, proteases and activated MMP gelatinases when stimulated (17, 58). Further, VILI is markedly attenuated by TRPV4 antagonists and in TRPV4⁻/⁻ mice, while reintroduction of TRPV4⁺/⁺ macrophages into TRPV4⁻/⁻ mice reconstitutes the lung injury response to this mechanical stress (17, 18). Activation of alveolar macrophages or lung microvascular endothelial cells by mechanical stress induces release of MMP2 and MMP9 (19, 36). MMPs can be stored in granules ready to be secreted
upon stimulus (12, 36, 44, 55). Since a localized increase in subplasmalemmal Ca$^{2+}$ is a critical requirement for fusion of secretory granules with the plasma membrane prior to exocytosis (20), and mechanical stretch and/or GSK1016790A both elicit TRPV4-mediated Ca$^{2+}$ transients (18, 22, 45), it is plausible that TRPV4 activation provides the Ca$^{2+}$ stimulus needed to promote MMP2 and MMP9 release. Indeed, our data support such a conclusion, as there was no gelatinase upregulation in response to GSK1016790A in TRPV4$^{-/-}$ mice.

Our study design does not allow us to specifically shed light on the discrete roles of MMP2 vs MMP9. However, we do note that the balance of potentially contributing factors within the alveolar septal compartment is likely key, due to localized patterns of TRPV4 expression and cell sources of MMPs. TRPV4-induced lung injury is localized to the alveolar septal compartment, where TRPV4 is expressed in pulmonary microvascular endothelial cells, alveolar macrophages, and type II alveolar epithelium (2, 17). In the normal lung, MMP2 and MMP9 are expressed by fibroblasts, alveolar macrophages, neutrophils, endothelial cells, and alveolar type II epithelial cells (21, 27, 34), all of which may be found in the alveolar septal compartment.

The picture becomes more complex due to potential for paracrine signaling, whereby TRPV4 activation in one cell type can give rise to a signal promoting secretion of MMP2 and/or MMP9 from another cell type. Notably, mechanical stretch in cultured endothelial cells and in intact lung can lead to increased expression and shedding of the extracellular matrix metalloproteinase inducer CD147 or EMMPRIN, which elicits increased MMP release (14, 19). Release of EMMPRIN can be abrogated by chelation of intracellular Ca$^{2+}$ (39). Thus, Ca$^{2+}$ influx through TRPV4 may facilitate this process, as membrane blebbing and vacuolization are noted in capillary endothelial cells after TRPV4 activation in rat and mouse lungs (2). While beyond the
The scope of this study, links between TRPV4 and EMMPRIN may provide further mechanistic clues for understanding TRPV4-mediated lung injury.

In this study, we have documented contributions of MMP2 and MMP9 in the acute lung injury response to TRPV4 activation. TRPV4-induced lung injury was significantly attenuated by concomitant pharmacological inhibition of MMP2 and MMP9. We conclude that TRPV4 activation and the subsequent Ca\(^{2+}\) transient initiates a rapid cascade of events leading to release and activation of the gelatinase MMPs. Coordinated impact of these MMPs on their targets within the alveolar septal compartment contributes to lung injury, evidenced by increased lung water and appearance of protein in bronchoalveolar lavage fluid. While available evidence in the literature suggests EMMPRIN activation and release as a potential link between the TRPV4-mediated Ca\(^{2+}\) transient and increased gelatinase activity, definitive evidence for such a link shall remain a focus for further study.
ACKNOWLEDGEMENTS

The authors thank Dr. David Weber (The University of South Alabama) for the use of lab equipment and software. This work was supported by grants from the NIH (HL066299 and R01 HL093052). PV was supported in part by NHLBI training grant T32 HL076125.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.


Figure 1. Activation of TRPV4 promotes increases in BALF total protein and lung W/D in TRPV4^{+/+}, but not TRPV4^{+-}, mouse lungs. TRPV4^{+/+} (n ≥ 4 per group) mouse lungs were perfused with vehicle or increasing doses of the specific TRPV4 agonist GSK1016790A. (A) BALF total protein levels were increased at 300, 1000, and 3000 nM GSK1016790 versus control, while (B) lung W/D was increased at 100, 300, 1000, and 3000 nM GSK1016790A. (C) TRPV4^{-/-} mouse lungs (n ≥ 5 per group) were perfused with vehicle, 30, or 1000 nM GSK1016790A. The high agonist dose, which did promote increases in BALF total protein and lung W/D in TRPV4^{+/+} animals, had no effect on these parameters in TRPV4^{-/-} animals. All values expressed as mean ± SEM; *p < 0.05 versus control (one-way ANOVA and Dunnett’s multiple comparison test).

Figure 2. TRPV4 activation increases active MMP2 and MMP9 isoforms in mouse lung. Representative Western blot images of pro and active isoforms of MMP2 (A, 72 and 64 kD, respectively) and MMP9 (B, 110 and 87/68/59/54 kD, respectively) in TRPV4^{+/+} and TRPV4^{-/-} mouse lungs treated with vehicle (Veh) or GSK1016790A. No changes in MMP2 or MMP9 were observed in TRPV4^{-/-} mouse lungs. All lanes for these panels are from a single blot; each lane is a representative of triplicates; white spaces indicate non-consecutive sections of lanes. Densitometry shows that in TRPV4^{+/+} animals, TRPV4 activation with GSK1016790A leads to increased expression of active MMP2 (C) and MMP9 (D) isoforms, referenced to β-actin (n=3-5 lungs per group). Note that for MMP9, the sum of densitometry for all active isoforms
was assessed for each lung, then data referenced to β-actin. All values expressed as mean ± SEM; *p< 0.05 versus vehicle control (one-way ANOVA and Dunnett’s posthoc test).

**Figure 3.** In concert with lung injury, expression of active MMP14 is decreased with TRPV4 activation, while MMP8 remains unchanged. Representative Western blot images of the active isoforms of MMP14 (A, 57 kD) and MMP8 (B, 53 kD) in TRPV4+/+ mouse lungs treated with vehicle (Veh) or varying doses of GSK1016790A (GSK). All lanes for these panels are from a single blot; each lane is a representative of triplicates; white spaces indicate non-consecutive sections of lanes. Densitometry (referenced to β-actin) shows that TRPV4 activation with 1000 nM GSK1016790A leads to decreased expression of active MMP14 (C), whereas active MMP8 expression (D) was unchanged (n= 5 lungs per group). All values expressed as mean ± SEM; *p< 0.05 versus vehicle control (one-way ANOVA and Dunnett’s posthoc test).

**Figure 4.** In concert with lung injury, TIMP2 is decreased after TRPV4 activation.

Representative Western blot images of (A) TIMP2 (21 kD) and (B) TIMP1 (28 kD) in TRPV4+/+ and TRPV4−/− mouse lungs. No changes in TIMP2 or TIMP1 levels are observed in TRPV4−/− lungs. (C) TIMP2 levels in the lung are decreased at 150 and 1000 nM GSK1016790A compared to vehicle control; (D) TIMP1 levels remain unchanged. Bands representing TIMP2 and TIMP1 were analyzed by densitometry and referenced to β-actin (n= 5 per group). All lanes for each panel (A) and (B) are from a single blot; each lane is a representative of triplicates; white spaces indicate non-consecutive sections of lanes. All values expressed as mean ± SEM; *p< 0.05 versus vehicle control (one-way ANOVA and Dunnett’s posthoc test).
Figure 5. Concomitant inhibition of MMP2 and MMP9 offers protection against TRPV4-induced lung injury. Lung BALF total protein (A) and W/D (B) were significantly decreased in TRPV4+/+ lungs pretreated with SB-3CT (1µM) then treated with GSK1016790A (150 nM) versus GSK1016790A alone (n ≥ 5 per group). All values expressed as mean ± SEM; p < 0.05, significant, *vs vehicle control; # vs 1 µM SB-3CT alone; % vs 150 nM GSK1016790A alone (One-way ANOVA and Tukey’s post hoc test).
Figure 1
Figure 2

(A) Gel blots showing the effect of GSK1016790A on proMMP2 and active MMP2 in TRPV4+/+ and TRPV4−/− cells. The graphs show the quantification of the bands, with bars indicating the total number of pixels and error bars representing standard deviation.

(B) Similar gel blots for proMMP9 and active MMP9.

(C) Bar graph showing the relative expression of MMP2 (64 kD) compared to β-actin for different GSK concentrations (0, 30, 150, 1000 nM) in TRPV4+/+ cells. The asterisk (*) denotes statistically significant differences.

(D) Bar graph for MMP9/β-actin ratio for the same conditions as (C).
Figure 3
Figure 4

A

GSK1016790A (nM)

TRPV4+/+

TIMP2

β-actin

TRPV4-/-

TIMP2

β-actin

B

GSK1016790A (nM)

TRPV4+/+

TIMP1

β-actin

TRPV4-/-

TIMP2

β-actin

C

TIMP2/β-actin Total Pixels

Veh 30 150 1000

D

TIMP1/β-actin Total Pixels

Veh 30 150 1000
Figure 5

A

BALF Total Protein (mg/mL)

GSK  -  +  -  +
SB3CT  -  -  +  +

B

Lung W/D

GSK  -  +  -  +
SB3CT  -  -  +  +