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Activation of Transient Receptor Potential Ankyrin-1 (TRPA1) in Lung Cells by Wood Smoke Particulate Material

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Abstract

Cigarette smoke, diesel exhaust, and other combustion-derived particles activate the calcium channel transient receptor potential ankyrin-1 (TRPA1), causing irritation and inflammation in the respiratory tract. It was hypothesized that wood smoke particulate and select chemical constituents thereof would also activate TRPA1 in lung cells, potentially explaining the adverse effects of wood and other forms of biomass smoke on the respiratory system. TRPA1 activation was assessed using calcium imaging assays in TRPA1-overexpressing HEK-293 cells, mouse primary trigeminal neurons, and human adenocarcinoma (A549) lung cells. Particles from pine and mesquite smoke were less potent agonists of TRPA1 than an equivalent mass concentration of an ethanol extract of diesel exhaust particles; pine particles were comparable in potency to cigarette smoke condensate, and mesquite particles were the least potent. The fine particulate (PM<2.5 μm) of wood smoke were the most potent TRPA1 agonists and several chemical constituents of wood smoke particulate: 3,5-*ditert*-butylphenol, coniferaldehyde, formaldehyde, perinaphthenone, agathic acid, and isocupressic acid were TRPA1 agonists. Pine particulate activated TRPA1 in mouse trigeminal neurons and A549 cells in a concentration-dependent manner, which was inhibited by the TRPA1 antagonist HC-030031. TRPA1 activation by wood smoke particles occurred through the electrophile/oxidant-sensing domain (i.e., C621/C641/C665/K710), based on the inhibition of cellular responses when the particles were pre-treated with glutathione; a role for the menthol-binding site of TRPA1 (S873/T874) was demonstrated for 3,5-*ditert*-butylphenol. This study demonstrated that TRPA1 is a molecular sensor for wood smoke particulate and several chemical constituents thereof, in sensory neurons and A549 cells, suggesting that TRPA1 may mediate some of the adverse effects of wood smoke in humans.

Keywords

Wood smoke particulate material; combustion-derived particulate material; transient receptor potential ankyrin-1 (TRPA1)

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Supporting Information Available:
Supplementary figures showing the smoke particle collection devices, chromatograms, and qualitative results for aldehyde and ketone analysis are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Introduction

Wood smoke particulate material (WSPM) is a common and often unavoidable combustion-derived indoor and outdoor air pollutant.[1,2] Where primitive wood burning stoves are the mainstay for cooking and heating, long-term exposure to high concentrations of WSPM and other forms of biomass smoke PM (BSPM) has been linked to progressively deteriorating respiratory function, exacerbation of pre-existing respiratory conditions such as asthma and heart disease, increased rates of respiratory infections, the development of chronic obstructive pulmonary disease and emphysema, and premature death.[1–5] Adverse effects on the respiratory system also have been reported by studies of short-term and seasonal elevations in WSPM/BSPM due to forest fires, crop burning, and home heating.[1,2,5]

While it is clear that wood and biomass smoke particles impact human health, the molecular and cellular processes underlying many of the commonly reported adverse effects are not completely understood. Similar to other combustion-derived particulate materials (cdPM), WSPM is comprised of carbon soot coated with chemicals including polycyclic aromatic hydrocarbons (PAHs), aldehydes and ketones, and other redox active oxygenated hydrocarbons.[6,7] WSPM has been shown to increase the production of pro-inflammatory mediators (e.g., IL-8, TNF-α, others) in lung cells via oxidative stress.[6,8,9] Additionally, WSPM has been shown to promote lipid peroxidation and oxidative damage to DNA *in vitro* and *in vivo*.[7,10] However, not all of the biological effects of WSPM can be directly attributed to oxidative stress.

An alternate mechanism by which WSPM may exert pulmonary toxicity could involve transient receptor potential-ankyrin 1 (TRPA1). TRPA1 is a cation channel that is a molecular sensor for electrophiles and oxidants in the respiratory tract, including H_2O_2 and hypochlorite,[11,12] aldehydes in cigarette smoke and cigarette smoke condensate (CSC),[13,14] and electrophiles on diesel exhaust particles (DEP).[15,16] TRPA1 is abundantly expressed on C-fibers originating in the trigeminal and vagal ganglia, which innervate the upper airways (i.e., the nose and mouth), conducting airways (i.e., the trachea, bronchi, and terminal bronchioles), and the respiratory bronchioles, alveolar ducts and alveoli. C-fibers frequently “sense” the presence of potentially toxic inhaled irritants and toxicants.[11,12,17] In airway C-fibers, TRPA1 is co-expressed with transient receptor potential vanilloid-1 (TRPV1), calcitonin-gene related peptide, neurokinin A, and substance P. When these neurons are stimulated by agonists of TRPV1 or TRPA1, they decrease respiratory drive, trigger cough and bronchoconstriction, and neurogenic inflammation. TRPA1 is also expressed by non-neuronal cells including human lung adenocarcinoma (A549) and small airway epithelial cells (SAEC), smooth muscle cells, and fibroblasts. When treated with TRPA1 agonists such as CSC, acrolein, allyl-isothiocyanate (AITC), 4-hydroxynonenal (4-HNE), and crotonaldehyde, these cells produce interlekin-8 (CXCL1/KC) and other pro-inflammatory mediators that promote non-neurogenic inflammation.[18,19] In addition to its role as a molecular sensor for pulmonary irritants, TRPA1 has also been shown to play a central role in the development of ovalbumin-induced airway hypersensitivity in mice.[20] These characteristics make TRPA1 an attractive target to study as a potential mediator of WSPM-related health effects.

The hypothesis of this study was that TRPA1 would be preferentially activated by WSPM in lung cell models, based on similarities in the chemical composition of WSPM to other cdPM (i.e., CSC and DEP) that have been shown to activate TRPA1. Using calcium imaging (Fluo-4 and Fura-2) in TRPA1-overexpressing HEK-293 cells, wild-type and mutant forms of TRPA1, the TRPA1 antagonist HC-030031, and different samples of WSPM and select constituents thereof, it has been demonstrated that TRPA1 is a molecular sensor of WSPM

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in cells that represent key cellular targets and mediators of pulmonary toxicity for multiple types of particulate materials.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Agathic acid, dihydroagathic acid, isocupressic acid, abietic acid, dehydroabietic acid, and tetrahydroagathic acid were provided by the USDA Poisonous Plants Research Laboratory, Logan, UT.

Preparation of WSPM

Size-fractionated WSPM was produced by burning ~10 g fresh Austrian pine or dry mesquite wood in a laboratory furnace and collecting the particulate material (PM) using an Anderson cascade impactor (Supplementary Figure 1). Briefly, an electric furnace (Blue M, New Columbia, PA) was fitted with a 90 × 2.7 cm i.d. steel tube open at one end to allow insertion of wood and intake of combustion air. The tube was heated to 750°C to induce flaming combustion within seconds of inserting split wood, ~3 to 10 mm thick. Downstream of the combustion tube, room air (4:1) was added to dilute and cool the smoke and allow for the condensation of semi-volatile components into/onto PM, mimicking the process that naturally occurs in smoke plumes. PM was collected using a 10-stage Andersen cascade impactor (ThermoAndersen, Smyrna GA), providing size-fractionated PM between 0.49 to 10 μm in aerodynamic diameter. Total flow through the impactor was monitored using flow-meters and manually regulated at 1L/min. The WSPM was a mixture of tar and solid carbonaceous particles (shown in Figure 1), which were recovered by washing the impactor stages with a minimal volume of 100% ethanol. The suspension was then dried under a stream of filtered air and stored at −20°C in the dark.

Preparation of Other cdPM

DEP was collected from an on-road “black smoker” 2004 Ford F350 truck and was extracted with ethanol (DEP-EtOH) to produce an oily, tar-like material enriched in TRPA1 agonists and similar in consistency to the WSPM and CSC used herein, as previously described by our group.[15] CSC was isolated from equilibrated 3R4F reference cigarettes (University of Kentucky Reference Cigarette Program, Lexington, KY) using a single-port smoking machine operated essentially as described by the Massachusetts Standard smoking Regimen, but without blocking the vent holes (Supplementary Figure 2).

Preparation of Particle Treatment Solutions

Each particle was re-suspended in DMSO to a concentration of 115 mg/mL and subsequently diluted to the final working concentrations in LHC-9 media (Invitrogen). For all cell treatments, the final concentration of DMSO in the treatment solutions was <1% (v/v). For screening TRP channel activation by WSPM, a concentration of up to 2.3 mg/mL was used. For kinetic studies, a concentration of 1.15 mg/mL was used, which allowed for differences in TRPA1 activation (i.e., potency) and kinetics of the calcium flux response to be differentiated between WSPM, CSC, and DEP-EtOH. For mechanistic studies, concentrations of 0.09 mg/mL and 0.19 mg/mL pine and mesquite PM were used, respectively. These concentrations produced robust responses without saturation of the response or overwhelming the capacity of glutathione (GSH) to inhibit the response.

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Cloning, Expression, and Site-Directed Mutagenesis

Human TRPA1 and the TRPA1-3CK mutant were cloned and over-expressed in human embryonic kidney (HEK-293) cells, as previously described.[15] Construction of the TRPA1-ST mutant, a loss of function mutant for menthol,[21] was performed using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers were: TRPA1-S873V/T874L (+) 5′-GTTGGAGGTAATTTTGAAAACTTTGTTGAGGGTTTTAGTTGTATTTATCTTCCTTC TT CTGGCTTTT-3′; and (−) 5′-AAAAGCCAGAAGAAGGAA GATAAATACAACTAAAACCCTCAACAAAGTTTTCAAAATTACCTCCAA C-3′.

Cell Culture

Cells were maintained in a humidified cell culture incubator at 37°C with a 95% air:5% CO_2 atmosphere. HEK-293 cells (ATCC; Rockville, MD) and human TRPA1 over-expressing HEK-293 cells[15] were cultured in DMEM:F12 media containing 5% fetal bovine serum and 1x penicillin/streptomycin; for TRPA1-overexpressing cells, Geneticin (300 μg/mL) was also included. Cells were sub-cultured using trypsin. Transient transfection of HEK-293 cells with TRPA1 mutant plasmids (i.e., TRPA1-3CK and TRPA1-ST) using Lipofectamine 2000 (Invitrogen) was also performed as previously described.[15,22] Human adenocarcinoma (A549) cells (ATCC; Rockville, MD), were cultured in DMEM containing 5% FBS and 1x penicillin/streptomycin, and were sub-cultured using trypsin.

Calcium Imaging Assays

Cells were plated in 96-well plates (coated with 1% gelatin for HEK-293 cells) and grown to 80–90% confluence before loading with Fluo 4-AM, a fluorescent calcium indicator, using the Fluo-4 Direct assay kit (Invitrogen). The Fluo 4-AM loading solution was diluted 1:1 in LHC-9 (HEK-293 cells) or calcium buffer (1X HBSS, 20 mM HEPES, pH 7.3; A549 cells) and applied to cells for 60 min at 37°C (HEK-293 cells) or room temperature (A549 cells) in the dark. Thirty minutes prior to analysis, the loading solution was replaced with LHC-9 (HEK-293 cells) or calcium buffer (A549 cells), containing 1 mM probenecid and 0.75 mM trypan red (ATT Bioquest). Treatment-induced changes in cellular fluorescence were quantified from fluorescence micrographs or using a NOVOStar fluorescence plate reader (BMG Labtech; Offenberg, Germany), as previously described.[15,22] All agonist/particle treatment solutions were prepared in LHC-9 or calcium buffer at 3X concentration, and added to cells at room temperature. For A549 cells, the TRPA1 antagonist HC-030031 was added the wash buffer 30 min before assaying using the plate reader. All data were corrected for non-specific responses, if any, observed with HEK-293 cells, and then normalized to the maximum attainable change in fluorescence elicited by ionomycin (10 μM). Additional normalization to a maximum stimulatory concentration of AITC (150 μM), a positive control for TRPA1, was performed in selected experiments, as noted in the figure legends.

Mouse Trigeminal Neuron Calcium Imaging Assays

Experimental procedures were approved by the University of Utah Animal Care and Use Committee. Mouse TG ganglia were isolated from 3-week old C57Bl/6 mice anesthetized with isoflurane and euthanized by cervical dislocation. The TG neurons were cultured as described for mouse dorsal root ganglia (DRG) neurons.[23] Isolated TG were prepared for imaging by loading with Fura 2-AM and assayed as previously described.[15] Cells were imaged and data was obtained using the Meta Imaging Series Metafluor program (Universal Imaging). Agonists and antagonists were sequentially assayed by exchanging the treatment solutions using two pipettes, one to remove and one to add the solutions, with a buffer wash between treatments. Pine PM was prepared in LHC-9 containing 0.2% v/v DMSO and 0.2% v/v ethanol. Following treatment with pine PM (0.023, 0.073, or 0.23 mg/mL), cells were

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washed, followed by a treatment with 50 μM AITC to identify TRPA1-expressing neurons, washed again, and then treated with KCl (50 mM) to identify all viable neurons. For studies using the TRPA1 antagonist HC-030031, cells were pre-treated with 50 μM HC-030031 for 30 s prior to co-treatment with particles (0.073 mg/mL), AITC, and KCl. Only viable cells responding to KCl were considered in the data analysis and the data are represented as the percentage of cells responding to the agonist relative to KCl.

Analysis of WSPM Aldehydes and Ketones

Formaldehyde, 5-hydroxymethylfurfural, acrolein, acetone, furfural, 4-hydroxybenzaldehyde, vanillin, coniferaldehyde, 2-butanone, 1,2-naphthoquinone, perinaphthenone, benzaldehyde, *o*-anisaldehyde, and glyoxal (i.e., chemical constituents of cdPM including WSPM) were assayed as (2,4-dinitrophenyl)hydrazone derivatives (i.e., hydrazones) using liquid chromatography-negative ion electrospray ionization tandem mass spectroscopy (LC/MS^2), essentially as described.[24,25] LC/MS^2 was performed using a Thermo-Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo, San Jose, CA). Standards were prepared by reacting 25 mg pure chemical with 3 mL Brady's reagent (37 mg/mL 2,4-dinitrophenylhydrazine in 75% methanol containing 7.5% (v/v) concentrated sulfuric acid). The resulting hydrazone derivatives were collected by centrifugation, washed 3X with 20% (v/v) methanol in water, and dried under a stream of air. The analytes were chromatographically separated using a Gemini C_{18} HPLC column (150 × 2 mm, 5 μ) eluted at 0.3mL/min with the following stepwise gradient of acetonitrile and water at 40°C: 40→50% acetonitrile from 0 to 26 minutes; 50→100% acetonitrile from 26 to 35 minutes; 100% acetonitrile from 35 to 37.5 minutes. A representative chromatogram showing the precursor-to-product ion transitions used to detect each analyte is provided as Supplementary Figure 3. Analysis of these aldehydes and ketones in WSPM achieved by reacting 115 μg WSPM from each impactor stage fraction (from Figures 1 and 3) with 250 μL Brady's reagent, and processing as described above. For comparison of specific aldehydes and ketones in the various size fractions of pine and mesquite WSPM, peak intensity was used (Supplementary Figure 4). For quantitative analysis of formaldehyde and coniferaldehyde (i.e., TRPA1 agonists), standard curves were used.

qPCR Analysis of TRPA1 Expression in A549 cells

Cells were cultured in 25 cm^2 flasks and grown to 90% density. Total RNA was extracted from cells using the RNeasy mini kit (QIAGEN, Valencia, CA), and 2.5 μg of the total RNA was converted to cDNA using iScript (BioRad, Hercules, CA). The resulting cDNA was diluted 1:5 for analysis by quantitative real-time PCR (qPCR). qPCR was performed using LightCycler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN) with a Light-Cycler 480 System as previously described.[22] Values for TRPA1 were normalized to β2-macroglobulin (β2M). Primer sequences were: β2M (+) 5′ – GATGAGTATGCCTGCCGTGTG – 3′ and (−) 5′ – CAATCCAAATGCGGCATCT – 3′; human TRPA1 (+) 5′ – TCACCATGAGCTAGCAGACTATTT – 3′ and (−) 5′ – GAGAGCGTCCTTCAGAATCG – 3′.

Statistical Analysis

Values represent the mean ± SEM unless otherwise stated. One-way or two-way ANOVA with post-testing at the 95% confidence interval was used to determine significance, as indicated in each figure legend.

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Results

Production and collection of WSPM

An image showing the size distribution of pine PM collected on the stages of the Anderson cascade impactor plates is provided as Figure 1. The majority of material deposited on stages 5 and 6 corresponding to PM 0.43 to 2.1 μm; the WSPM appears as dark colored material on the circular, silver impactor stage plates. An identical deposition pattern was observed for mesquite PM, except that the PM was less oily (data not shown). Pine and mesquite PM from stage 5 (1.1–2.1 μm) was used for initial TRP channel activation studies due to the relative abundance of material in this fraction.

Comparison of particle induced calcium flux in TRPA1-overexpressing HEK-293 cells

The concentration-response relationships for TRPA1 activation by various cdPM are shown in Figure 2A. TRPA1-overexpressing HEK-293 cells were treated with pine and mesquite PM, CSC, and DEP-EtOH, which contains TRPA1 agonists,[15] at concentrations of 0, 0.58, 1.15, and 2.3 mg/mL. The rank order for potency was DEP-EtOH > pine PM and CSC > mesquite PM. Kinetic curves comparing the change in intracellular calcium content due to TRPA1 activation by the WSPM (1.15 mg/mL concentration) are shown in Figure 2B. As in Figure 2A, the rank order was the DEP-EtOH > pine PM and CSC > mesquite PM. DEP was the only PM that reached a maximum change in fluorescence (ΔF) during the 72 s measurement period, but pine PM and CSC ultimately produced the same maximum at longer time periods; mesquite did not reach a maximum at the 1.15 mg/mL treatment concentration.

The relative capacity of the different size fractions of pine PM (0.43 μm to 10 μm) to activate TRPA1 was also evaluated. The change in intracellular calcium resulting from TRPA1 activation in TRPA1-overexpressing HEK-293 cells treated with 0.73 mg/mL PM from the various impactor stages/size fractions are represented in Figure 3. Pine PM <1.1 μm activated TRPA1 equal to a maximum stimulating concentration of the prototypical TRPA1 agonist AITC (150 μM). As particle size increased, the ability to activate TRPA1 decreased. On a per mass basis, PM <1.1 μm was most potent, followed by PM 1.1 to 4.7 μm; PM >4.7 μm did not activate TRPA1. Comparable to the pine PM, mesquite PM <2.1 μm was the most potent and PM > 4.7 μm did not activate TRPA1 (data not shown). WSPM from stage 5 (1.1–2.1 μm) was used for the remainder of the studies due to abundance and relative potency of this fraction.

Specificity of TRPA1 activation by pine PM in TG neurons and A549 cells

The selectivity of pine PM for TRPA1 was studied using isolated mouse trigeminal ganglia neurons (TG) as a model for studying interactions between WSPM and TRPA1-expressing sensory neurons in human and animal airways. A cellular response comparable in magnitude to that of the prototypical TRPA1 agonist AITC (50 μM) was observed in neurons treated with pine PM at 0.23 mg/mL (Figures 4A). Responses of TG neurons to the pine PM were inhibited ~90% by co-treatment with the TRPA1 antagonist HC-030031 (50 μM), which was proportionally greater than the ~75% inhibition observed for AITC (Figure 4B).

The selectivity of pine PM for TRPA1 was also studied using A549 cells as a general model for non-neuronal lung cells that express TRPA1.[18,19] Expression of TRPA1 mRNA by A549 cells was confirmed using quantitative PCR (Figure 5A). Consistent with this result, the TRPA1 agonist AITC (200 μM; EC_{50}~145 μM in A549 cells) and pine PM promoted concentration-dependent calcium flux, which was inhibited by co-treating cells with the TRPA1 antagonist HC-030031 (Figure 5B). However, at 2.3 mg/mL pine PM, HC-030031 was unable to completely inhibit calcium flux, suggesting either a non-specific response of

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A549 cells to this concentration of pine PM, or the activation of additional WSPM-sensitive calcium channels. TRPV1, M8, and V4 are also expressed by A549 cells, but HEK-293 cells over-expressing these channels did not respond to either pine or mesquite PM at the 2.3 mg/mL concentration (data not shown).

Chemicals involved in TRPA1 activation by WSPM

Chemicals representing several major classes of combustion by-products found in wood smoke particles (i.e., fatty acids, aldehydes, ketones, resin acids, furans, etc.) were selected for screening as TRPA1 agonists based on their relative abundance in wood smoke emissions.[26–31] The structures and results for TRPA1 activation by various chemical constituents of WSPM (250 μM) are shown in Table 1. Coniferaldehyde, 3,5-*ditert*-butylphenol, and perinaphthenone activated TRPA1. However, the aldehydes furfural, 5-hydroxymethylfurfural, glyoxal, 4-hydroxybenzaldehyde, and vanillin did not. The fatty acid, palmitic acid, also failed to activate TRPA1. The resin acids agathic acid and isocupressic acid were TRPA1 agonists, but structurally related abietic acid, dehydroabietic acid, dihydroagathic acid, and tetrahydroagathic acid were not. Isopimaric acid caused extensive calcium flux in both HEK-293 and TRPA1-overexpressing HEK-293 cells and, thus, was not concluded to be a specific TRPA1 agonist.

Vanillin, 4-hydroxybenzaldehyde, coniferaldehyde, 5-hydroxymethylfurfural, furfural, glyoxal, perinaphthenone and several other aldehydes and ketones reported to be constituents of WSPM [26–31] were verified in the pine and mesquite PM samples as their 2,4-dinitrophenylhydrazone conjugates. The relative abundance of individual aldehydes and ketones from Table 1, those previously reported to be TRPA1 agonists (e.g., formaldehyde and acrolein),[15] and others that are constituents of WSPM, is shown graphically in Supplemental Figure 4 as well as in the chromatograms in Supplemental Figure 5. In general, the content of aldehydes and ketones (i.e., 2,4-dinitrophenylhydrazine-reactive substances) in the pine and mesquite PM increased as the size of the particles decreased. Additionally, pine PM contained greater total quantities of 2,3-dinitrophenylhydrazine-reactive substances, particularly PM>4.7 μm, since hydrazone pellets were not recovered from mesquite PM collected from stages 1 and 2. A quantitative comparison of the TRPA1 agonists coniferaldehyde (Table 1) and formaldehyde,[15] for the various size fractions of pine and mesquite PM is shown in Figure 6. Pine PM contained up to ~700 ± 80 ng/mg coniferaldehyde, which was near the limits of quantification in the mesquite PM. This quantity could produce a concentration of ~4 μM coniferaldehyde in a 1 mg/mL sample of pine PM. Pine PM also contained up to 1,300 ± 200 ng/mg formaldehyde, while mesquite PM contained up to 700 ± 200 ng/mg. These quantities could produce concentrations of ~43 and 23 μM formaldehyde in 1 mg/mL samples of pine and mesquite PM, respectively. Perinaphthenone was inconsistently detected in both the pine and mesquite PM samples. Isopimaric acid, dehydroabietic acid, and abietic acid were also quantified using gas chromatography-mass spectroscopy by the USDA Poisonous Plants Research Laboratory, Logan Utah. In the pine stage 5 sample, isopimaric acid, dehydroabietic acid, and abietic acid were ~270 ng/mg, ~785 ng/mg, and ~7,350 ng/mg, respectively, potentially producing concentrations of ~0.9, 3, and 24 μM in a 1 mg/mL sample of pine PM. However, only trace quantities of isopimaric and abietic acid were present in the same size fraction of mesquite PM. For all WSPM constituents, the values were consistent with prior reports of emission rates from pine and other hardwoods [26–33].

Mechanisms of TRPA1 activation by WSPM and potential chemical constituents

The relative contributions of the electrophile/oxidant-sensing site (i.e., C621, C641, C665 and K710)[34,35] and the menthol-binding site (i.e., S873 and T874)[21] in TRPA1 activation by WSPM and select chemical constituents thereof, was also assessed. Responses of wild-

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type TRPA1, the TRPA1-C621A/C641A/C655A/K710R (TRPA1-3CK) mutant, and the TRPA1-S873V/T874L (TRPA1-ST) mutant were compared (Figure 7). The TRPA1-3CK mutant was not activated by any of the agonists, suggesting that the TRPA1-3CK protein had limited function. As such, glutathione (GSH) pre-treatment of WSPM and WSPM-associated chemicals was used to selectively inhibit TRPA1 activation by electrophiles. Samples of pine PM, mesquite PM, agathic acid, and 3,5-*ditert*-butylphenol were prepared at 0.09 mg/mL, 0.19 mg/mL, 75 μM, and 250 μM, respectively, and incubated for 10 min at room temperature, in the presence or absence of GSH (20 mM) in treatment media. Pre-incubation of the pine and mesquite PM with GSH significantly and comparably reduced the activation of both wild-type TRPA1 and the TRPA1-ST mutant (Figure 7), and there was no significant difference between activation of TRPA1 and the TRPA1-ST mutant for either pine or mesquite PM alone. Wild-type TRPA1 and TRPA1-ST mutant activation by agathic acid was also inhibited ~90% by GSH, similar to the WSPM samples. Conversely, GSH did not reduce wild-type TRPA1 activation by 3,5-*ditert*-butylphenol, but significant (~60–70%) reduction in response was observed with the TRPA1-ST mutant.

Discussion

Ambient cdPM, particularly WSPM and BSPM, is increasingly being recognized as a cause of many adverse health effects in humans. Increased hospitalization rates due to exacerbation of pre-existing diseases such as asthma, chronic bronchitis, respiratory infection, development of chronic obstructive pulmonary disease, and premature death have been reported by numerous epidemiological studies investigating the effects of PM on human health.[1,2,4,5] The work presented here identifies TRPA1 as a selective molecular sensor for WSPM and chemical constituents thereof, in cells representative of airway sensory neurons, epithelial, and other non-neuronal cells that express TRPA1. Thus, it is proposed that TRPA1 may play an important role in regulating airway cell and respiratory responses to WSPM and related cdPM, highlighting the possibility of therapeutic modulation of TRPA1 to protect unusually sensitive individuals and/or high-risk populations (e.g., asthmatics, children, elderly) from developing adverse effects due to acute high-level and/or chronic exposure to WSPM and similar cdPM.

Environmental particulate matter is a complex mixture of solids, absorbed gasses, and liquid. The adverse health effects of environmental PM generally correlate with $PM_{2.5}$,[1,2,5] which is primarily produced from the inefficient combustion of fossil fuels (e.g., gasoline, oil, diesel, coal) and biomass (e.g., wood, grass, dung).[1,2,36,37] The WSPM collected in this study was an oily, tar-like material mostly <2.1 μm in size. The WSPM was also soluble in DMSO, which is a property of environmental WSPM.[1,2,38] WSPM in the environment is generally the result of seasonal wildfires and home heating.[1,2,5,36,37] It has been shown to be stable and capable of transporting long distances from its source while retaining its ability to affect humans.[1,2,36,37] Source apportionment studies of environmental PM, often regardless of the location of PM collection, routinely report the presence of chemical tracers for wood/biomass smoke PM.[1,2,26–31] Thus, it is a possibility that TRPA1 may contribute to the development of adverse health effects associated with episodic high pollution events, particularly if the event is precipitated by a forest or range fire, or weather that increases the use of inefficient wood/biomass fireplaces and stoves.[1,2,5]

In the respiratory tract, TRPA1 is expressed by C-fibers sensory neurons originating from the trigeminal and vagal ganglia, which when activated result reduce respiratory drive, trigger cough and bronchoconstriction, and the release of substance P, neurokinin A, and calcitonin-gene related peptide, which cause neurogenic inflammation. Mouse TG neurons were used as a general model of TRPA1-expressing primary sensory neurons. It was determined that pine PM activated calcium flux in TG neurons in a TRPA1-dependent

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manner, as previously shown for DEP[15,16] and CSC.[13] The similarities between DEP, CSC, and WSPM in TG neurons is most likely do to the presence of similar chemical agonists present in these types of cdPM, including chemicals like acrolein, 3,5-*ditert*-butylphenol, formaldehyde, PAHs, aldehydes and ketones.

TRPA1 is also expressed by primary human lung cells such as fibroblasts, small airway epithelial, and smooth muscle cells, where activation by TRPA1 agonists has been shown to promote non-neurogenic inflammation.[18,19] Using A549 cells as a model of TRPA1-expressing lung cells, it was also demonstrated that WSPM selectively activated TRPA1 at lower concentrations, which more closely mimic potential human exposure levels, particularly higher levels of exposure that may occur in fire fighters or those living in primitive dwellings where wood- and biomass burning is essential. The latter group of individuals are exposed to high concentrations of cdPM such as WSPM over longer periods of time, thus it is possible that TRPA1 may play an important role in some of the adverse effects on the respiratory system that are most pronounced in these populations.[1,2,5]

While the collective data presented by this study support a role for TRPA1 in mediating WSPM pulmonary toxicity, literature overwhelmingly implies a mechanism involving oxidant-mediated injury whereby reactive oxygen species and/or redox-cycling molecules present in WSPM promote pulmonary inflammation and injury.[6,7,9,10,39] Although not specifically tested, it is possible that TRPA1 may also be central to the oxidative injury process. In addition to being directly activated by WSPM, several by-products of oxidative damage, including 4-HNE[40] and 4-oxononenal,[17] are TRPA1 agonists. Thus, further research is needed to differentiate the precise contributions of direct versus indirect activation of TRPA1, at multiple levels of exposure, in order to better elucidate the specific mechanisms by which WSPM and similar cdPM ultimately produce short- and long-term effects on human health, particularly respiratory and cardiovascular health.

Finally, TRPA1 has three distinct mechanisms for activation: The first involves an electrophile/oxidant-sensitive site;[34,35] the second involves a site selective for menthol[21] and structurally similar chemicals (e.g., propofol and 3,5-*ditert*-butylphenol); and the third involves an undefined mechanosensitive component that has been shown to be responsive to insoluble components of various forms of PM.[15,22] Here, it was shown that WSPM and specific chemical components of WSPM, presumably acting on TRPA1 as a complementary mixture, predominantly activated TRPA1 through the electrophile binding site, with only 3,5-*ditert*-butylphenol acting through the menthol site. Based on these results, and those previously published for DEP[15] and CSC,[13] it is probable that the majority of cdPM that activate TRPA1 will do so via this mechanism since cdPM generally contain similar types of electrophiles. However, this generalization must be cautiously interpreted. While many cdPM contain the same chemicals,[2,26–31] the chemical composition of all types of cdPM is not identical. Thus, the biological activity of different cdPM in different cell, organ, animal models, or in humans will vary. For example, CSC, DEP, WSPM from two different types of wood, and even different size fractions of PM from a single type of wood, have strikingly different potencies as TRPA1 agonists. These data indicate that small differences in the chemical composition can markedly affect the ability of the aggregate material to activate TRPA1, and presumably other mediators of biological effects. Thus, it is emphasized that, in addition to evaluating the expression of different PM/PM constituent-sensitive ion channels such as TRPA1,[13–16,19] TRPM2,[42] TRPM8,[15] TRPV1,[22,43] and TRPV4 [15,44] in cell, organ, and animal models, and humans, that the chemical composition of the PM being studied, is more carefully assessed, such that more accurate mechanisms of biological activity can be ascertained.

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Supplementary Material

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Abbreviations

WSPM	wood smoke particulate material
BSPM	biomass smoke particulate material
cdPM	combustion-derived particulate material
PAHs	polycyclic aromatic hydrocarbons
IL-8 or CXCL1/KC	interleukin-8, TNF-α, tumor necrosis factor-alpha
TRPA1	transient receptor potential ankyrin-1
CSC	cigarette smoke condensate
DEP	diesel exhaust particulate
TRPV1	transient receptor potential vanilloid-1
SAEC	small airway epithelial cells
AITC	allyl-isothiocyanate
4-HNE	4-hydroxynonenal
PM	particulate material
DEP-EtOH	ethanol extract of diesel exhaust particles
TRPV4	transient receptor potential vanilloid-4
TRPM8	transient receptor potential melastatin-8
TRPM2	transient receptor potential melastatin-2

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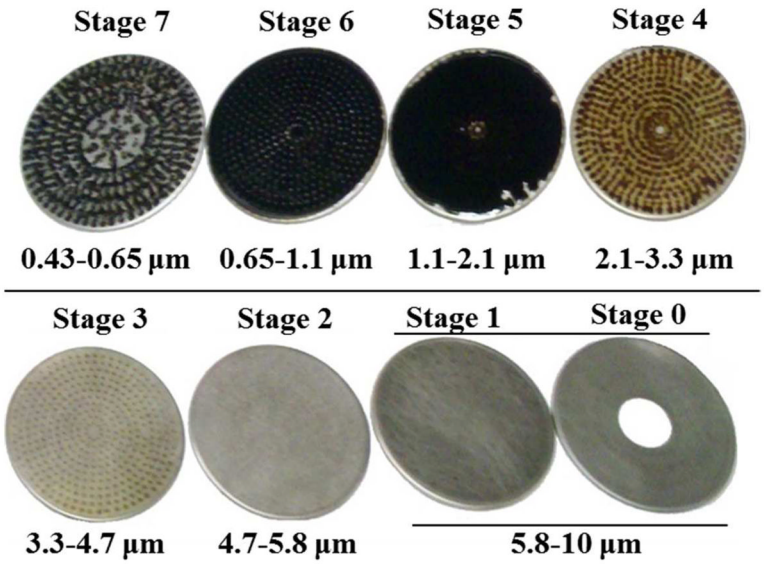

Figure 1.

Anderson cascade impactor stages (silver collection disks) showing the relative mass and size distribution of pine PM collected for this study. The residue on the stages indicate deposited WSPM with the black denoting highest concentrations of particle, brown (medium), and silver is the lowest.

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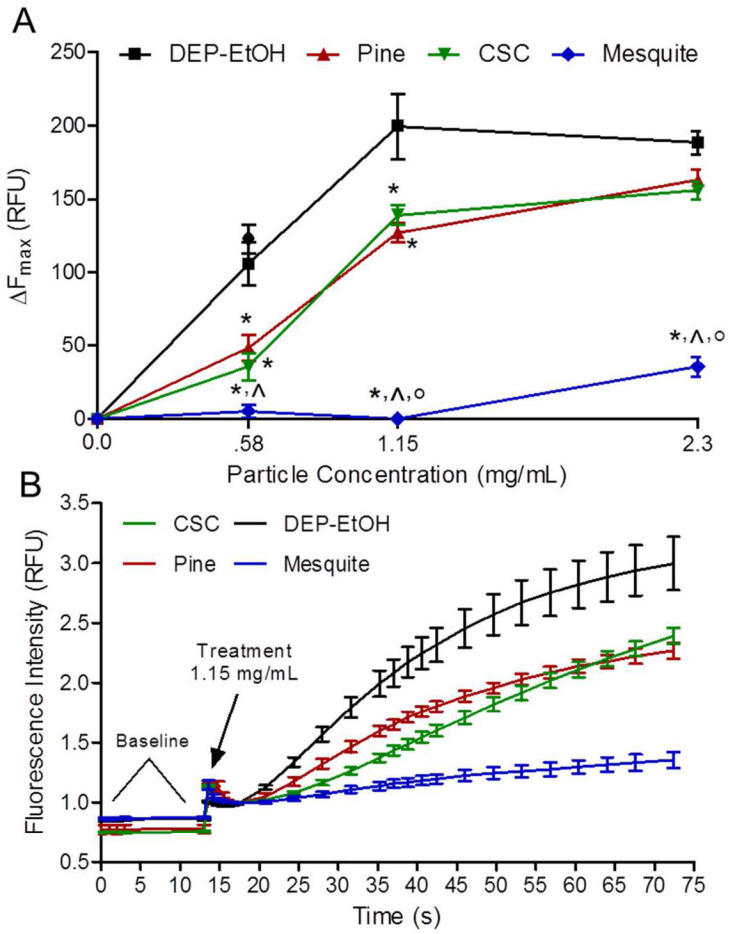

Figure 2.

(A) Quantitative comparison of TRPA1-mediated calcium flux in human TRPA1-overexpressing HEK-293 cells using pine PM, mesquite PM, cigarette smoke condensate (CSC), and diesel exhaust-ethanol extract (DEP-EtOH) at different concentrations. Data were collected using a NOVOstar plate reader. (*) Represents a statistical difference relative to DEP-EtOH, (^) indicates a difference relative to pine PM, and (○) represents a difference relative to CSC using two-way ANOVA with Bonferroni post-test, $p<0.05$. **(B)** Kinetic comparison of TRPA1 activation by 1.15 mg/mL pine PM, mesquite PM, CSC, and DEP-EtOH.

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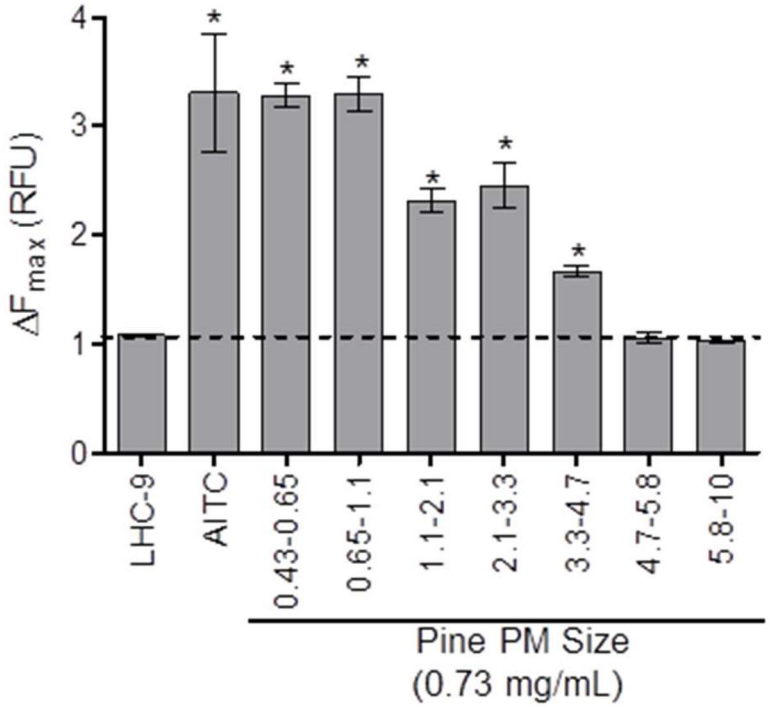

Figure 3.

Comparison of TRPA1 activation by different size fractions of pine PM, ranging from 0.43 μm (left) to 10 μm (right), at 0.73 mg/mL in TRPA1-overexpressing HEK-293 cells. LHC-9 represents the vehicle control. AITC (150 μM) was used as the positive control and to determine the maximum value for TRPA1-dependent calcium flux. Data were collected using a NOVOstar plate reader. *Indicates a significant response ($p<0.01$ using one-way ANOVA and Dunnett's post-test) compared to vehicle control

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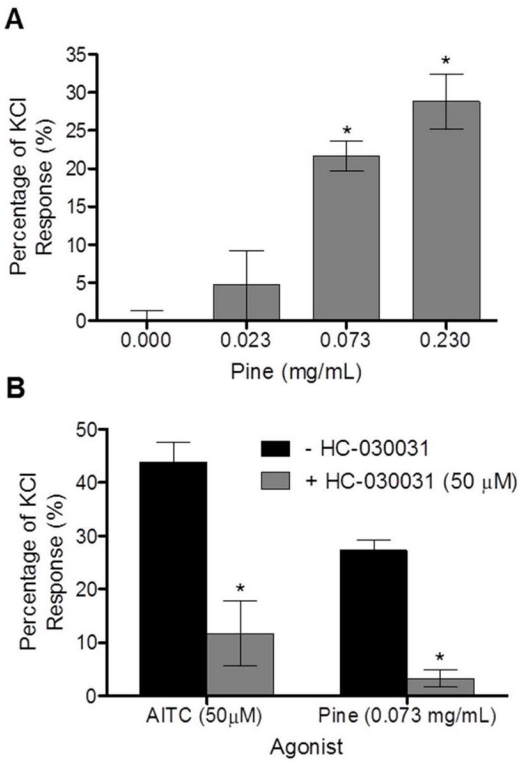

Figure 4.

(A) Concentration-response analysis for pine PM-induced calcium flux, using Fura-2, in isolated mouse TG neurons. Each data point represents the mean ± SEM value from TG neurons isolated from ≥ three animals. *Indicates a significant increase ($p<0.01$ using one-way ANOVA and Dunnett's post-test) in response versus vehicle control. **(B)** Inhibition of AITC (50 μM)- and pine PM (0.073mg/mL)-induced calcium flux by the selective TRPA1 antagonist HC-030031 (50 μM). Data are expressed as percentage of maximum response in viable neurons determined using KCl (50 mM). Each data point represents the mean ± SEM response from ≥ three animals. *Indicates a significant inhibition of calcium flux by HC-030031 ($p<0.01$ using two-way ANOVA with Bonferroni post-test).

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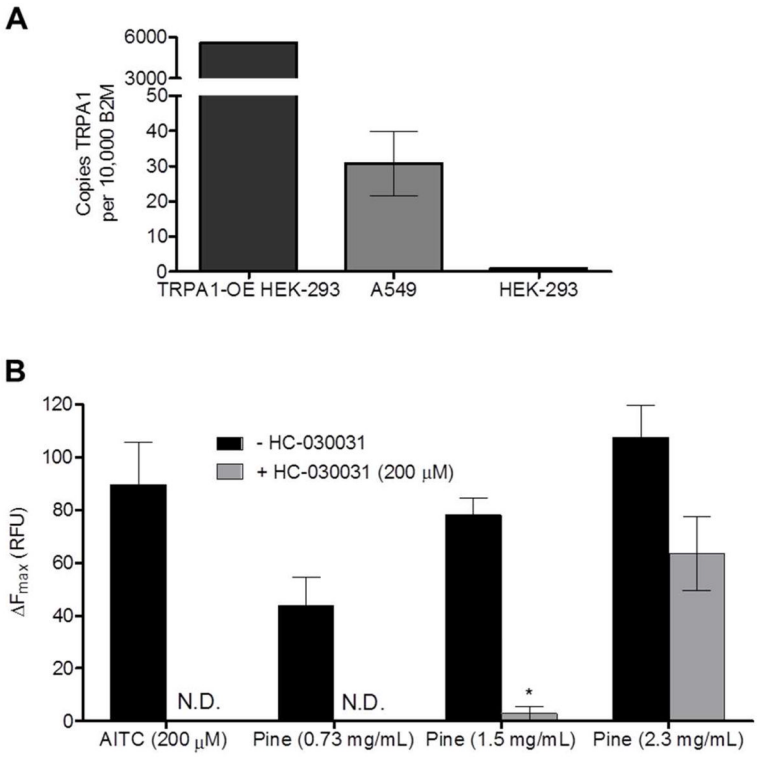

Figure 5.

(A) Expression of TRPA1 mRNA in A549 cells. qPCR analysis of TRPA1 expression in TRPA1-overexpressing (TRPA1-OE) HEK-293, A549, and HEK-293 cells. N.D.=none detected. **(B)** Inhibition of AITC (200 μM)- and pine PM (0.73, 1.5, and 2.3 mg/mL)-induced calcium flux in A549 cells by HC-030031 (200 μM). Data were collected using a NOVOstar plate reader. *Indicates a significant inhibition of calcium flux by HC-030031 ($p<0.05$ using two-way ANOVA with Bonferroni post-test).

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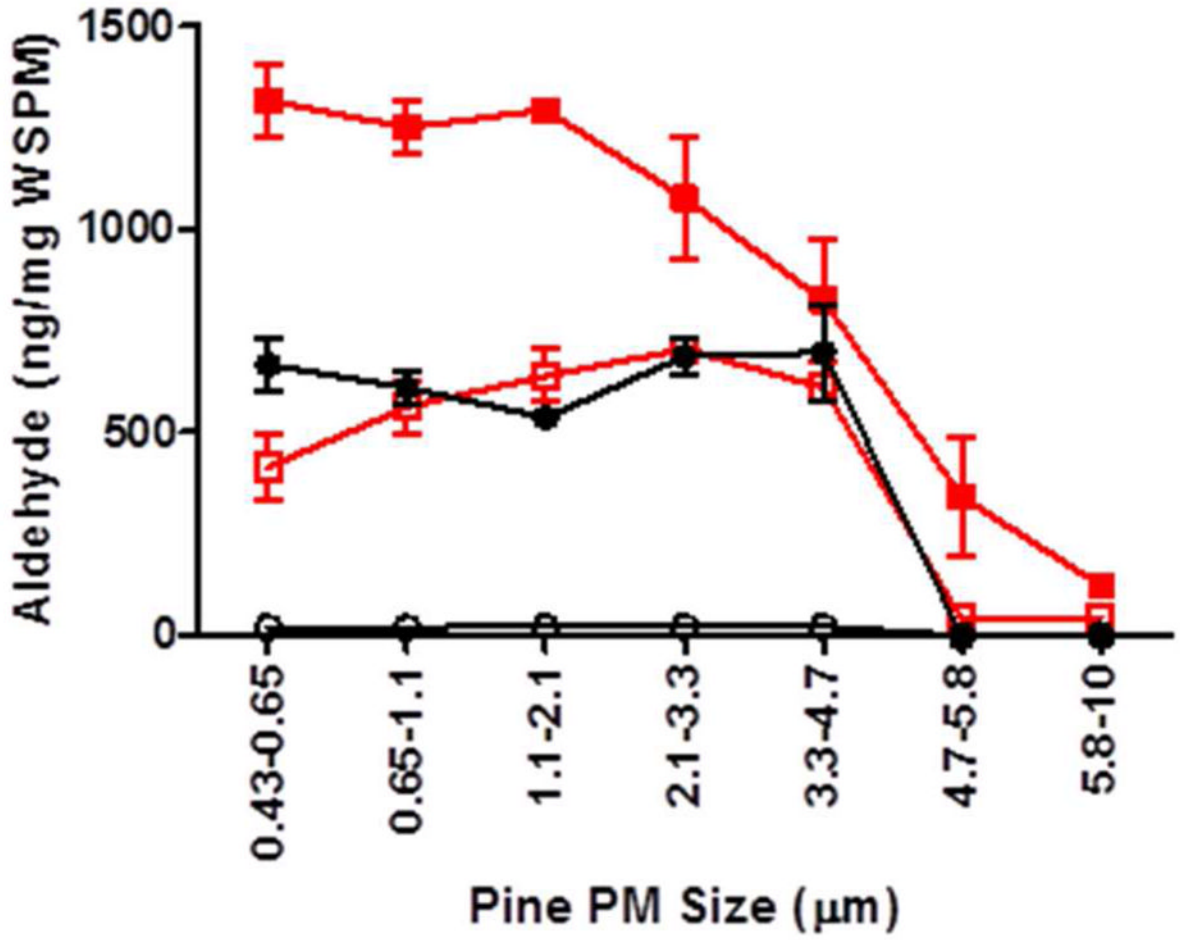

Figure 6.
Comparison of coniferaldehyde and formaldehyde in pine and mesquite PM of various sizes. Data are the mean ± SEM representative of three replicates. Formaldehyde in pine PM is represented as red squares, and open red squares for mesquite PM. Coniferaldehyde in pine PM is represented as black circles, and open circles for mesquite PM.

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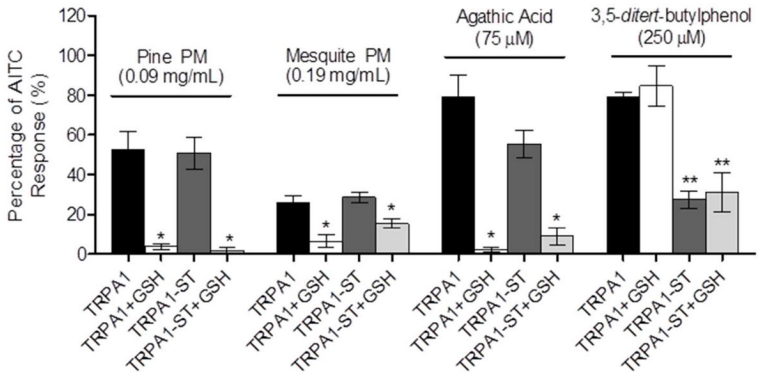

Figure 7.

Elucidation of the mechanism of activation of TRPA1 by pine PM (0.09 mg/mL), mesquite PM (0.19 mg/mL), agathic acid (75 μM), and 3,5-*ditert*-butylphenol (250 μM). Responses were compared using HEK-293 cells transiently transfected with either wild-type TRPA1 or TRPA1-ST mutant plasmids. Treatments were with and without pre-incubation of the PM/agonist for 10 min with 20 mM GSH. Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μM) and normalized to the positive control for TRPA1, AITC (150 μM). *Indicates significant reduction in calcium flux due to GSH pre-treatment ($p<0.05$ using one-way ANOVA with Bonferroni post-test). **Indicates a significant reduction in calcium flux between wild-type TRPA1 and the TRPA1-ST mutant ($p<0.05$ using one-way ANOVA and Bonferroni post-test).

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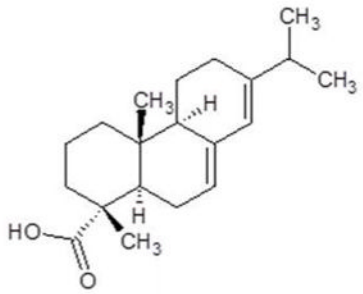
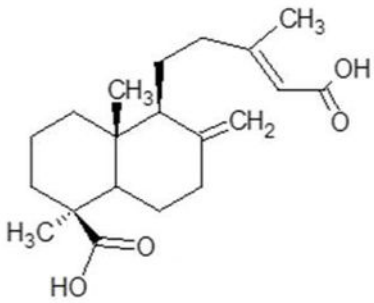
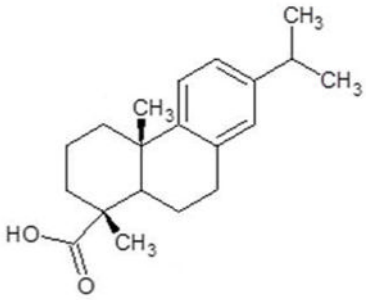
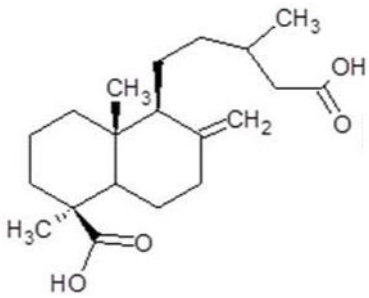
Table 1

Quantitative analysis of TRPA1 activation (calcium flux) in TRPA1-overexpressing HEK-293 cells by representative chemical components of WSPM (250 μM).

Identity	Structure	* TRPA1 Activity
Abietic acid†		N.D.
Agathic acid		80±10^Δ
Dehydroabietic acid		N.D.
Dihydroagathic acid		N.D.

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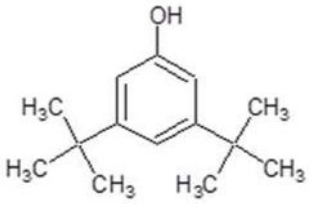
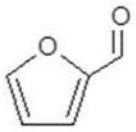
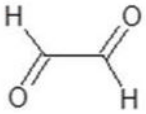
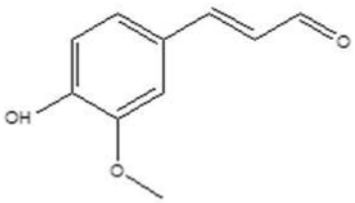
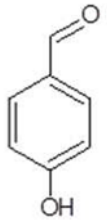
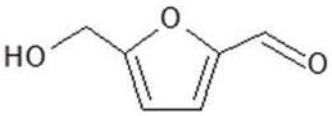
Identity	Structure	* TRPA1 Activity
3,5-*Ditert*-butylphenol		40±23^
Furfural		N.D.
Glyoxal		N.D.
Coniferaldehyde		14±7^
4-Hydroxybenzaldehyde		N.D.
5-Hydroxymethyl-furfural		N.D.

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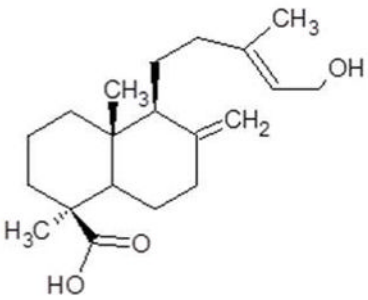
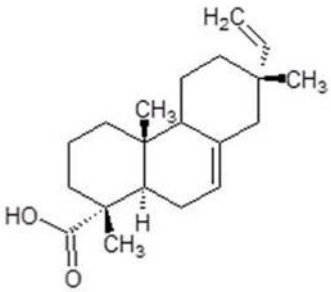
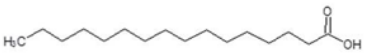
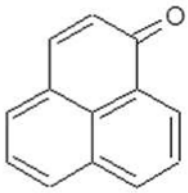
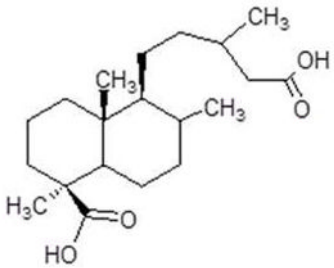
Identity	Structure	* TRPA1 Activity
Isocupressic acid		6±3
Isopimaric acid		110±12^Λ
Palmitic acid		N.D.
Perinaphthenone		34±6^Λ
Tetrahydroagathic acid		N.D.

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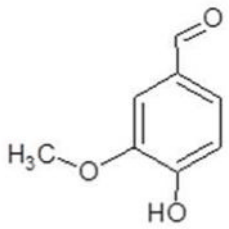
Identity	Structure	* TRPA1 Activity
Vanillin		N.D.

*Changes in cellular fluorescence were determined microscopically and are the percentage of cellular response elicited by ionomycin (10 μM) and normalized to the positive control for TRPA1, AITC (150 μM).

N.D. = none detected.

Non-specific indicates a comparable response between TRPA1-overexpressing HEK-293 and normal (control) HEK-293 cells.

^Indicates significant difference between HEK-293 and TRPA1 over-expressing HEK-293 cells ($p<0.05$ using two-way ANOVA with Bonferroni post-test).