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Abstract

Transient receptor potential protein (TRP) ion channels TRPA1 and TRPV1 may be important in mediating airway tissue injury and inflammation. This study was designed to clarify the role of TRPA1 and TRPV1 channels in cigarette smoke extract (CSE)-induced damage to bronchial and alveolar epithelial cells. Bronchial (Beas-2b) and alveolar epithelial (A549) cell lines were treated with CSE in the presence and absence of a TRPA1 inhibitor (100μM, A967079), a TRPV1 inhibitor (100μM, AMG9810) or both. DCFH-DA and Mito SOX RED probes were used to assay intracellular and mitochondrial oxidative stress, respectively. The mRNA levels of inflammatory mediators (IL-1β, IL-8, IL-18, IL-33) and antioxidants (HO-1, NQO1, MnSOD, Catalase) and the protein expression levels of mitochondrial and inflammasome factors (MFN2, OPA1, DRP1, MFF, NLRP3, Caspase-1) were detected by Western Blot. The results were validated in TRPA1 shRNA and TRPV1 shRNA cells. In both cell types, 10% CSE increased intracellular and mitochondrial oxidative stress, induced Ca²⁺ influx, increased inflammatory gene expression and reduced antioxidant gene expression. 10% CSE increased the expression of mitochondrial fission proteins (MFF and DRP1), Caspase-1 and NLRP3 protein expression and decreased that of mitochondrial fusion proteins (MFN2 and OPA1). Both inhibitors and gene-knockout of TRPA1 and TRPV1 reduced oxidative stress, blocked Ca²⁺ influx, decreased inflammatory and increased antioxidant gene expression. They also reversed the changes in mitochondrial fission and fusion proteins induced by CSE. Both TRPA1 and TRPV1 mediate CSE-induced damage of airway and alveolar epithelial cells via modulation of oxidative stress, inflammation and mitochondrial damage and their inhibition should be considered as potential therapy for COPD.

Keywords	TRPA1, TRPV1, Cigarette smoke, oxidative stress, mitochondria damage, COPD
Taxonomy	Molecular Biology, Physiology, Biochemistry
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Dear Editor,

Enclosed for your consideration is an original research article entitled " Roles of TRPA1 and TRPV1 in Cigarette smoke -induced Airway Cell Injury Model ", which has not been published previously, in any language, in whole or in part, and is not currently under consideration elsewhere.

All authors of this research paper have directly participated in the design, execution, or analysis of this study. They have read and approved the final version submitted. Approval for the study was obtained by the hospital's ethics committee. This work was supported by the Key International (Regional) Cooperative Project of National Natural Science Foundation of China(No.51420105010), Project of National Nature Science Foundation of China (No. 81870031), and Natural Science Foundation of Anhui Province(KJ2018A0208).

Using CSE-induced cell lung inflammation model, this study suggests that Both TRPA1 and TRPV1 mediate CSE-induced damage of airway and alveolar epithelial cells via modulation of oxidative stress, inflammation and mitochondrial damage and their inhibition should be considered as potential therapy for COPD.

The result is encouraging and might be interesting for readers of Free Radical Biology and Medicine.

We'll be very grateful if you can review and publish it in your distinguished journal. Many Thanks!

Best Wishes.

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Roles of TRPA1 and TRPV1 in Cigarette smoke -induced Airway Cell Injury Model

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Running title: TRPA1/TRPV1 in airway cell injury

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ABSTRACT:

Transient receptor potential protein (TRP) ion channels TRPA1 and TRPV1 may be important in mediating airway tissue injury and inflammation. This study was designed to clarify the role of TRPA1 and TRPV1 channels in

cigarette smoke extract (CSE)-induced damage to bronchial and alveolar epithelial cells.

Bronchial (Beas-2b) and alveolar epithelial (A549) cell lines were treated with CSE in the presence and absence of a TRPA1 inhibitor (100μM, A967079), a TRPV1 inhibitor (100μM, AMG9810) or both. DCFH-DA and Mito SOX RED probes were used to assay intracellular and mitochondrial oxidative stress, respectively. The mRNA levels of inflammatory mediators (IL-1β, IL-8, IL-18, IL-33) and antioxidants (HO-1, NQO1, MnSOD, Catalase) and the protein expression levels of mitochondrial and inflammasome factors (MFN2, OPA1, DRP1, MFF, NLRP3, Caspase-1) were detected by Western Blot. The results were validated in TRPA1 shRNA and TRPV1 shRNA cells.

In both cell types, 10% CSE increased intracellular and mitochondrial oxidative stress, induced Ca²⁺ influx, increased inflammatory gene expression and reduced antioxidant gene expression. 10% CSE increased the expression of mitochondrial fission proteins (MFF and DRP1), Caspase-1 and NLRP3 protein expression and decreased that of mitochondrial fusion proteins (MFN2 and OPA1). Both inhibitors and gene-knockout of TRPA1 and TRPV1 reduced oxidative stress, blocked Ca²⁺ influx, decreased inflammatory and increased antioxidant gene expression. They also reversed the changes in mitochondrial fission and fusion proteins induced by CSE.

Both TRPA1 and TRPV1 mediate CSE-induced damage of airway and alveolar epithelial cells via modulation of oxidative stress, inflammation and mitochondrial damage and their inhibition should be considered as potential therapy for COPD.

KEY WORDS: TRPA1, TRPV1, Cigarette smoke, oxidative stress, mitochondria damage, COPD

Introduction

Chronic obstructive pulmonary disease (COPD), characterized by persistent

respiratory symptoms and airflow limitation, is a major health problem that is to become the third leading cause of mortality worldwide in 2030 by WHO [1]. Oxidative stress, due to the excessive presence of reactive oxygen species (ROS) or impaired endogenous antioxidant defenses, is a main predisposing factor in the pathogenesis of COPD [2]. ROS may be either exogenous from external sources such as cigarette smoke (CS), the most important risk factor for COPD, as it contains a high concentration of oxidants [3], or endogenous from cellular sources such as mitochondria, dihydronicotinamide-adenine dinucleotide phosphate (NADPH) oxidase (NOX) and the xanthine/xanthine oxidase system [2].

As the primary intracellular source of ROS, mitochondria are involved in the pathogenesis of COPD [4]. Mitochondria are double-membrane subcellular organelles whose structure and function are regulated by the fusion/fission process [5]. Mitochondrial fusion is regulated by mitofusions 1 and 2 (MFN1 and MFN2) and optic atrophy 1 (OPA1), while mitochondrial fission is regulated by Fission1 (FIS1), dynamin-related protein 1 (DRP1) and mitochondrial fission factor (MFF). The imbalance of mitochondrial fusion and fission can indicate mitochondrial damage and decreased oxidative phosphorylation [6]. Functionally, mitochondria can regulate the cellular redox state and redox signaling, cell proliferation and apoptosis, innate and adaptive immune responses and NLRP3 inflammasome activation [7]. NLRP3 inflammasome contains the adaptor protein, apoptosis-associated speck-like protein (ASC), and the proinflammatory caspase-1. Recent data indicate that mitochondria plays a key role in activating NLRP3 inflammasome leading to caspase-1-induced processes such as the generation of mature IL-1 β and IL-18 from their precursor forms [8, 9].

Transient receptor potential (TRP) ion channels are a large family of ion channel proteins, which are located in the cell membrane and widely distributed throughout the respiratory tract [10]. TRP channels are Ca²⁺-permeable cation channels which regulate cell function by directly allowing

Ca²⁺ influx into the cell in response to various stimuli, or through depolarizing the membrane potential due to cation influx [11]. Among the TRP channels, TRPA1 and TRPV1 play a major role in inflammation and tissue damage and are respectively recognized as a “gatekeeper of inflammation” [12] and a “molecular gateway to the pain pathway” [13].

We hypothesized that CS exposure activates TRPA1 or TRPV1 in the airway epithelial cells, which leads to mitochondrial damage and dysfunction which ultimately lead to airway inflammation. Therefore, in the present study, we evaluated the role of TRPA1 and TRPV1 pathways in CS- induced lung epithelial cell injury using pharmacological agents and gene deletion.

Materials and Methods

Cell culture

A549 cells (Shanghai Institutes for Biological Sciences, China Academy of Science, Shanghai) were cultured in high-glucose Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) with 10% fetal bovine serum (BioInd, Kibbutz Beit Haemek, Israel), 100U/ml penicillin and 100µg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in 5% CO₂. Beas-2b cells (Shanghai Institutes for Biological Sciences, China Academy of Science) were cultured in 1640 Medium (Hyclone) with 10% fetal bovine serum (BioInd), 100 U/ml penicillin and 100µg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C under an atmosphere of 95% air and 5% CO₂.

Cigarette smoke extract preparation

Cigarette Smoke Extract (CSE) was freshly prepared on the day of each experiment [14, 15]. In brief, two burning cigarettes (Marlboro Red Label, Longyan Tobacco Industrial co.LTD, Fujian, China) without filters were sucked

at a constant flow rate (8 mL/s) into a syringe and then bubbled into a tube containing 10mL serum-free medium. The CSE solution was sterilized using a 0.22µm filter (Millipore, Bedford, MA) and the pH was adjusted to 7.4. This CSE solution was considered 100% CSE and was further diluted with serum-free medium to the desired concentrations that were then used to treat cells for different time duration.

Cell culture and treatment of CSE and antagonists

Following observations from a preliminary study, A549 cells and Beas-2B cells were treated with 10% CSE to cause cell injury. Cells were pretreated with 100µM of TRPA1 antagonist (A967079, Abcam, Cambridge, MA, USA) or 100µM of TRPV1 antagonist (AMG9810, Abcam) or both for 1h and then cultured with vehicle or CSE for another 24h.

TRPA1 shRNA and TRPV1 shRNA cells.

Human TRPA1 and TRPV1 shRNA interference sequences were designed by BIO-biotechnologies Shanghai Co. LTD. (Shanghai, China). Cells were transiently transfected with the TRPA1 or TRPV1 plasmids using Lipofectamine TM 2000 (Invitrogen) according to the manufacturer's protocol. Both cells were cultured in six well culture plates 24 hrs prior to transfection. Then, 4 µl of Lipofectamine TM 2000 were incubated with 100 pmol of shRNA (2 µg plasmids) or negative control sequences in 500 µl of Opti-MEM® (Invitrogen) for 20 min at room temperature. Cells were transfected by replacing the medium with 2 ml of Opti-MEM containing the shRNA (plasmids) or negative control sequences and Lipofectamine TM 2000, and incubated at 37°C, 5% CO₂ for 6 hrs. Then, the Opti-MEM was replaced with 2 ml of fresh culture medium for 24 or 48 hrs, the cells were collected and Western blot assays were performed.

Cell viability assay

The effect on cell proliferation by vehicle (culture medium) or CSE was measured by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells were subcultured in a 96-well plate with a density of 5×10^4 cells/ml, incubated overnight at 37°C, and then treated with vehicle or CSE. After 24h incubation, cells were washed twice with PBS and incubated with CCK8 working solution for 2hrs at 37°C following the manufacturer's protocol. The absorbance at 450 nm was measured by microplate reader iMark (Molecular Devices, Sunnyvale, USA).

Measurement of intracellular ROS and mitochondrial ROS

Intracellular ROS generation was detected using the DCFH-DA (D6883, Sigma-Aldrich, St. Louis, MO, USA) with a fluorescence plate reader and fluorescence microscopy. Briefly, the cells were seeded in 96-well black plates with a density of 5×10^4 /ml with 6 parallel wells in each group. Cells were stained with 10 μ M DCFH-DA at 37°C in the dark for 15 min. Then cells were washed with serum-free DMEM for three times, the level of ROS was determined by Flexstation®2 fluorescence plate reader (Molecular Devices, San Jose, CA, USA) at 488/525 nm and by microscopy (Leica, Germany).

Mitochondrial ROS activity was measured with Mito SOX Red (Invitrogen, Life Technologies, Carlsbad, CA, USA) assay, a redox-sensitive fluorescent probe that is selectively targeted to the mitochondria. Cells were seeded in 96-well black plates with a density of 5×10^4 /ml with 6 parallel wells in each group. Cells were incubated with 5mmol/L Mito SOX Red probe for 10 minutes at 37°C. The cells were washed twice with PBS, and red fluorescence was determined at 510/580 nm using Flexstation®2 fluorescence plate reader.

Measuring Ca²⁺ influx.

Intracellular Ca²⁺ levels were determined using a Fluo-4 AM Calcium Assay Kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well black plates with a density of 5×10⁴ /ml with 6 parallel wells in each group. The experimental group were pretreated with antagonist for 1h, then all groups were exposed to CSE for 2 min. Cells were washed three times and stained with 5μM Fluo-4 AM at 37°C in the dark for 30 min. Then cells were washed with PBS three times, and the level of fluorescence was determined by Flexstation®2 fluorescence plate reader at 488/516 nm and by microscopy (Leica).

Reverse transcription-polymerase chain reaction (RT-PCR).

Cytokines (IL-1β, IL-8, IL-18 and IL-33) and antioxidants (HO-1, NQO1, MnSOD, Catalase) were measured by RT-PCR. (IL-1β:5'-ATGATGGCTTATTACAGTGGCAA-3' (forward), 5'-GTCGGAGATTCGTAGCTGGA-3' (reverse), IL-8:5'-ACTGAGAGTGATTGAGAGTGGAC-3' (forward), 5'-AACCCTCTGCACCCAGTTTTTC-3' (reverse), IL-18:5'-TCTTCATTGACCAAGGAAATCGG-3' (forward), 5'-TCCGGGGTGCATTATCTCTAC-3' (reverse), IL-33:5'-GTGACGGTGTTGATGGTAAGAT-3' (forward),5'-AGCTCCACAGAGTGTTTCCTTG-3' (reverse), (HO)-1:5'-CTGCCCCAACCACCTTCTGTT-3' (forward), 5'-ATAAGAAGGCCTCGGTGGAT-3' (reverse), NQO1:5'-CAGTGGCATGCACCCAGGGAA-3' (forward), 5'-GCATGCCCCCTTTTAGCCTTGGCA-3' (reverse), MnSOD:5'-ACAGGCCTTATTCCACTGCT-3' (forward), 5'-

CAGCATAACGATCGTGGTTT-3' (reverse), Catalase:5'-TAAGACTGACCAGGGCATC-3' (forward), 5'-CAAACCTTGGTGAGATCGAA-3' (reverse).)Total RNA was extracted from cells using TRIzol reagent (TaKaRa, Dalian, Liaoning, China) . The concentration of the total RNA was determined using an ultraviolet spectrophotometer. Reverse transcription was conducted using a Prime Script™ RT Master Mix Kit (TaKaRa) according to the manufacturer's instruction. RT-PCR was performed using a Power Green qPCR Mix (TaKaRa) and an ABI ViiATM 7 System. The specific primers (Table 1) for β -actin, IL-1 β , IL-8, IL-18, IL-33, (HO)-1, NQO1, MnSOD, Catalase were generated by BioTNT (Shanghai, China). All samples were assayed in triplicate, and the values were normalized to β -actin.

Western blot analysis.

The expression of DRP1, MFF, OPA1, MFN2, NLRP3 and Caspase-1 were examined by Western Blot assay. Total proteins were extracted from the cells in each group using RIPA buffer (Beyotime). Protein concentrations were determined with a BCA protein assay kit (Beyotime). Total protein samples were separated using 10% SDS PAGE, transferred to a PVDF membrane, blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST, BioTNT, Shanghai, China), and incubated with primary antibodies against DRP1, MFF, OPA1, MFN2, Caspase-1, NLRP3 and β -Tubulin (all 1:1000, Cell Signaling Technology, Boston, MA, USA) overnight at 4°C. The secondary antibodies, HRP-conjugated goat anti-rabbit IgG (1:15000, Cell Signaling Technology), were incubated with the membranes for 2hrs at room temperature. After the membranes were washed in TBST, the bands were visualized with the ECL detection system.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7; GraphPad Software, Inc., San Diego, CA). One-way ANOVA with Bonferroni's post hoc test (forequal variance) or Dunnett's T3 post hoc test (for unequal variance) was performed for comparisons among multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of CSE and TRPA1/TRPV1 inhibitors on cell viability and inflammatory gene expression

There was a CSE-dose-dependent loss of viability in both cell types (Supplement 1A, B). There was no significant effect of CSE, the TRPA1 inhibitor A967079 or the TRPV1 inhibitor AMG9810 alone, or in combination, on cell viability as determined by CCK8 expression in A549 or in Beas-2B cells after 24hrs (Fig. 1A, B). CSE exposure for 24 hours increased the mRNA expression of the inflammatory cytokines IL-1 β , IL-8, IL-18 and IL-33 compared with basal levels in A549 cells (Fig. 1C-F). Pretreatment with A967079 significantly reduced CSE-induced IL-18 and IL-33 mRNA expression (Fig. 1E, F) whilst pretreatment with AMG9810 significantly reduced CSE-enhanced IL-8 and IL-18 (Fig. 1D, E) mRNA levels. Combined A967079 and AMG9810 pretreatment significantly reduced CSE-induced expression of IL-1 β , IL-8 and IL-18 mRNAs (Fig. 1C, E). CSE-enhanced IL-1 β , IL-8, IL-18 and IL-33 mRNA was decreased in CSE-exposed TRPA1 shRNA A549 (Supplement 2B) cells and in TRPV1 shRNA A549 cells (Supplement 2B).

In Beas-2b cells, CSE exposure also enhanced IL-1 β , IL-8, IL-18 and IL-33 mRNA expression compared with baseline (Fig. 1G, J). Pretreatment with A967079 decreased IL-1 β mRNA expression (Fig. 1G) whilst pretreatment with AMG9810 significantly reduced IL-8, IL-18 and IL-33 mRNA levels (Fig.

1H, J). The combination of A967079 and AMG9810 significantly attenuated CSE-induced mRNA expression of IL-1 β , IL-18 and IL-33 (Fig. 1G, I, J). CSE-enhanced IL-1 β , IL-8, IL-18 and IL-33 mRNA was decreased in CSE-exposed TRPA1 shRNA Beas-2b cells and in TRPV1 shRNA Beas-2b cells (Supplement 3B).

Effect of CSE and TRPA1/TRPV1 inhibitors on anti-oxidant gene expression

In A549 cells, CSE exposure for 24 hours significantly reduced the mRNA expression of HO-1, NQO1, MnSOD and Catalase compared with basal levels (Fig. 2A, D). Pretreatment with A967079 significantly increased CSE-attenuated expression of NQO1, MnSOD and Catalase mRNAs (Fig. 2B, D). Pretreatment with AMG9810 significantly increased CSE-depleted HO-1 and MnSOD mRNA expression (Fig. 2A, C). Pretreatment with A967079 and AMG9810 together increased CSE-suppressed HO-1 and MnSOD mRNA expression (Fig. 2A, C, D). The mRNA levels of HO-1, NQO1, MnSOD and Catalase were increased in CSE-exposed TRPA1 shRNA A549 cells and TRPV1 shRNA A549 cells (Supplement 2C).

In Beas-2b cells, CSE reduced HO-1, NQO1, MnSOD and catalase mRNA expression (Fig. 2E, H). Pretreatment with A967079 increased CSE-suppressed NQO1 and catalase mRNA expression (Fig. 2F, H). AMG9810 pretreatment only significantly increased the expression of CSE-attenuated catalase mRNA (Fig. 2H). Finally, the combination of A967079 and AMG9810 significantly enhanced CSE-suppressed HO-1 and MnSOD mRNA expression (Fig. 2E, G). The mRNA levels of HO-1, NQO1, MnSOD and catalase were increased in CSE-exposed TRPA1 shRNA Beas-2b cells and TRPV1 shRNA Beas-2b cells (Supplement 3C).

Effect of CSE and TRPA1/TRPV1 inhibitors on intracellular and

mitochondrial ROS

To determine whether there was a functional consequence of the loss in anti-oxidant genes, we examined intracellular and mitochondrial ROS levels. CSE exposure for 24 hours increased intracellular ROS levels in A549 cells and Beas-2b cells (Fig. 3A, B). Pretreatment with A967079 or AMG9810 inhibited the level of CSE-enhanced intracellular ROS in A549 cells (Fig. 3A). Pretreatment with combined A967079 and AMG9810 was more effective than either A967079 or AMG9810 pretreatment alone in reducing CSE-enhanced intracellular ROS (Fig. 3A). Similarly, pretreatment with either A967079 or AMG9810 or combined A967079/AMG9810 inhibited the level of CSE-induced intracellular ROS in Beas-2b cells (Fig. 3B). The same results shown in CSE-exposed TRPA1 shRNA A549 cells and TRPV1 shRNA A549 cells compared to CSE-exposed wild type A549 cells (Supplement 4A, C).

CSE exposure for 24 hours increased mitochondrial ROS levels in Beas-2B cells (Fig. 3C). Pretreatment with either A967079 or AMG9810 or both in combination inhibited the level of CSE-induced mitochondrial ROS (Fig. 3C). CSE exposure for 24 hours also increased mitochondrial ROS levels in Beas-2B cells (Fig. 3D). Pretreatment with A967079 or AMG9810, and with both together, inhibited the level of CSE-enhanced mitochondrial ROS (Fig. 3D). The same results shown in CSE-exposed TRPA1 shRNA Beas-2B cells and TRPV1 shRNA Beas-2B cells compared to CSE-exposed wild type Beas-2B cells (Supplement 4B, D).

Effect of CSE and TRPA1/TRPV1 inhibitors on mitochondrial fission and fusion protein expression

To examine further the mechanism of these changes in mitochondrial function, we examined the expression of mitochondrial fission and fusion proteins that regulate mitochondrial function. Western blot analysis of the mitochondrial

fusion and fission proteins OPA1, MFN2, DRP1 and MFF in A549 cells after exposed to CSE or pretreatment with inhibitors were shown (Fig. 4A). Western blot analysis of the mitochondrial fusion and fission proteins OPA1, MFN2, DRP1 and MFF in Beas-2b cells after exposed to CSE or pretreatment with inhibitors were shown (Fig. 4B).

In A549 cells, CSE exposure for 24 hours decreased the expression of the mitochondrial fusion proteins OPA1 and MFN2, and enhanced the expression of the mitochondrial fission proteins DRP1 and MFF (Fig. 4C-F). Pretreatment with A967079 or AMG9810 or both together significantly increased CSE-suppressed OPA1 (Fig. 4C) and MFN2 protein expression (Fig. 4D). Pretreatment with A967079 decreased the CSE-induced expression of DRP1 (Fig. 4E), whilst pretreatment with AMG9810 or both A967079 and AMG9810 together decreased CSE-induced DRP1 (Fig. 4E) and MFF (Fig. 4F) protein levels. Compared to CSE-exposed wild type A549 cells, CSE-exposed TRPA1 shRNA A549 cells showed increased OPA1 and MFN2 (Supplement 2D) and reduced DRP1 and MFF (Supplement 2D) protein expression. Similar results were seen in CSE-exposed TRPV1 shRNA A549 cells which showed increased OPA1 and MFN2 protein (Supplement 2D) and reduced DRP1 protein (Supplement 2D).

A similar pattern was observed in Beas-2b cells. CSE exposure for 24 hours suppressed OPA1 and MFN2 expression, and enhanced DRP1 and MFF expression (Fig. 4G-J). Pretreatment with A967079 or AMG9810 or both in combination increased CSE-suppressed OPA1 (Fig. 4G) and MFN2 protein expression (Fig. 4H). Pretreatment with A967079 or combined use of A967079 and AMG9810 decreased CSE-enhanced DRP1 (Fig. 4I) and MFF (Fig. 4J) proteins. Pretreatment with AMG9810 decreased CSE-enhanced MFF protein expression (Fig. 4J). Compared to CSE-exposed wild type Beas-2b cells, the protein level of OPA1 significantly increased in TRPV1 shRNA Beas-2b cells and MFN2 significantly increased in TRPA1 shRNA Beas-2b cells (Supplement 3D). The protein levels of DRP1 and MFF were markedly

decreased in TRPA1 shRNA Beas-2b cells and TRPV1 shRNA Beas-2b cells (Supplement 3D).

Effect of CSE, TRPA1/TRPV1 inhibitors, TRPA1/TRPV1 blocked on intracellular Ca²⁺ levels in A549 cells and Beas-2B cells

10% CSE had a time-dependent effect on intracellular Ca²⁺ mobilization in both A549 and Beas-2b cells that peaked at 2 min (Supplement 1C, D). Intracellular Ca²⁺ level was increased 2 min after CSE exposure compared to basal state in A549 cells (Fig. 5A). CSE-induced increases in Ca²⁺ levels were inhibited by pretreatment with A967079 or AMG9810 alone and their combination (Fig. 5A). CSE also significantly enhanced intracellular Ca²⁺ in Beas-2b cells (Fig. 5B) and this was significantly reduced by both A967079 and AMG9810 alone and in combination (Fig. 5B).

There were no difference in intracellular Ca²⁺ level between CSE exposure and control shRNA-treated+CSE exposed A549 cells (Fig. 5C). There were significantly reduced levels of intracellular Ca²⁺ in CSE-exposed TRPA1 shRNA A549 cells and CSE-exposed TRPV1 shRNA A549 cells compared to CSE –exposed wild type A549 cells (Fig. 5C). Similar results were observed in TRPA1 shRNA Beas-2b cells and CSE-exposed TRPV1 shRNA Beas-2b cells compared to CSE–exposed wild type Beas-2b cells (Fig. 5D).

Effect of CSE, TRPA1/TRPV1 inhibitors, TRPA1/TRPV1 blocked on inflammasome activation in A549 cells and Beas-2B cells

Western blot analysis of NLRP3 and Caspase-1 in A549 cells after exposed to CSE or pretreatment with inhibitors were shown (Fig. 6A). Western blot analysis of NLRP3 and Caspase-1 in Beas-2b cells after exposed to CSE or pretreatment with inhibitors were shown (Fig. 6B).

CSE increased NLRP3 and caspase-1 protein expression in A549 cells (Fig. 6C, D). Pretreatment with A967079 or AMG9810 or their combination decreased both CSE-attenuated protein expression of NLRP3 and caspase-1 (Fig. 6C, D). In Beas-2b cells, CSE also induced the expression of both NLRP3 and caspase-1 proteins (Fig. 6E, F). Pretreatment with A967079, but not AMG9810, decreased NLRP3 protein expression (Fig. 6E) whereas pretreatment with AMG9810 decreased caspase-1 protein expression (Fig. 6F). Pretreatment with combined A967079 and AMG9810 resulted in a significant decrease in CSE-enhanced NLRP3 and caspase-1 protein expression (Fig. 6E, F).

The negative control shRNAs for TRPA1 and TRPV1 had no effect on the ability of CSE to enhance NLRP3 and caspase-1 protein expression in A549 cells (Fig. 6G, H). Compared to CSE-exposed wild type A549 cells, CSE-exposed TRPA1 shRNA A549 cells and TRPV1 shRNA A549 cells showed reduced NLRP3 and caspase-1 (Fig. 6G, H) protein expression. These results were replicated in Beas-2b cells (Fig. 6I, J). There was no effect of the control shRNA-treated on the ability of CSE to induce NLRP3 or caspase-1 proteins. The protein levels of NLRP3 and caspase-1 were markedly decreased in CSE-exposed TRPA1 shRNA Beas-2b cells and TRPV1 shRNA Beas-2b cells (Fig. 6I, J).

DISCUSSION

In the present study, we demonstrated that cigarette smoke induced intracellular and mitochondrial oxidative stress and an inflammatory response in airway epithelial cells. Pretreatment with either a pharmacological inhibitor or gene-knock-out of the TRPA1 and TRPV1 receptors significantly decreased the level of intracellular and mitochondrial ROS, mitochondrial damage and the downstream inflammatory response. In A549 and Beas-2b cells, CSE induced Ca^{2+} influx, decreased antioxidant gene expression, increased the intracellular

and mitochondrial ROS and disrupted the balance between mitochondria fission and fusion proteins. CSE exposure also increased the expression of NLRP3 and caspase-1 proteins. The results suggested that TRPA1 and TRPV1 pathways play an important role in cigarette smoke -induced airway epithelial cell injury.

TRPA1 was originally cloned from human fibroblasts and then identified in a subset of C-fibers. Apart from sensory nerve fibers, TRPA1 was also found in airway epithelial cells and smooth muscle cells [16]. CSE contains crotonaldehyde [17], acetaldehyde [18] and nicotine [19], which all are TRPA1 agonists. This suggests that TRPA1 may play a role in COPD as these stimulants that are found in tobacco can promote inflammatory mediator release through TRP channel activation in epithelial and other cells. TRPV1 channel was initially believed to be confined to nociceptive neurons, but has now been identified in a range of tissues and organs including airway and lung. TRPV1 can be activated by diverse stimuli such as irritant chemicals, acidic pH, hot temperatures ($>43^{\circ}\text{C}$) and endogenous mediators [20].

Many studies relating to TRPV1 focus on its role on sensory nerves, which stimulate the cough reflex. McLeod et al. [21] demonstrated that TRPV1 activation was involved in capsaicin-induced cough in guinea pigs which was inhibited by TRPV1 antagonists. Additional studies showed that patients with an upper respiratory tract infection, asthma or COPD have an increased incidence of cough in response to capsaicin [22, 23]. These findings in man were recapitulated in animal models of respiratory diseases. For example, exposure of animals to TRPV1 agonists such as allergens, CSE or viral infection causes cough hypersensitivity and morphological changes in airway [24-28].

Oxidative stress, characterized by an altered redox regulation with increased ROS, is a major mechanism underlying CSE-induced lung injury. In both A549 and Beas-2b cells, inhibitors of both TRPA1 and TRPV1 reduced oxidative stress induced by CSE exposure. Compared to A967079 and

AMG9810 alone, the effects of dual inhibition were generally better in reducing intracellular, but not mitochondrial ROS. The effects of pharmacological inhibition were replicated in TRPA1 shRNA and TRPV1 shRNA A549 and Beas-2b cells. The overall similarity of the effects of the two inhibitors in the two cell types in combination with the genetic knock down data indicates that TRPA1 and TRPV1 pathways in the lung provide an important link among extracellular ROS, the resultant intracellular ROS and mitochondrial dysfunction associated with downstream inflammatory responses. We have shown mitochondrial dysfunction, excessive mitochondrial ROS and intracellular ROS upon CSE exposure contributes to the pro-inflammatory properties of lung and airway epithelial cells. This acquired mitochondrial dysfunction might be important in the development of COPD [29].

CSE caused increased intracellular Ca^{2+} levels via ion influx. Exposure to CSE caused a rapid, time-dependent increase in Ca^{2+} influx via activation of lung and airway epithelial TRPA1 and TRPV1 pathways [30]. Pretreatment with A967079 and AMG9810 reduced the intracellular Ca^{2+} level in A549 and Beas-2b cells. In addition, TRPA1/TRPV1 blocked prevented CSE-induced Ca^{2+} influx. Previous studies have reported that Ca^{2+} signaling mediated by various Ca^{2+} channels regulate the activity of cellular NADPH oxidase, which in turn also results in an elevation of the intracellular ROS [31].

CSE also reduced the expression of antioxidant gene mRNA levels. In A549 cells, preventive treatment with A967079 and AMG9810 only partially enhanced CSE-suppressed antioxidant expression, whilst co-treatment with the two inhibitors or knock down of TRPA1 and TRPV1 effectively increased antioxidant gene expression. In Beas-2b cells, A967079 and AMG9810 gave a greater increase in antioxidant expression and TRPA1 shRNA and TRPV1 shRNA Beas-2b cells gave a greater effect than seen with pharmacological inhibitors.

These differences in response may reflect intrinsic differences in the oxidant/antioxidant capacity in these cells or a differential response to the

single dose of inhibitor used. Future experiments should examine the effect of a full concentration response curve on these intracellular and mitochondrial responses seen with cigarette smoke exposure and ideally looking at different time points to find an optimum for each readout. However, despite this limitation, it is evident that in these two lung epithelial cells both TRPA1 and TRPV1 pathways play an important role in controlling the oxidative stress induced by cigarette smoke. These drugs may not only be useful in chronic cough [21-23] but may also have anti-oxidant and anti-inflammatory actions in COPD.

Mitochondrial structure is important in controlling their function [32] and is abnormal in airway epithelial cells from patients with COPD [33] as well as in asthma [34]. Our data demonstrates that CSE exposure destroys the balance of mitochondrial fission and fusion protein expression in both A549 and Beas-2b cells. Western blot analysis indicated that pretreatment with A967079 and AMG9810, or genetic ablation of TRPA1 and TRPV1 expression, reduced CSE-induced alterations in the expression of mitochondrial fission/fusion proteins. Although not formally studied here, this would have a marked effect on mitochondrial structure and function. Fission/fusion protein mismatch results in mitochondrial dysfunction, permeabilization of the outer mitochondrial membrane, release of apoptotic proteins and finally, it contributes to cell death [35]. Inhibitors of TRPA1 and TRPV1 pathways should be examined in in vivo models of COPD to determine whether induced mitochondrial changes and the resultant effects on cell death, inflammation and epithelial cell function can be reversed.

CSE exposure increased NLRP3 and caspase-1 protein expression in both A549 and Beas2b cells. Inhibited or knock down of TRPA1/TRPV1 pathways reduced these enhanced NLRP3 and caspase-1 levels back towards baseline. Similarly, we saw an induction of IL-1 β and IL-18 mRNA by CSE in these cells which again was attenuated by TRPA1 and TRPV1 inhibition or knock down. Although this data is indicative of an effect of CSE on activation of

the inflammasome as reported elsewhere [36, 37], it is important to demonstrate inflammasome activity by measurement of IL-1 β and IL-18 protein release. In in vitro studies, using alveolar (A549 cells) and bronchial epithelial (Beas-2B cells, 16-HBE cells) cells, ROS have been shown to cause increased gene expression of inflammatory mediators [38, 39], such as IL-8 [40] and IL-33 [41].

We posit a scenario whereby CSE, as a direct oxidant, induces oxidative stress and lung inflammation: ROS acts on TRPA1 and TRPV1 pathways to prime NLRP3 inflammasome, which stimulates the secretion of pro-inflammatory cytokines followed by the caspase-1 activation, leading to the secretion of IL-1 β and IL-18 [42]. NLRP3 requires a dual hit for full activation and production of IL-1 β and IL-18 proteins [43]. This suggests that NLRP3 may play a key role in the pathogenesis of COPD [44], however, recent evidence has shown that NLRP3 is primed but not activated in stable COPD [45, 46] and clinical trials suggests little benefit of anti-IL-1 therapy in COPD [47]. These studies did not differentiate between current and ex-smokers and so clinical results may reflect a failure to obtain the correct second hit essential for inflammasome activation to occur in stable ex-smoking disease or a failure to distinguish the correct COPD sub-phenotype associated with inflammasome activation.

Our study highlighted the importance of the TRPA1 and TRPV1 pathway in regulating airway inflammation associated with oxidative stress. Inhibitors of these pathways might be a potential therapeutic approach to COPD. The enhanced effect seen with the combination of TRPA1 and TRPV1 inhibitors showed the interaction between them may play an important role in regulating their actions. We did not examine the expression of TRPA1 and TRPV1 on A549 and Beas-2b cells but previous studies suggest that the co-expression of the TRPV1 and TRPA1 channels can explain the TRPA1-mediated responses in sensory neurons [21]. Endogenous inflammatory mediators, although not being a TRPV1 or TRPA1 activator themselves, can enhance the sensitivity of

these channels [48]. Hence, both two channels may be expressed and activated contemporaneously during airway inflammation. Importantly, an interaction between TRPA1 and TRPV1 channels may play an important role in regulating the sensitivity and function of cellular response [49], and this interaction may also play an important role in the function and regulation of sensory neurons excitatory of lung and this may impact upon airway inflammation, but the underlying mechanism of this is not understood [49].

There are no effective anti-inflammatory or anti-oxidant therapies available for COPD at present but pathway-targeted TRPA1 and TRPV1 inhibitors represent a promising novel approach to the treatment of COPD. These would have the benefit of reversing inflammatory and remodeling processes in the airway as well as targeting cough.

Acknowledgments

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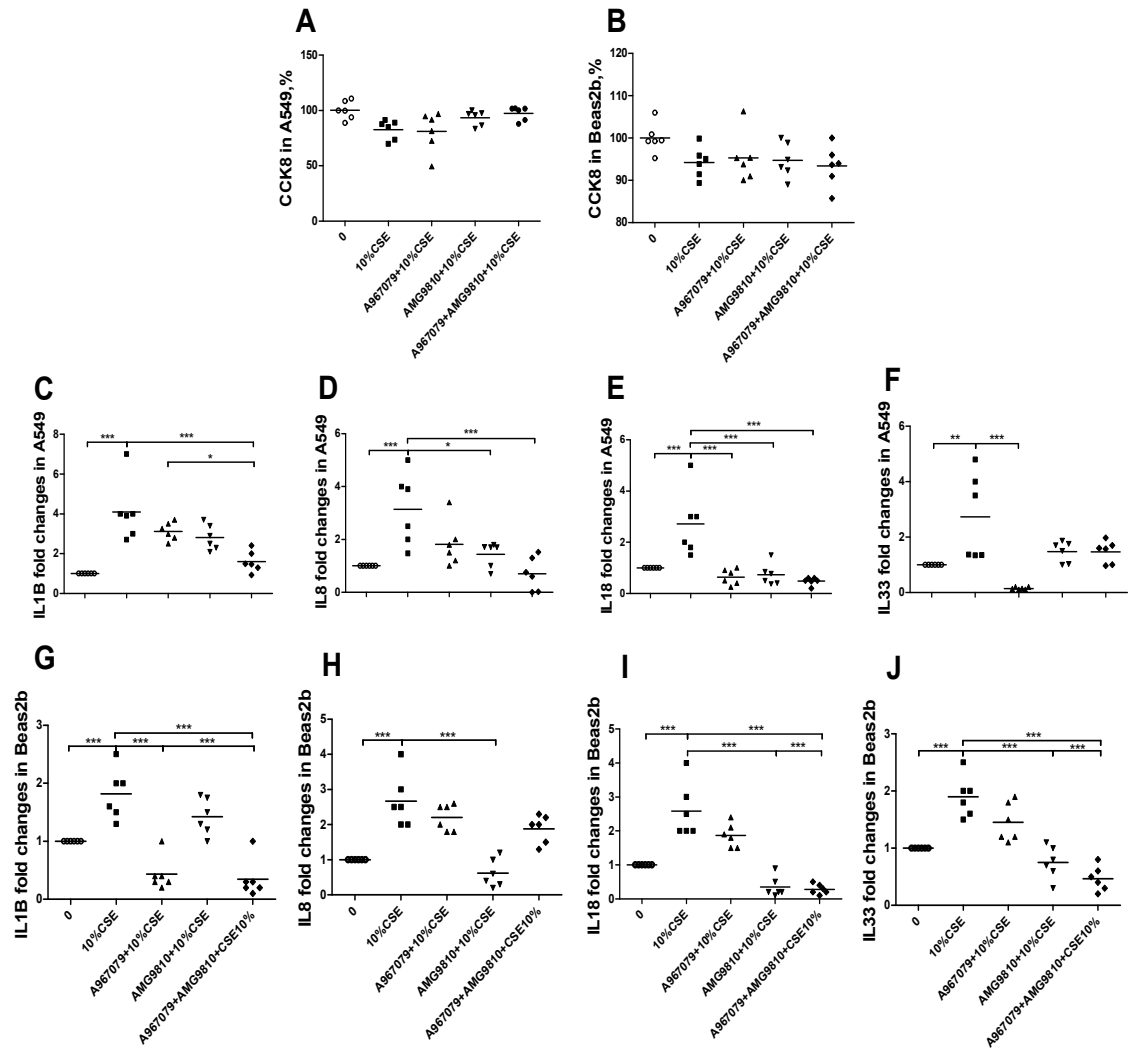


Figure 1. Effect of CSE and TRPA1/TRPV1 inhibitors on cell viability and inflammatory gene expression. There was no significant effect of 10% CSE, the TRPA1 inhibitor (A967079, 100μM) or the TRPV1 inhibitor (AMG9810, 100μM) or both on cell viability in A549 cells (A) and Beas-2b cells (B). Pretreatment of cells with both A967079 and AMG9810 prevented the induction of inflammatory gene expression by 10% CSE. Fold changes in mRNA expression of IL-1β (C, G), IL-8 (D, H), IL-18 (E, I) and IL-33 (F, J) in A549 (C-F) and Beas-2b (G-J) cells as measured by quantitative RT-PCR. Mean values and data from individual experiments are shown. *P<0.05, **P<0.01, ***P<0.001.

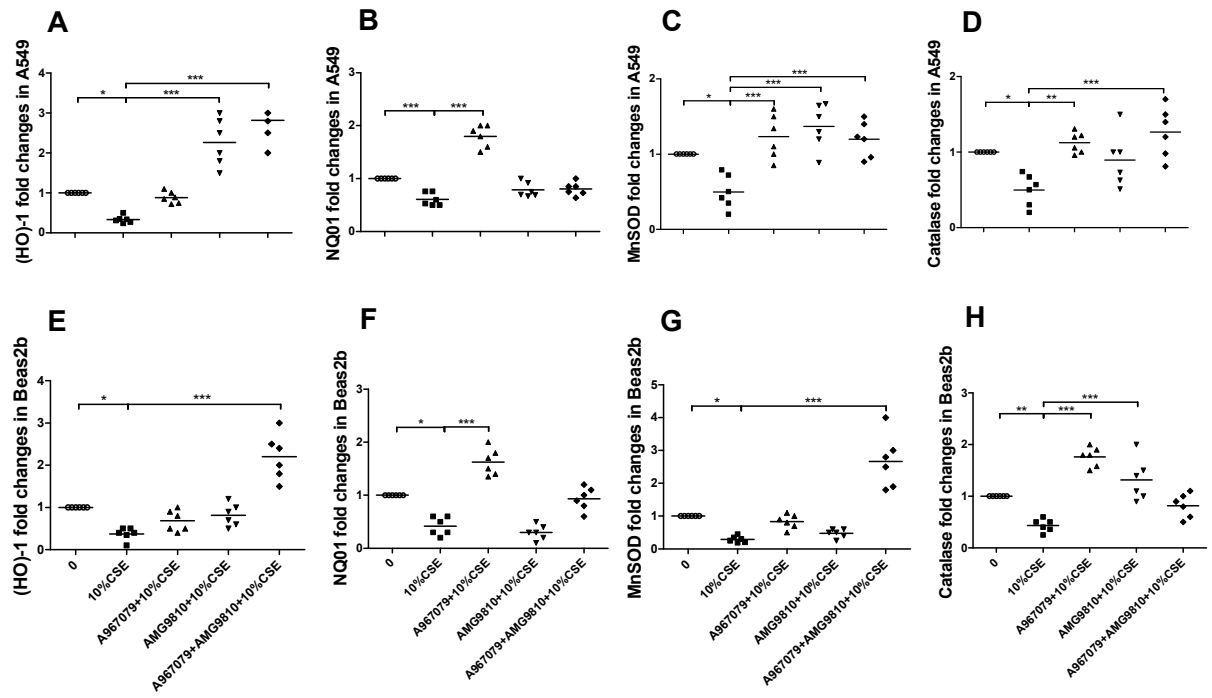


Figure 2. Effect of CSE and TRPA1/TRPV1 inhibitors on anti-oxidant gene expression. Pre-treatment of cells with the TRPA1 inhibitor (A967079, 100 μ M) and the TRPV1 inhibitor (AMG9810, 100 μ M) prevented the effects of 10% CSE. Fold changes in mRNA expression of heme oxygenase (HO)-1 (A, E), NQO1 (B, F), MnSOD (C, G) and Catalase (D, H) in A549 (A-D) and Beas-2b (E-H) cells as measured by quantitative RT-PCR. Mean values and data from individual experiments are shown. *P<0.05, **P<0.01, ***P<0.001.

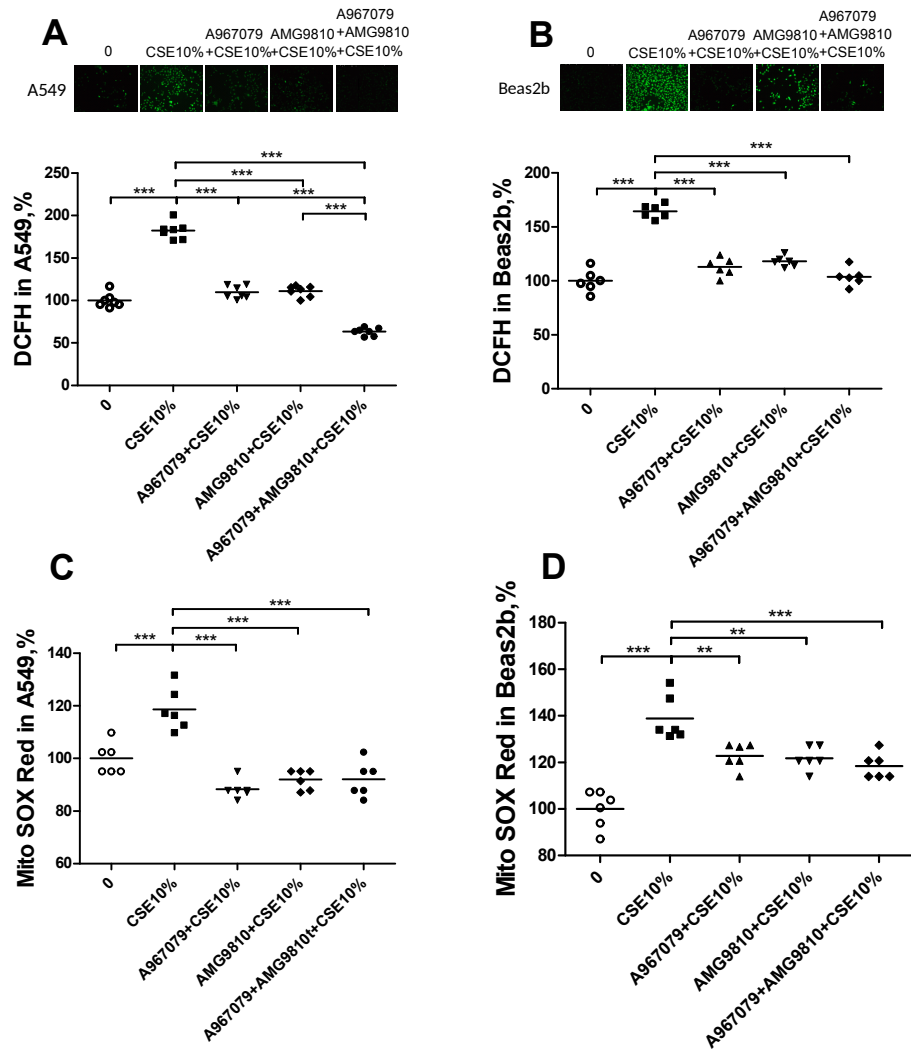


Figure 3. Effect of CSE and TRPA1/TRPV1 inhibitors on intracellular and mitochondrial ROS. The level of intracellular ROS was increased by exposure to 10% CSE for 24hrs as detected by DCFH in A549 cells (A) and in Beas-2b cells (B) using fluorescence microscopy (upper panel) and fluorescence plate reader (lower panel). Pretreatment of cells with the TRPA1 inhibitor (A967079, 100 μ M) and the TRPV1 inhibitor (AMG9810, 100 μ M) prevented the effects of CSE. Representative microscopy images are shown (x100) with mean values and data from individual plate reader experiments shown graphically below. Blocking TRPA1 and/or TRPV1 with inhibitors reduced CSE-induced mitochondrial ROS as measured by MitoSOX Red fluorescence in A549 cells (C) and Beas-2b cells (D). The mean values and data from individual experiments are shown graphically. *P<0.05, **P<0.01, ***P<0.001.

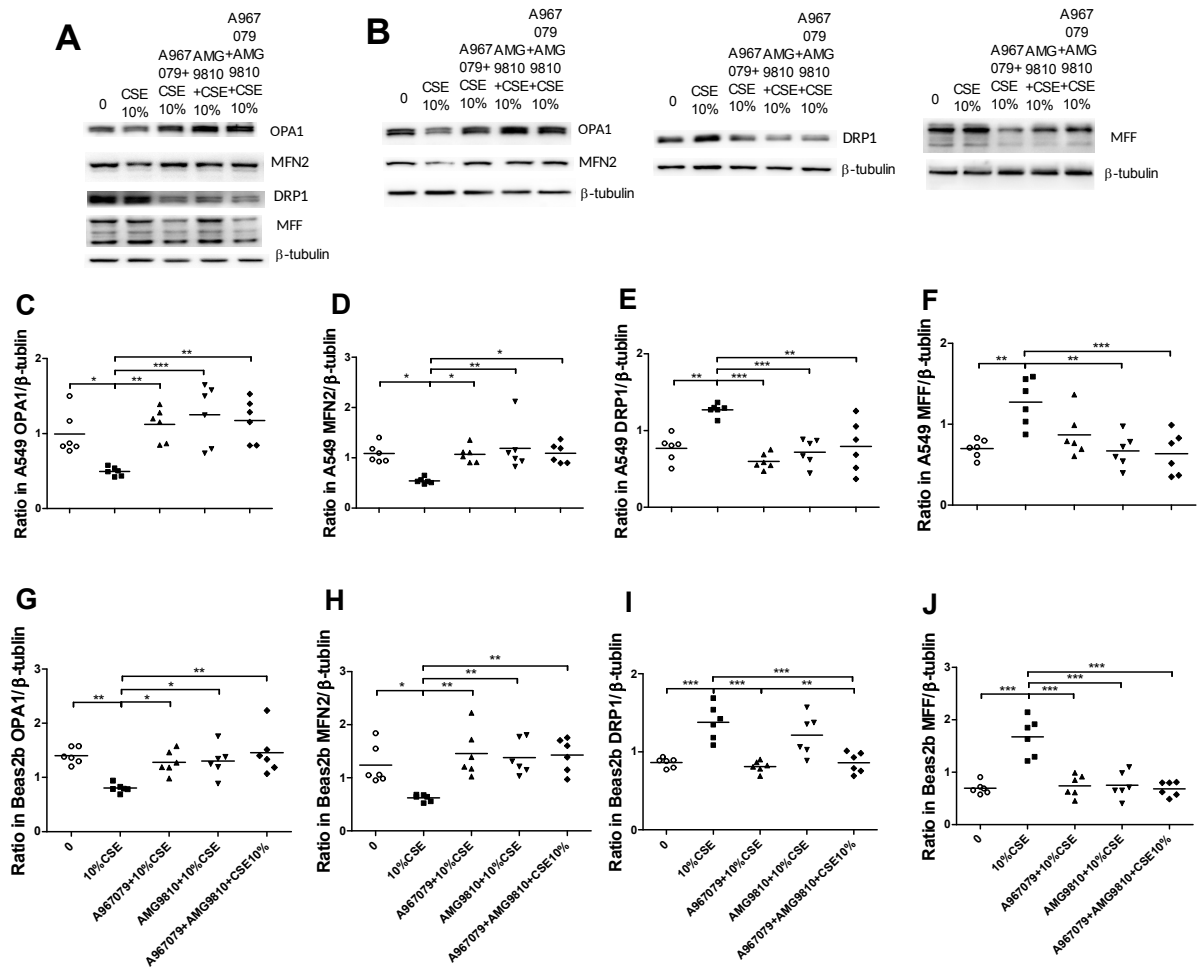


Figure 4. Effect of CSE and TRPA1/TRPV1 inhibitors on mitochondrial fission and fusion protein expression. Western blot analysis of the mitochondrial fusion and fission proteins Optic Atrophy 1 (OPA1), Mitofusin 2 (MFN2), dystrophin-related protein 1 (DRP1) and Mitochondrial Fission Factor (MFF) in A549 cells after exposed to CSE or pretreatment with inhibitors were shown (A). Western blot analysis of the mitochondrial fusion and fission proteins Optic Atrophy 1 (OPA1), Mitofusin 2 (MFN2), dystrophin-related protein 1 (DRP1) and Mitochondrial Fission Factor (MFF) in Beas-2b cells after exposed to CSE or pretreatment with inhibitors were shown (B). Pre-treatment of cells with the TRPA1 inhibitor (A967079, 100 μ M) and the TRPV1 inhibitor (AMG9810, 100 μ M) prevented the effects of 10% CSE. The ratio of Optic Atrophy 1 (OPA1), a Mitochondrial Dynamin-Like GTPase (C), Mitofusin 2 (MFN2, D), dystrophin-related protein 1 (DRP1, E) and of Mitochondrial Fission Factor (MFF, F) to β -tubulin as determined by Western blot analysis in A549 cells. The ratio of OPA1 (G), MFN2 (H), DRP1 (I) and of MFF (J) to β -

tubulin as determined by Western blot analysis in Beas-2b cells. Each panel shows a representative Western Blot with mean values and data from individual experiments presented graphically below. *P<0.05, **P<0.01, ***P<0.001.

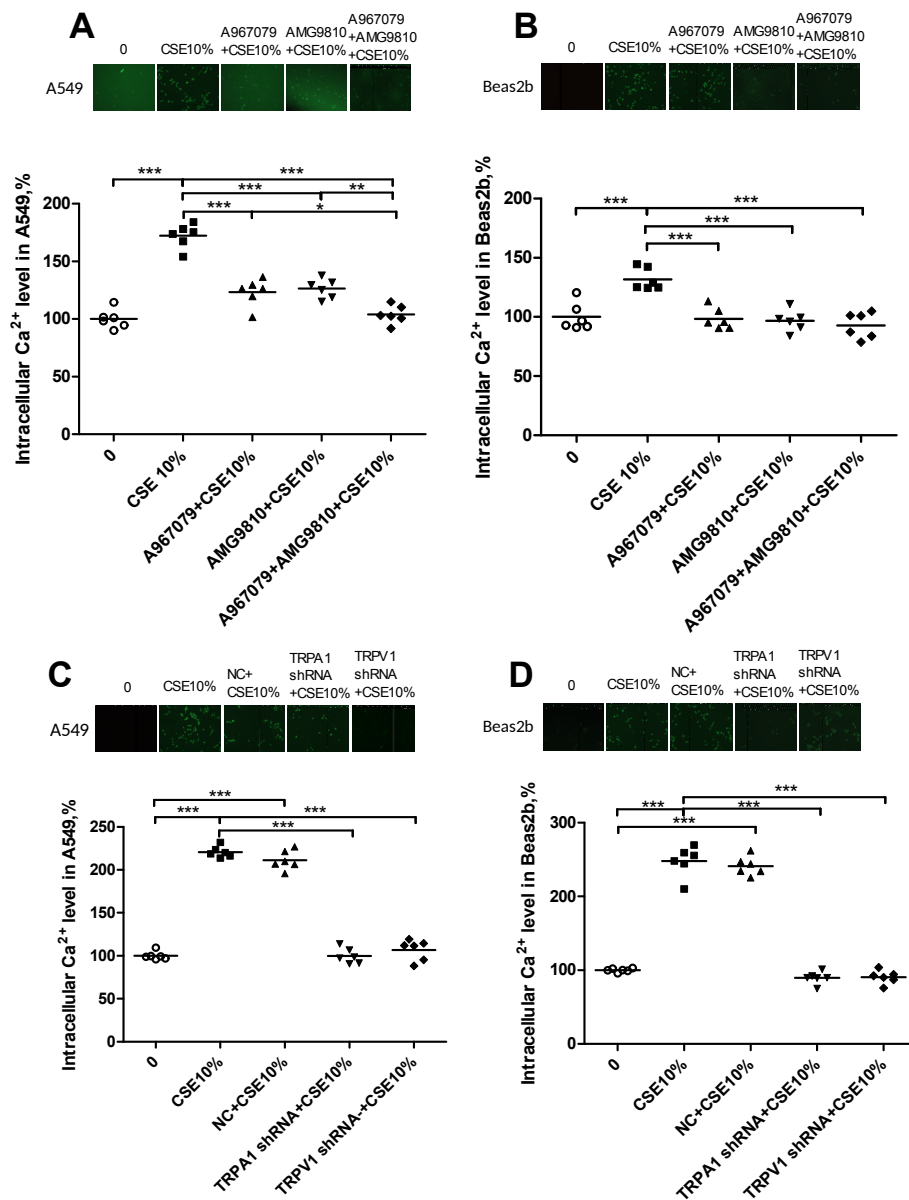


Figure 5. Effect of CSE, TRPA1/TRPV1 inhibitors, TRPA1/TRPV1 knockdown on

intracellular Ca^{2+} levels in A549 cells and Beas-2b cells. TRPA1 and TRPV1 inhibitors attenuated CSE-induced intracellular Ca^{2+} levels determined by fluorescence plate reader in A549 cells (A) and Beas-2b cells (B). Intracellular Ca^{2+} was enhanced by CSE and reduced in TRPA1 shRNA and TRPV1 shRNA A549 cells (C) and TRPA1 shRNA and TRPV1 shRNA Beas-2b cells (D). Representative microscopy images are shown (x100) with mean values and data from individual plate reader experiments shown graphically below. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NC = non-specific control shRNA.

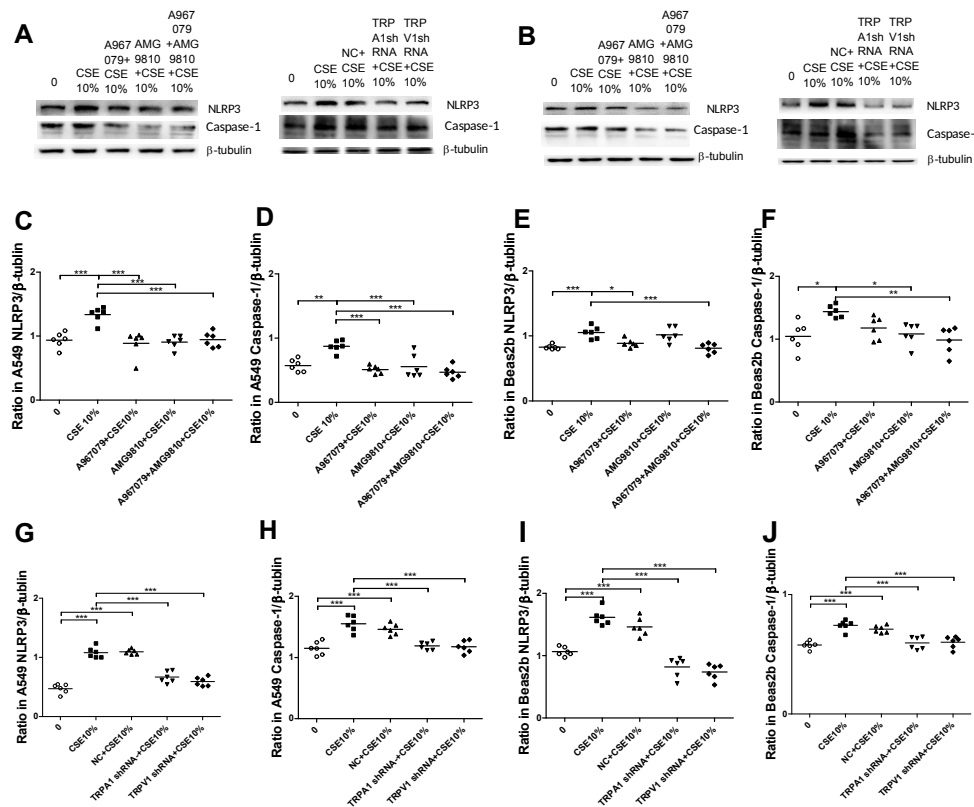
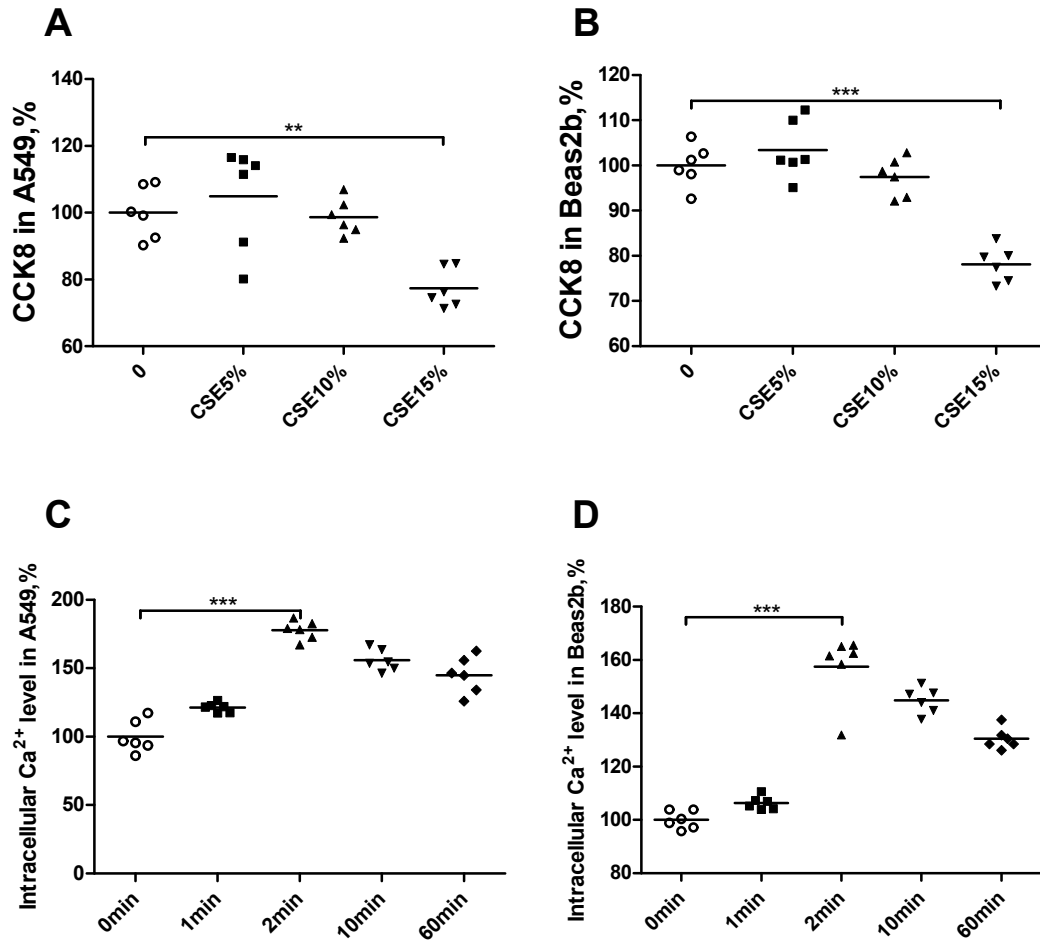


Figure 6. Effect of CSE, TRPA1/TRPV1 inhibitors, TRPA1/TRPV1 knockdown on inflammasome activation in A549 cells and Beas-2b cells. Western blot analysis of NLRP3 and Caspase-1 in A549 cells after exposed to CSE or pretreatment with inhibitors were shown (A). Western blot analysis of NLRP3 and Caspase-1 in Beas-2b cells after exposed to CSE or pretreatment with inhibitors were shown (B). Western Blot analysis showing the reduction in CSE-induced NLRP3 (A and C) and Caspase-1 (B and D) expression compared to β -tubulin in

A549 (A and B) and in Beas-2b cells (C and D). TRPA1 (TRPA1 shRNA) and TRPV1 (TRPV1 shRNA) blocked reverse the induction of NLRP3 (E and G) and of Caspase-1 (F and H) by CSE in A549 (E and F) and in Beas-2b (G and H) cells. Each panel shows a representative Western Blot with the mean values and data from individual experiments shown below. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NC = non-specific control shRNA.



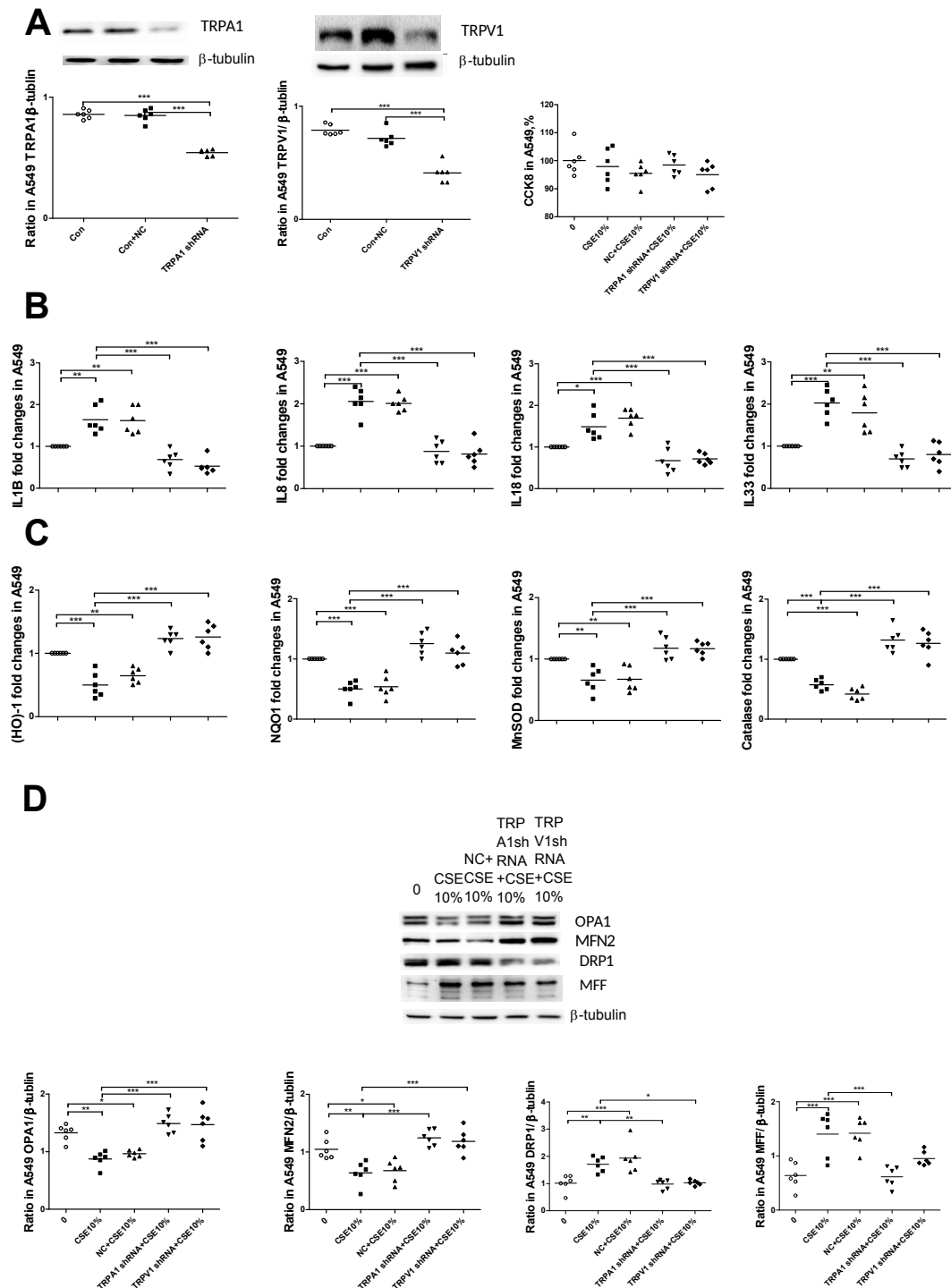
Supplement 1. Dose dependent effect of CSE on cell viability and intracellular Ca^{2+} levels.

Cell viability was reduced by increasing doses of CSE in A549 cells (A) and Beas-2b cells (B).

Time-dependent effect of 10% CSE on intracellular Ca^{2+} concentrations in A549 (C) and Beas-2b

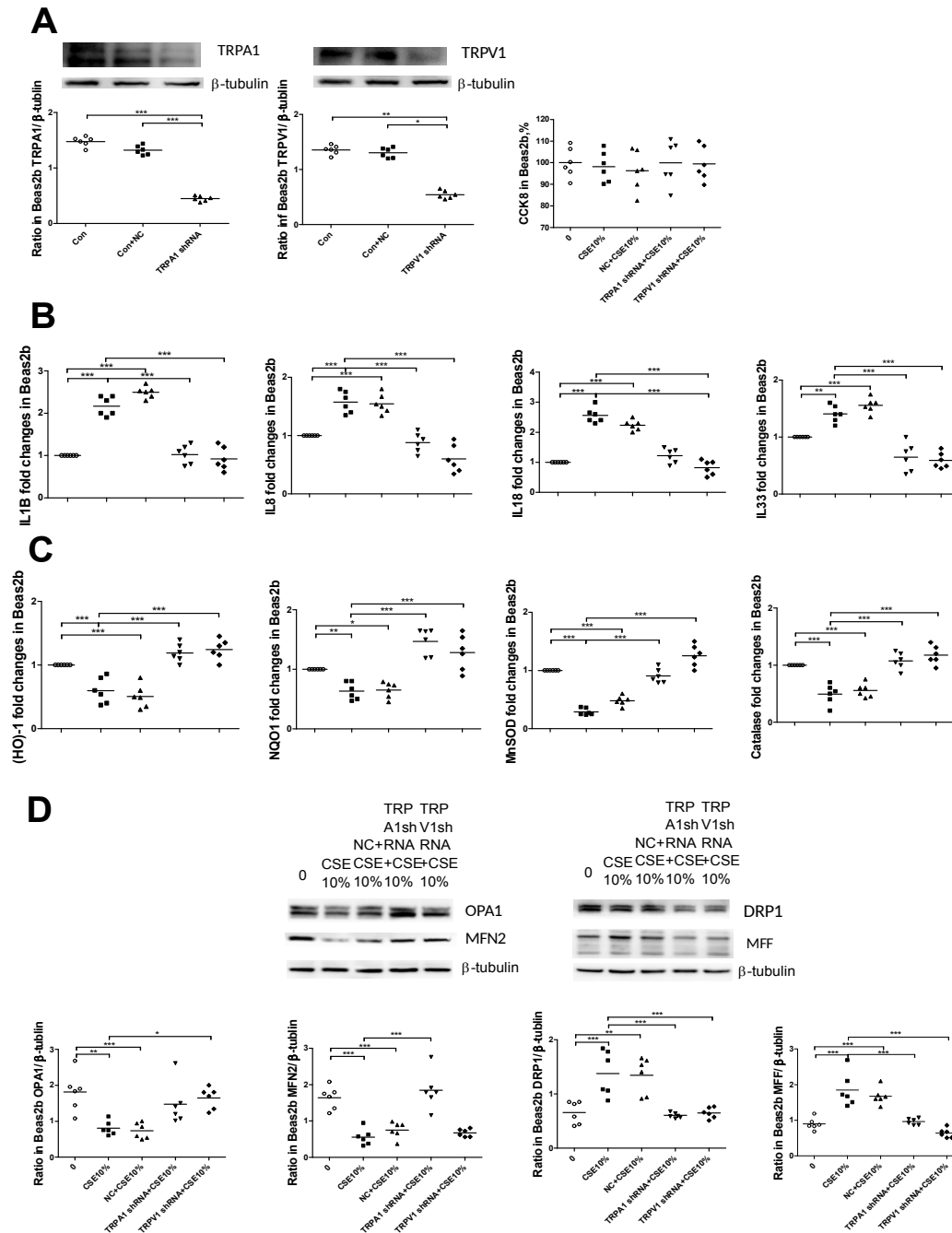
(D) cells. Mean values and data from individual experiments are shown. * $P < 0.05$, ** $P < 0.01$,

*** $P < 0.001$.



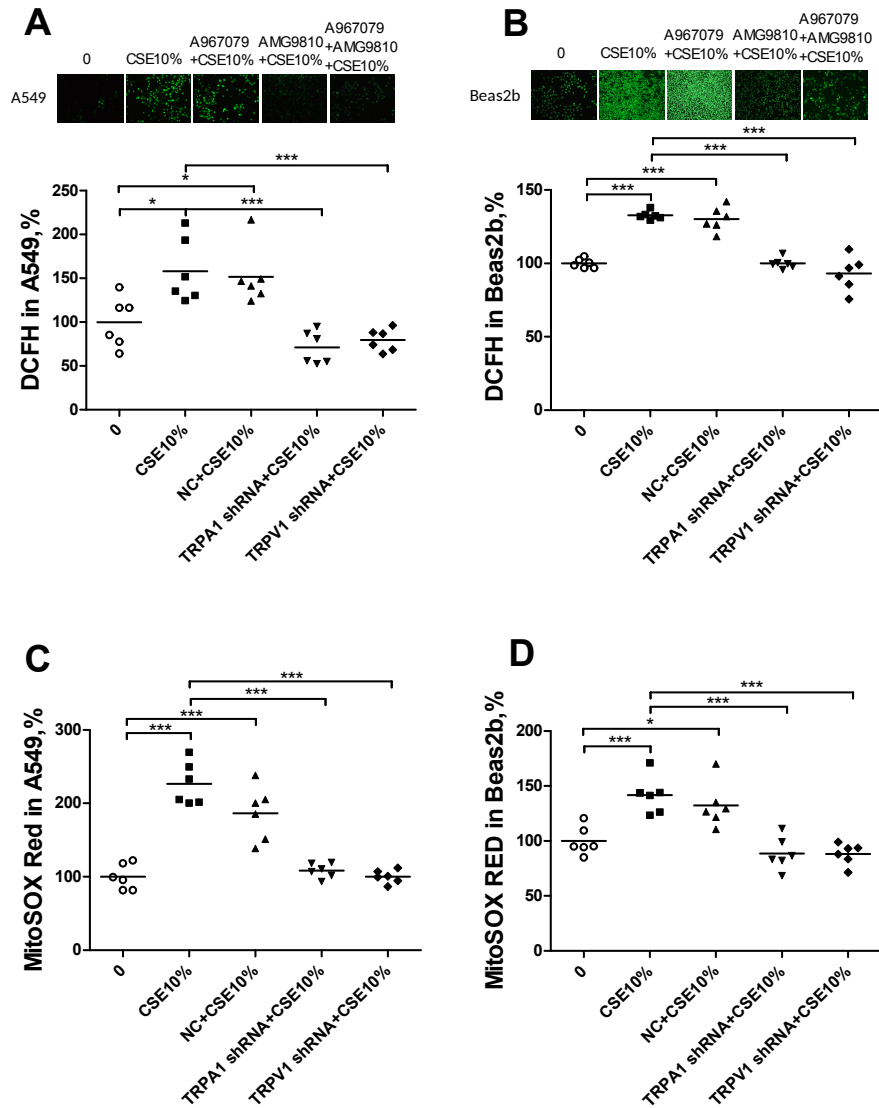
Supplement 2. Effect of CSE and TRPA1/TRPV1 knockdown on inflammatory, anti-oxidant and mitochondrial gene expression in A549 cells. Cell viability was measured using the CCK8 assay in A549 cells (A) and was not significantly affected by 10% CSE or pretreatment of cells with the TRPA1 inhibitor (A967079, 100μM) and the TRPV1 inhibitor (AMG9810, 100μM).

The ability of selective short hairpin (sh)RNAs (A) to attenuate TRPA1 (TRPA1 shRNA, left panel) and TRPV1 (TRPV1 shRNA, middle panel) protein expression in A549 cells. Neither CSE nor knock down of TRPA1 or TRPV1 affected cell viability (right panel). Functional analysis of 10% CSE and TRPA1 and TRPV1 knockdown on fold changes in (B) interleukin (IL)-1 β , IL-8, IL-18 and IL-33 mRNA expression and (C) the mRNA expression of the anti-oxidant genes heme oxygenase (HO)-1, NAD(P)H quinone dehydrogenase 1 (NQO1), superoxide dismutase 2 (MnSOD) and catalase genes. (D) The effect of CSE on the expression of the mitochondrial fusion and fission proteins Optic Atrophy 1 (OPA1), Mitofusin 2 (MFN2), dystrophin-related protein 1 (DRP1) and Mitochondrial Fission Factor (MFF) compared to β -tubulin as measured by Western blotting in TRPA1 shRNA and TRPV1 shRNA cells. Representative blots are shown with the mean values and data from individual experiments given below. *P<0.05, **P<0.01, ***P<0.001. NC = non-specific control shRNA.



Supplement 3. Effect of CSE and TRPA1/TRPV1 knockdown on inflammatory, anti-oxidant and mitochondrial gene expression in Beas-2b cells. Cell viability was measured using the CCK8 assay in Beas-2b cells (A) and was not significantly affected by 10% CSE or pretreatment of cells with the TRPA1 inhibitor (A967079, 100 μ M) and the TRPV1 inhibitor (AMG9810, 100 μ M). The ability of selective short hairpin (sh)RNAs (A) to attenuate TRPA1 (TRPA1 shRNA, left panel) and TRPV1 (TRPV1 shRNA, middle panel) protein expression in A549 cells. Neither

CSE nor knock down of TRPA1 or TRPV1 affected cell viability (right panel). Functional analysis of 10% CSE and TRPA1 and TRPV1 knockdown on fold changes in (B) interleukin (IL)-1 β , IL-8, IL-18 and IL-33 mRNA expression and (C) the mRNA expression of the anti-oxidant genes heme oxygenase (HO)-1, NAD(P)H quinone dehydrogenase 1 (NQO1), superoxide dismutase 2 (MnSOD) and catalase genes. (D) The effect of CSE on the expression of the mitochondrial fusion and fission proteins Optic Atrophy 1 (OPA1), Mitofusin 2 (MFN2), dystrophin-related protein 1 (DRP1) and Mitochondrial Fission Factor (MFF) compared to β -tubulin as measured by Western blotting in TRPA1 shRNA and TRPV1 shRNA cells. Representative blots are shown with mean values and data from individual experiments given below. *P<0.05, **P<0.01, ***P<0.001. NC = non-specific control shRNA.



Supplement 4. Effect of CSE and TRPA1/TRPV1 knockdown on intracellular and mitochondrial ROS in A549 cells and Beas-2b cells. Intracellular ROS was increased by 10% CSE for 24hrs in TRPA1 shRNA or TRPV1 shRNA A549 cells (A) and TRPA1 shRNA or TRPV1 shRNA Beas-2b cells (B) as detected by DCFH using fluorescence microscopy (upper panel) and fluorescence plate reader (lower panel). Representative microscopy images are shown (x100) with mean values and data from individual plate reader experiments reported graphically below. The level of mitochondrial ROS as detected by MitoSOX Red was enhanced by CSE and reduced in TRPA1 shRNA and TRPV1 shRNA A549 cells (C) and TRPA1 shRNA and TRPV1 shRNA Beas-2b cells (D). The mean values and data from individual experiments are shown graphically. *P<0.05, **P<0.01, ***P<0.001. NC = non-specific control shRNA.

RESULTS

Dose dependent effect of CSE on cell viability and intracellular Ca²⁺ levels

Initial experiments examined the effect of CSE on cell survival in A549 and in Beas-2b cells using CCK8 analysis (Supplement 1A, B). There was a dose-dependent loss of viability that was significant at 15% CSE in both cell types. 10% CSE had a time-dependent effect on intracellular Ca²⁺ mobilization in both A549 and Beas-2b cells that peaked at 2 min (Supplement 1C, D). This suggests that calcium channel activation may occur with exposure to CSE. 10% CSE was used in all subsequent experiments.

Effect of CSE and TRPA1/TRPV1 blocked on inflammatory, anti-oxidant and mitochondrial gene expression in A549 cells

Transfection of A549 cells with TRPA1 shRNA plasmids (Supplement 2A left panel) or TRPV1 shRNA plasmids (Supplement 2A middle panel) for 48hrs resulted in a 50-60% reduction in their respective proteins. Transfection of control shRNA plasmids or control media did not affect TRPA1 or TRPV1 protein expression. These cells were designated TRPA1 shRNA A549 and TRPV1 shRNA A549 cells and used in subsequent studies. There was no effect on cell viability of TRPA1 or TRPV1 knockdown or of CSE exposure in these cells (Supplement 2A right panel).

The induction of inflammatory cytokine (Supplement 2B) and repression of anti-oxidant gene (Supplement 2C) mRNAs by CSE exposure was unaffected in vehicle or shRNA controls in A549 cells. CSE-enhanced IL-1 β , IL-8, IL-18 and IL-33 mRNA was decreased in CSE-exposed TRPA1 shRNA A549 (Supplement 2B) cells and in TRPV1 shRNA A549 cells (Supplement 2B). In contrast, the down-regulation of anti-oxidant genes by CSE was completely

reversed and reached levels above baseline. The mRNA levels of HO-1, NQO1, MnSOD and catalase were increased in CSE-exposed TRPA1 shRNA A549 cells (Supplement 2C) and TRPV1 shRNA A549 cells (Supplement 2C).

CSE reduced OPA1 and MFN2 and enhanced DRP1 and MFF protein expression in wild type A549 cells. These effects were not affected by transfection vehicle or shRNA controls (Supplement 2D). Compared to CSE-exposed wild type A549 cells, CSE-exposed TRPA1 shRNA A549 cells showed increased OPA1 and MFN2 (Supplement 2D) and reduced DRP1 and MFF (Supplement 2D) protein expression. Similar results were seen in CSE-exposed TRPV1 shRNA A549 cells which showed increased OPA1 and MFN2 protein (Supplement 2D) and reduced DRP1 protein (Supplement 2D).

Effect of CSE and TRPA1/TRPV1 blocked on inflammatory, anti-oxidant and mitochondrial gene expression in Beas-2b cells

Transfection of Beas-2b cells with TRPA1 shRNA plasmids (Supplement 3A left panel) or TRPV1 shRNA plasmids (Supplement 3A middle panel) for 48hrs resulted in a 50-60% reduction in their respective proteins. Transfection of control shRNA plasmids or control media did not affect TRPA1 or TRPV1 protein expression. These cells were designated TRPA1 shRNA Beas-2b and TRPV1 shRNA Beas-2b cells and used in subsequent studies. There was no effect on cell viability of TRPA1 or TRPV1 knockdown or of CSE exposure in these cells (Supplement 3A right panel).

The induction of inflammatory cytokine (Supplement 3B) and repression of anti-oxidant gene (Supplement 3C) mRNAs by CSE exposure was unaffected by vehicle or shRNA controls in wild type Beas-2b cells. CSE-enhanced IL-1 β , IL-8, IL-18 and IL-33 mRNA was decreased in CSE-exposed TRPA1 shRNA Beas-2b (Supplement 3B) cells and in TRPV1 shRNA Beas-2b cells (Supplement 3B). In contrast, the down-regulation of anti-oxidant genes by CSE was completely reversed and reached levels above baseline. The mRNA

levels of HO-1, NQO1, MnSOD and catalase were increased in CSE-exposed TRPA1 shRNA Beas-2b cells (Supplement 3C) and TRPV1 shRNA Beas-2b cells (Supplement 3C).

CSE reduced OPA1 and MFN2 and enhanced DRP1 and MFF protein expression in wild type Beas-2b cells. These effects were not affected by transfection vehicle or shRNA controls (Supplement 3D). Compared to CSE-exposed wild type Beas-2b cells, the protein level of OPA1 significantly increased in TRPV1 shRNA Beas-2b cells and MFN2 significantly increased in TRPA1 shRNA Beas-2b cells (Supplement 3D). The protein levels of DRP1 and MFF were markedly decreased in TRPA1 shRNA Beas-2b cells and TRPV1 shRNA Beas-2b cells (Supplement 3D).

Effect of CSE and TRPA1/TRPV1 blocked on intracellular and mitochondrial ROS in A549 cells and Beas-2B cells

CSE exposure for 24 hours enhanced intracellular ROS production in A549 (Supplement 4A) and Beas-2b (Supplement 4B) cells. There were no differences in the induction of intracellular ROS between wild type cells exposed to CSE and cells pretreated with a negative control shRNA control in A549 (Supplement 4A) or Beas-2b cells (Supplement 4B). There were reduced levels of intracellular ROS in CSE-exposed TRPA1 shRNA A549 cells and CSE-exposed TRPV1 shRNA A549 cells compared to CSE-exposed wild type A549 cells (Supplement 4A). Reduced levels of intracellular ROS were also observed in CSE-exposed TRPA1 shRNA Beas-2b cells and CSE-exposed TRPV1 shRNA Beas-2b cells compared to CSE-exposed wild type Beas-2b cells (Supplement 4B).

There were no differences in the mitochondrial ROS response to CSE exposure between wild type cells and control shRNA treated A549 cells (Supplement 4C) or Beas-2b cells (Supplement 4D). There were reduced levels of mitochondrial ROS in CSE-exposed TRPA1 shRNA A549 cells and

CSE-exposed TRPV1 shRNA A549 cells compared to CSE-exposed wild type A549 cells (Supplement 4C). There were reduced levels of mitochondrial ROS in CSE-exposed TRPA1 shRNA Beas-2b cells and CSE-exposed TRPV1 shRNA Beas-2b cells compared to CSE-exposed wild type Beas-2b cells (Supplement 4D).