Activation of Transient Receptor Potential Ankyrin-1 by Insoluble Particulate Material and Association with Asthma

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Abstract

Inhaled irritants activate transient receptor potential ankyrin-1 (TRPA1), resulting in cough, bronchoconstriction, and inflammation/edema. TRPA1 is also implicated in the pathogenesis of asthma. Our hypothesis was that particulate materials activate TRPA1 via a mechanism distinct from chemical agonists and that, in a cohort of children with asthma living in a location prone to high levels of air pollution, expression of uniquely sensitive forms of TRPA1 may correlate with reduced asthma control. Variant forms of TRPA1 were constructed by mutating residues in known functional elements and corresponding to single-nucleotide polymorphisms in functional domains. TRPA1 activity was studied in transfected HEK-293 cells using allyl-isothiocyanate, a model soluble electrophilic agonist; 3,5-*di*-*tert* butylphenol, a soluble nonelectrophilic agonist and a component of diesel exhaust particles; and insoluble coal fly ash (CFA) particles. The N-terminal

variants R3C and R58T exhibited greater, but not additive, activity with all three agonists. The ankyrin repeat domain-4 single nucleotide polymorphisms E179K and K186N exhibited decreased response to CFA. The predicted N-linked glycosylation site residues N747A and N753A exhibited decreased responses to CFA, which were not attributable to differences in cellular localization. The pore-loop residue R919Q was comparable to wild-type, whereas N954T was inactive to soluble agonists but not CFA. These data identify roles for ankyrin domain-4, cell surface N-linked glycans, and selected pore-loop domain residues in the activation of TRPA1 by insoluble particles. Furthermore, the R3C and R58T polymorphisms correlated with reduced asthma control for some children, which suggest that TRPA1 activity may modulate asthma, particularly among individuals living in locations prone to high levels of air pollution.

Keywords: TRPA1; diesel; CFA; lung; SNPs

Particulate air pollution (PM) is a complex mixture that is derived from many sources, including the combustion of fossil fuels, attrition of roads, tires, brakes, and suspended geological material. PM is associated with multiple acute and chronic adverse health effects in humans, including exacerbation of asthma, but the requisite

physical and chemical features of PM and associated biochemical mechanisms linking particle inhalation to specific toxicological processes are not fully understood.

It has been shown that components of urban PM, such as diesel exhaust PM (DEP), activate airway neurons that express transient receptor potential (TRP) ankyrin-1

(TRPA1) and TRP vanilloid-1 (TRPV1) (1–8). TRPA1 and TRPV1 are members of the TRP family of ion channels. This family of receptors appears to function as environmental sensors and mediate protective responses in the respiratory tract that include stimulation of the cough reflex, suppression of respiratory drive, and the

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Clinical Relevance

This manuscript presents novel data demonstrating the mechanical activation of transient receptor potential ankyrin-1 (TRPA1) by coal fly ash and the identification of functional domains and single-nucleotide polymorphisms that alter activation of TRPA1 by particulate material. We hypothesize that particulate materials activate TRPA1 via a mechanism distinct from chemical agonists and that, in a cohort of children with asthma living in a location prone to high levels of air pollution, expression of uniquely sensitive forms of TRPA1 may correlate with reduced asthma control.

initiation of immune responses; TRPA1 and TRPV1 also trigger pulmonary edema via the release of substance P and neurokinin A (9). TRPA1 has also been implicated in cardiovascular dysfunction associated with pulmonary DEP exposure in rats (8). TRPA1 and TRPV1, as well as other TRP channels, are expressed by various airway epithelial and alveolar cells in addition to airway neurons. Activation of TRPA1 (10, 11), TRPV1 (7, 12–17), TRPV4 (18), and transient receptor potential cation channel, subfamily M, member 8 (TRPM8) (19) in these cell types by prototypical agonists, select environmental pollutants, and PM also has been demonstrated to cause proinflammatory cytokine/chemokine production and, in some cases, apoptosis.

Recent studies have demonstrated an essential role of TRPA1 as a mediator of acute respiratory responses to the electrophilic and oxidizing chemicals acrolein and crotonaldehyde, which are common by-products of combustion (20, 21); H_2O_2 and $HOCl$ (22), which activate TRPA1 through covalent modification of cysteine (C621, C641, and C665); and lysine (K710), referred to as the 3CK agonist site (23, 24). Other nonelectrophilic compounds associated with DEP, including 3,5-*d*-tert-butylphenol (DTBP), activate TRPA1 through the menthol binding site (S873 T874; abbreviated as TRPA1-ST) (25). TRPA1 is activated by soluble chemicals associated with DEP, solvent-“stripped” insoluble soot material from DEP (2), and wood

smoke PM (3). Previous studies investigating the mechanical activation of TRPV1 suggest that PM may interact with proteoglycans on the surface of the protein (1). However, this mechanism of TRPA1 activation by insoluble PM has not been explored. The evolving role for TRPA1 as a target for combustion-derived electrophiles, oxidants, and insoluble PM, coupled with the ability of TRPA1 to mediate pulmonary reflex responses and neurogenic inflammation/pulmonary edema, make TRPA1 an attractive candidate for mediating at least some of the toxicological effects of air pollutants and a potential regulator of environmentally affected lung diseases such as asthma (26). Salt Lake City, which is prone to developing high levels of combustion-derived PM (e.g., DEP, wood smoke, and coal fly ash [CFA]), is an ideal environment for the study of environmental impacts on asthma.

The objectives of this study were (1) to identify unique sites of the TRPA1 protein that were involved in the mechanical activation of TRPA1 by insoluble PM and (2) to determine if naturally occurring variants of TRPA1, having altered activation properties using representative environmental stimuli as agonists, were

associated with changes in measures of asthma severity.

Materials and Methods

Chemicals

Allyl-isothiocyanate (AITC), DTBP, ruthenium red, EGTA, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Particles

Information regarding CFA and DEP source, size, and composition can be found in prior publications by our group and are referred to as CFA1 and DEP “black smoker” (1, 2). Solvent-stripped DEP/CFA was prepared by recovering the insoluble components of the DEP/CFA after repeated extraction/washing with ethanol and *n*-butyl chloride:hexane (1:1) as previously described (2).

Site-Directed Mutagenesis

Human TRPA1 was cloned as previously described (2). The mutants were generated using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) (2, 3). The primers are listed in Table E1 in the online supplement.

Figure 1. Structure of transient receptor potential ankyrin-1 depicting mutation sites and chemical/mechanical agonists. DEP, diesel exhaust particulate matter.

Cell Culture

HEK-293 cells (ATCC, Rockville, MD) were cultured in DMEM:F12 media containing 5% FBS and 1× penicillin/streptomycin. Transient transfection of HEK-293 cells with TRPA1 mutant plasmids was achieved using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) (2, 3). Transfection efficiency and relative mRNA expression was comparable for all mutants, and no overt changes in cellular morphology/properties were observed.

Calcium Imaging Assays

Imaging assays were performed in 96-well plates using the Fluo-4 Direct assay kit (Invitrogen). Treatment-induced changes in cellular fluorescence were quantified using the average value for change in fluorescence over a 2-minute period, normalized to the maximum response elicited by ionomycin (10 μ M) (2, 3). In some instances the data were also normalized to the prototypical TRPA1 agonist AITC. Data were also corrected for nonspecific responses, if any, observed with HEK-293 cells. All agonist/particle treatment solutions were prepared in LHC-9 at 3× concentration and were added to cells at room temperature. Specific information regarding particle concentrations can be found in the figure legends and in the online supplement.

Pediatric Asthmatic DNA Sample Collection and Purification

As part of an unrelated, ongoing clinical study, saliva samples were obtained prospectively from children 2 to 17 years of age with a physician-confirmed diagnosis of asthma. A description of the study population is provided in the online supplement. DNA was extracted as previously described (27).

Characterization of Asthma Phenotypes

Each patient's level of chronic asthma control was determined using a questionnaire (Table E2) based upon guidelines modified from the National Heart Lung and Blood Institute Expert Panel Report 3 (28) as described by Stockmann and colleagues (27). Each subject's asthma control score was calculated as the sum of all five question in Table E2, providing a score ranging from 0 (optimally controlled) to 15 (poorly controlled). This is

Figure 2. Activation of transient receptor potential ankyrin-1 (TRPA1) by soluble and insoluble particulate air pollution. HEK-293 cells transiently transfected with TRPA1-wild type, TRPA1-3CK, and TRPA1-ST were treated with DEP, DEP-ethanol (EtOH), the residual solvent-stripped insoluble DEP soot material (DEP-Core), coal fly ash (CFA), CFA-EtOH, and the residual solvent-stripped insoluble CFA soot material (CFA-Core) at 2.3 mg/ml. Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μ M), which were then normalized to the positive control for TRPA1 and allyl-isothiocyanate (AITC) (150 μ M). *Significant reduction in calcium flux compared with wild-type ($P < 0.05$ using one-way ANOVA with Bonferroni *post hoc* test). N.D., none detected; N.T., not tested.

the recommend approach of the American Thoracic Society and the European Respiratory Society (29, 30).

Single Nucleotide Polymorphism Genotyping

Single nucleotide polymorphism (SNP) genotyping was performed using TaqMan probe-based SNP genotyping assays for TRPA1 R3C (rs13268757) and R58T (rs16937976). TaqMan reactions were cycled 50 times (10 s at 95°C denaturation, annealed/elongated at 60°C for 1 min) on a Life Technologies QuantStudio 6 instrument. To confirm the results, five

wild-type (WT) and five SNP samples were sequenced.

Results

Activation of TRPA1 by Soluble and Insoluble Agonists

Figure 1 shows the structures of AITC, DTBP, DEP, and CFA and provides a graphical representation of TRPA1 with the locations of the mutants studied and known binding sites highlighted. HEK-293 cells transfected with human WT TRPA1 were responsive to both DEP and CFA, exhibiting approximately 65 and 15%,

Table 1. Subcellular Localization of the Glycosylation Site Mutants as Assessed by Using the Calcium Chelator/Cell Impermeable Pore Channel Blocker EGTA/Ruthenium Red

Mutation	Calcium Flux Results* (% wild-type)			
	AITC	AITC + EGTA/RR	CFA	CFA + EGTA/RR
WT	100 $\pm$ 3	14 $\pm$ 9	100 $\pm$ 8	35 $\pm$ 6
N747A	119 $\pm$ 8	12 $\pm$ 9	83 $\pm$ 13	24 $\pm$ 5
N753A	90 $\pm$ 8	15 $\pm$ 1	34 $\pm$ 14	14 $\pm$ 3
N747A_N753A	25 $\pm$ 9	1 $\pm$ 1	26 $\pm$ 11	20 $\pm$ 6

Definition of abbreviations: AITC, allyl-isothiocyanate; CFA, coal fly ash; RR, ruthenium red; WT, wild type. *Values are mean $\pm$ SEM.

respectively, of the response of the prototypical TRPA1 agonist AITC (Figure 2). DEP and CFA were also extracted with ethanol (EtOH), and the clarified solution was concentrated to produce a particle-free residue (DEP-EtOH or CFA-EtOH). DEP-EtOH activated WT TRPA1 at a rate roughly 75% that of AITC, comparable to the unfractionated DEP. CFA-EtOH did not activate TRPA1. Treatment of cells with the residual solvent-stripped insoluble DEP soot material (DEP-Core) as well as CFA and the residual solvent-stripped insoluble CFA soot material (CFA-Core) produced responses approximately 10 to 15% that of AITC.

In HEK 293 cells transfected with the electrophile/oxidant sensor-deficient TRPA1 mutant TRPA1-3CK, an approximately 75% decrease in response to DEP was observed, whereas the response to DEP-EtOH was eliminated. There was no change in response to the residual insoluble DEP-Core material, CFA, or CFA-Core. Treatment of the menthol/propofol binding site-deficient TRPA1 mutant TRPA1-ST reduced responses to DEP and DEP-EtOH by approximately 25 and 50%, respectively, but had no effect on responses to CFA. These data show that DEP-associated/extractable electrophiles and nonelectrophilic chemicals such as DTBP, as well as insoluble components of DEP and CFA, activate TRPA1. All subsequent experiments used AITC as a prototypical electrophilic agonist, DTBP as a surrogate for DEP activation of TRPA1 through the ST binding site, and CFA to assess mechanical activation.

Analysis of Contributions of Cell Surface TRPA1 to Activity

HEK-293 cells were transfected with TRPA1 and assayed for responses to AITC and CFA in the absence and presence of the cell-impermeable TRP channel inhibitor ruthenium red combined with the calcium chelator EGTA (Table 1). Responses of cells to AITC were reduced to $14 \pm 9\%$ that of the control by EGTA/ruthenium red, indicating that TRPA1 activity was largely associated with cell surface channel activation and calcium influx. Similarly, treatment of cells with CFA in the presence of EGTA/ruthenium red yielded a response

Figure 3. Mutation of the proposed TRPA1 glycosylation site reduces TRPA1 activation by CFA. HEK-293 cells transiently transfected with TRPA1-wild type (WT), N474A, N753A, and N747A/N753A were exposed to AITC (150 μ M) and CFA (2.3 mg/ml). Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μ M) and normalized to TRPA1-WT. *Significant reduction in calcium flux compared with WT ($P < 0.05$ using one-way ANOVA with Bonferroni *post hoc* test).

of $35 \pm 6\%$, a significant reduction from control, consistent with predominant activation of cell surface TRPA1 by CFA.

Assessment of Predicted N-Linked Glycosylation Site Residues on TRPA1 Activation

N747 and N753 are predicted N-linked glycosylation sites for TRPA1, which reside

on the extracellular loop between transmembrane helices 1 and 2. N-linked glycosylation sites of TRPV1 were shown to be involved in the activation of TRPV1 by CFA (1). Thus, N747A, N753A, and N747A/N753A mutants of TRPA1 were studied. The mRNA expression for the single mutants was $87 \pm 37\%$ and $112 \pm 24\%$ that of the WT control, whereas the double mutant was $59 \pm 7\%$. Neither the

Figure 4. The mutation N954T (white bar), in the putative pore-loop region, alters activation of TRPA1. HEK-293 cells transiently transfected with TRPA1-WT, R919Q, and N954T were exposed to AITC (150 μ M), CFA (2.3 mg/ml), and 3,5-di-tert butylphenol (DTBP) (250 μ M). Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μ M) and normalized to TRPA1-WT. *Significant change in calcium flux compared with WT ($P < 0.05$ using one-way ANOVA with Bonferroni *post hoc* test). ND, none detected.

N747A nor the N753A mutants exhibited a change in response to AITC or DTBP (Figure 3), but activation of the N753A mutant by CFA was $35 \pm 5\%$ that of WT, whereas the double mutant was $26 \pm 11\%$. Furthermore, the percentage of inhibition by EGTA and ruthenium red was similar to the inhibition of WT TRPA1 for the individual mutants ($\sim 60\text{--}90\%$). These data indicate a role for glycosylation primarily at N753 in the activation of TRPA1 by insoluble PM.

Assessment of Predicted Pore-Loop Region Residues on TRPA1 Activation

Two mutations to the putative pore-loop region of TRPA1 were constructed: R919Q (extracellular loop between transmembrane helices 5 and 6) and N954T (transmembrane helix 6 adjacent to the PIP₂ hydrolysis domain). Glutamate residues in this region were previously shown to be involved in the activation of TRPV1 by CFA (1), and N954 has been previously shown to be required for TRPA1 activation by AITC, with the N954A mutation forming a constitutively active channel resistant to further activation by AITC (31). The relative mRNA expression of R919Q and N954T mutants was $71 \pm 7\%$ and $104 \pm 13\%$ of WT. For R919Q, the response to all agonists was unchanged (Figure 4). As expected, the response of the N954T mutant to AITC and DTBP was negated; however, the response to CFA remained approximately 45% that of WT TRPA1. These data further demonstrate a fundamental difference in the mechanism of TRPA1 activation by soluble chemicals and insoluble PM.

Assessment of Intracellular Topical Domain and C-Terminal Domain Residues on TRPA1 Activation

The natural variant N855S (VAR_069737) located in the cytoplasm between transmembrane regions 4 and 5 (published as located in TM4) is considered a gain-of-function mutation and is associated with familial episodic pain syndrome (32). Responses to AITC and DTBP were unaltered and decreased approximately 50% for CFA (Figure 5). The SNP H1018R (rs959976), located in the intracellular C-terminal domain of TRPA1, is located near functional basic and acidic residues that modulate responses of TRPA1 to different stimuli. This protein exhibited approximately 70% increased response

Figure 5. Mutation of the cytoplasmic residue N855S and H1018S altered responses to CFA. N855S (gray bar) had reduced responses to CFA only, whereas H1018S (white bar) showed increased response to CFA only. HEK-293 cells transiently transfected with TRPA1-WT, N855S, and H1018S were exposed to AITC (150 μM), CFA (2.3 mg/ml), and DTBP (250 μM). Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μM) and normalized to TRPA1-WT. *Significant change in calcium flux compared with WT ($P < 0.05$ using one-way ANOVA with Bonferroni *post hoc* test).

to CFA relative to the WT channel, but the responses to AITC and DTBP were unchanged, again demonstrating that mechanical activation of TRPA1 is unique from chemical activation (Figure 5).

Assessment of N-Terminal Ankyrin Repeat Domain Residues in TRPA1 Activation

The N-terminal domain of human TRPA1 contains 15 ankyrin repeat domains. Two

SNPs occur in ankyrin domain 4 (amino acids 163–193), E179K (rs920829), and K186N (rs7819749), with minor allele frequencies of 21 and 18.4%. The E179K and K186N mutants exhibited $41 \pm 18\%$ and $34 \pm 22\%$ the response of WT TRPA1 in response to CFA, respectively, with a negligible ($<10\%$) change in response to AITC and DTBP (Figure 6). The relative levels of mRNA expression were $64 \pm 4\%$ and $89 \pm 5\%$ that of WT. The lack of

Figure 6. Mutation in the N-terminal ankyrin repeat domain 4 decreases activation of TRPA1 by CFA. HEK-293 cells transiently transfected with TRPA1-WT, E179K, and K186N were exposed to AITC (150 μM), CFA (2.3 mg/ml), and DTBP (250 μM). Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μM) and normalized to TRPA1-WT. *Significant change in calcium flux compared with WT ($P < 0.05$ using one-way ANOVA with Bonferroni *post hoc* test).

change in response to AITC and DTBP of these mutants, despite slightly lower levels of mRNA expression, suggests that E179K and K186N, and thus ankyrin domain 4, play an important role in mechanical, but not chemical, activation of TRPA1.

Assessment of N-Terminal Domain Residues in TRPA1 Activation

The N-terminal domain of TRPA1 consists of amino acids 1 to 719. There are two SNPs that occur in the N-terminal domain, R3C

and R58T. The following mutants were constructed: R3C, R58T, and R3C/R58T, with the latter selected based on the British 1958 Birth Cohort report of approximately 99% coinheritance of R3C and R58T. The R3C mutant exhibited roughly 2-fold greater sensitivity to AITC, DTBP, and CFA (Figure 7A). Quantification of mRNA in transiently transfected HEK-293 cells was also $200 \pm 47\%$ that of WT, suggesting that the increase in response to agonists was perhaps due to increased expression. The R58T mutant exhibited responses that were

approximately 170% that of WT TRPA1 for all three agonists with only $48 \pm 15\%$ relative mRNA expression suggesting a gain-of-function phenotype for this mutant. Analysis of the R3C/R58T mutant demonstrated an approximate 20% increase in response to all three agonists with $77 \pm 5\%$ relative mRNA expression, which is also suggestive of a gain of function effect for these SNPs for all types of TRPA1 agonists.

Assessment of Contributions of Gain-of-Function TRPA1 SNPs in Children with Asthma

Genomic DNA samples collected from children with asthma were successfully genotyped for the expression of R3C and R58T SNPs ($n = 989$ and 959 , respectively). The minor allele frequency of both R3C and R58T is reported to be approximately 10.2%, and was 12.9 and 11.1%, respectively, in this cohort. Individuals homozygous for either R3C or R58T were less likely to be well-controlled (asthma control score of 0–1) when compared with children who did not have these variant alleles (relative risk [RR], 0.21, $P = 0.06$; RR, 0.27, $P = 0.14$; and RR, 0.27, $P = 0.14$ for the R3C, R58T, and R3C/R58T variants, respectively) (Table 2; Figure 7B). These data suggest that variations in TRPA1 may have an adverse effect on the ability of some individuals to control their asthma symptoms, which may be attributable to frequent exposure to a broad range of environmental TRPA1 agonists, such as cigarette smoke, DEP, CFA, and wood smoke PM.

Discussion

A primary goal of this study was to identify the mechanisms by which insoluble PM agonists activate TRPA1. This was achieved by selectively mutating residues associated with glycosylation of the protein and by studying SNP-derived variants located in domains, such as the ankyrin 4 domain, that have been reported to or hypothesized to have the potential to alter the functional characteristics of TRPA1. Previous studies have identified N-terminal domain cysteine and lysine residues (C621, C641, C665, and K710) that AITC and other electrophilic agonists covalently modify to activate the channel (23, 24). Additionally, it has been shown that nonelectrophilic agonists of TRPA1, including menthol, propofol, and

Figure 7. (A) Mutation of the N-terminal residues R3C and R58T increases activation of TRPA1 by AITC, CFA, and DTBP. HEK-293 cells transiently transfected with TRPA1-WT, R3C, R58T, and R3C/R58T were exposed to AITC (150 μ M), CFA (2.3 mg/ml), and DTBP (250 μ M). Changes in cellular fluorescence were determined microscopically and are expressed as the percentage of cellular fluorescence elicited by ionomycin (10 μ M) and normalized to TRPA1-WT. *Significant change in calcium flux compared with WT ($P < 0.05$ using one-way ANOVA with Bonferroni *post hoc* test). (B) Children with asthma who were homozygous for either R3C or R58T exhibited poorer asthma control (denoted by higher asthma control scores). Green, red, and blue solid lines show patients homozygous for R3C, R58T, or R3C/R58T. Heterozygous individuals are depicted by dashed lines, and R3C/R58T^{-/-} individuals are represented by black lines.

Table 2. Effect of Transient Receptor Potential Ankyrin-1 Single Nucleotide Polymorphisms on Asthma Control Scores

	Well-Controlled (asthma control score of 0–1)	Less Well-Controlled (asthma control score of ≥ 2)	RR (95% CI)
R3C			
Homozygous	1 (0.4%)	14 (1.9%)	0.21 (0.01–1.20)
Heterozygous/wild type	243 (99.6%)	713 (98.1%)	
R58T			
Homozygous	1 (0.4%)	11 (1.6%)	0.27 (0.01–1.50)
Heterozygous/wild type	238 (99.6%)	691 (98.4%)	
R3C/R58T			
Homozygous	1 (0.4%)	11 (1.6%)	0.27 (0.01–1.49)
Heterozygous/wild type	235 (99.6%)	678 (98.4%)	

Definition of abbreviations: CI, confidence interval; RR, relative risk.

DTBP, bind to a different site (S873 or T874) (25). Both of these sites have been previously evaluated for their role in modulating TRPA1 activation by DEP and biomass smoke particles, with electrophilic compounds associated with these types of PM being principally responsible for TRPA1 activation (2, 3). Data presented in Figures 2 through 7, which are summarized in Table 3, expand upon results initially presented by Deering-Rice and colleagues (2) by conclusively showing that DEP and CFA activate TRPA1 via a third mechanism involving specific interactions with insoluble components

of the PM, similar to that reported for TRPV1 (1).

N-linked glycosylation plays a major role in the subcellular localization and function of TRP channels (33). Previous studies have shown that mutation of the TRPV1 glycosylation site, N604, altered responsiveness to capsaicin (34). When this TRPV1 mutant was studied using CFA as an agonist, it was found that responses were also decreased compared with WT TRPV1 (1). It was concluded that cell surface N-linked glycans on TRPV1 interacted with CFA, causing structural changes in the TRPV1 protein

that translated into calcium flux through the channel pore. Similarly, results in Figure 3 show that the two predicted sites for N-linked glycosylation of TRPA1 (N747 and N753), located on the extracellular loop between TM1 and TM2, also played a role in TRPA1 activation by CFA but not AITC or DTBP. The most pronounced effects were observed with the N753A mutant, suggesting that it is likely the site of glycosylation and that glycosylated N753 is involved in the mechanical activation of the TRPA1 channel. Importantly, the subcellular localization of these mutant channel proteins was not altered, as demonstrated using EGTA and ruthenium red to block calcium flux arising from the opening of TRPA1 on the cell surface.

A number of other variant proteins representing naturally occurring variants arising from SNPs were evaluated with the purpose of identifying mechanically sensitive sites on TRPA1 that could potentially cause altered sensitivity of individuals to respirable irritants such as smoke and airborne PM. Several SNP-derived variants of TRPA1 exhibited a selective decrease in function when stimulated with the mechanical activator CFA, identifying their role in the mechanism by which mechanical stimuli activate TRPA1 (specifically, E179K and K186N, located in the N-terminal ankyrin repeat domain). The ankyrin domains of TRPA1 and other proteins have been described as “gating springs” (35). Data in Figure 6 indicate that ankyrin domain 4 is involved in the mechanical activation of TRPA1 by particles, presumably through transducing mechanically induced structural changes in the TRPA1 protein at the cell surface into pore opening. As shown in Figure 4, the N954T mutant of TRPA1 also retained activity to CFA, but not AITC or DTBP as previously reported (31), further emphasizing key differences between mechanisms of TRPA1 activation by traditional chemical and atypical particle agonists.

The C-terminus of TRPA1 contains many domains important for regulation of the channel. Of note, acidic residues of the C-termini regulate channel activation and inactivation by calcium (36). Basic residues also play an important role in transducing signals to the gate and voltage-dependent activation (37). The SNP H1018R has not yet been identified as a functionally relevant

Table 3. Summary of Evidence for a Distinct Insoluble Particle Activation Mechanism for Transient Receptor Potential Ankyrin-1

	Mutation	AITC (3CK binding site)	DTBP (ST binding site)	CFA (mechanical)
Extracellular Glycosylation	N747A	—*	N.T.	↓ [†]
	N753A		N.T.	↓
	N747A/N753A		—	—
Pore loop	R919Q	—	—	—
TM6	N954T	L.O.F.	L.O.F.	↑ [‡]
Intracellular N-terminus	R3C	↑	↑	↑
	R58T	↑	↑	↑
	R3C/R58T	—	—	—
Ankyrin 4	E179K	—	—	↓
	K186N	—	—	↓
Loop (TM4–5)	N855S	—	—	↓
C-terminus	H1018S	—	—	↑

Definition of abbreviations: AITC, allyl-isothiocyanate; CFA, coal fly ash; DTBP, 3,5-ditert butylphenol; L.O.F., loss of function; N.T., not tested.

*No significant change.

[†]Significantly decreased.

[‡]Significantly increased.

basic residue, but in this study SNP H1018R was shown to have enhanced responses to CFA, suggesting yet another residue that may play a part in the mechanical activation of TRPA1.

The TRPA1-N855S SNP, which has been linked to altered pain sensitivity (specifically Familial Episodic Pain Syndrome [32]) was also studied. Although our SNP analysis did not identify any individuals with this SNP, experimental results suggest a decreased response to mechanical stimuli, providing insight into intramolecular binding interactions that control mechanical sensitivity. Using molecular modeling, Zayats and colleagues (38) showed that residue N855 formed a strong link between the transmembrane helices 4 and 5 and 1, connecting the N-terminus and the gate. As shown in Figure 5, the N855S protein was less sensitive to mechanical activation by CFA, suggesting that transduction of physical perturbations of at the cell surface through the TRPA1 protein are essential for TRPA1 activation by mechanical stimuli.

Several apparent gain-of-function mutations, including the SNPs R3C and R58T, were also identified. These SNPs are natural variants that are coinherited. When each variant protein was individually

expressed, an increase in calcium flux in response to all three agonists was observed (Figure 7A). When the double mutation was evaluated, as would likely be the case in a majority of people, minimal change in channel activation was observed, which we believe is the result of poor expression levels in transfected HEK-293 cells.

Therefore, if these mutations truly result in a more responsive channel, individuals expressing these SNPs may be uniquely sensitive to a broad range of inhaled irritants that activate TRPA1, such as DEP, cigarette smoke, and wood smoke.

A second goal of this study was to evaluate whether variant forms of TRPA1 exhibiting unique functional characteristics might have a modulatory effect on asthma, given that TRPA1 has been implicated in the pathogenesis of asthma and that many asthma exacerbating stimuli, such as environmental air pollution, are TRPA1 agonists. Analysis of the expression of these SNPs in a cohort of children with asthma demonstrated that patients homozygous for either or both R3C and/or R58T exhibited poorer asthma control (i.e., higher scores) (Figure 7), which is suggestive of a potential disease modifying effect of these SNPs for some individuals with asthma. Although not statistically significant, at the

population level, the presence of the R3C and R58T SNPs in some individuals was associated with greater odds for having intermediate to high asthma control scores compared with WT and heterozygous individuals. Thus, variations in TRPA1 that confer greater sensitivity to agonists present in the environment, such as environmental PM, might contribute to reduced effectiveness of asthma treatments and poorer asthma control in some individuals.

In summary, this study has identified several components of the TRPA1 protein that are important for mechanical activation by insoluble particulate materials, including N-linked glycosylation, which appears to be a common factor for the mechanical activation of both TRPA1 and TRPV1 by particles. The results of this study show that mechanical activation of TRPA1 is distinct from chemical activation, and it is possible that variations in TRPA1 that influence the sensitivity of TRPA1 to agonists may contribute to individual variability in sensitivity to asthma exacerbating agents, such as high levels of PM in ambient air. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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