Human airway epithelia function in barrier formation, defense against pathogens, and mucociliary clearance (Hogg 2004). They represent the first barrier against airborne environmental pollutants, and they coordinate recruitment of pivotal inflammatory cells in several pathologies, including chronic obstructive pulmonary disease (COPD) (Li et al. 2009; Mercer et al. 2006; Yang et al. 2005). The inhalation of diesel exhaust particles (DEP), produced by vehicular traffic contributing to urban smog, leads to serious respiratory diseases (e.g., COPD, emphysema, bronchial cancer, chronic asthma) (Torres-Duque et al. 2008). The particles’ carbonaceous cores are coated with thousands of organics and heavy metals. Because large numbers of hazardous chemicals are present on DEP, its pathological effects on human airways are pleiotropic. We and others have found that DEP evokes the secretion of matrix metalloproteinase-1 (MMP-1) from human bronchial epithelia (Amara et al. 2007; Li et al. 2009). Matrix metalloproteinase-1 (MMP-1) plays a role in tissue remodeling during development, inflammation, migration of inflammatory and malignant cells, and COPD and emphysema pathogenesis (Segura-Valdez et al. 2000). It also has neurotropic effects, possibly enhancing sensitization of airway-innervating sensory neurons, contributing to airway hypersensitization and chronic cough (Conant et al. 2004). We recently identified a novel pathway that results in DEP-induced MMP-1 activation and entails activation of RAS-RAF-MEK-extracellular signal–regulated kinase (ERK) signaling, dependent on β-arrestins (Li et al. 2009). From a global health perspective, one important finding was that the human MMP-1 polymorphism at position -1607(1G/2G) of the MMP-1 promoter yielded, after DEP exposure, either a diminutive (1G) or large (2G) response. The 2G polymorphism is found in 75% of humans. Against this background, we sought to identify critical elements upstream of RAS in human airways in response to DEP. The pathogenic component of DEP that activates MMP-1 is primarily retained in its organic extract (OE), so that DEP carbonaceous core particles shuttle water-insoluble OE to the ciliary plasma membrane. The DEP/OE initially activates proteinase-activated receptor 2 (PAR-2), which, via G12α-G-protein, phospholipase-C β3 (PLCβ3) and phosphatidylinositol 3 kinase (PI3-K), activates Ca2+-permeable TRPV4 (transient receptor potential vanilloid, family member 4) ion channels (Liedtke et al. 2000; Lorenzo et al. 2008; Sidhaye et al. 2008; Strotmann et al. 2000). A uniquely protracted Ca2+ influx through TRPV4 follows, which is critical for mitogen-activated protein kinase (MAPK)–mediated MMP-1 activation. Localization studies show that PAR-2, PLCβ3, and TRPV4 colocalize to cilia of human differentiated airway epithelia. DEP exposure greatly enhances protein–protein complex formation between these signaling molecules and calmodulin. Importantly, we observed that TRPV4P19S, a human genetic polymorphism previously identified as a COPD susceptibility locus (Zhu et al. 2009), increases MMP-1 activation via increased Ca2+ influx, providing a mechanistic link between human airway epithelia signaling, airway disease, and air pollution.

**Materials and Methods**

**DEP.** Particles were generated at the U.S. Environmental Protection Agency (EPA; Research Triangle Park, NC) from a Deutz four-cylinder diesel engine, running at 119–784–793 (2011). doi:10.1289/ehp.1002807 [Online 18 January 2011]
three defined engine loads before collection, as described previously (Li et al. 2009). For experiments, we used DEP at 100 μg/mL. DEP organic extract (OE) was prepared by washing organic chemicals off of DEP using methylene chloride, followed by solvent exchange with dimethyl sulfoxide (DMSO). In experiments, we used 20 μg/mL OE, which is equivalent to the organic compounds contained in 100 μg DEP. We used Degussa Printex 90 carbon nanospheres (P90; provided by W. Moeller, GSF, Munich, Germany) as controls.

**Chemicals.** We used the following compounds: pertussis toxin (G10o inhibitor; Sigma Chemical Co., St. Louis, MO), U73122 (PLC inhibitor; Tocris, Ellisville, MO), LY294002 and P1828 (PI3-K inhibitors; Tocris), 46-phorbol 12,13-diacecanote (46-PDD; TRPV4 activator; Tocris), GSK205 [TRPV4 blocker (Phan et al. 2009)], ruthenium red [TRP(V) blocker; Tocris], gadolinium(III) chloride [GdCl3 (Sigma); inhibitor of store-operated calcium entry (SOCE)] at 5 μM, and thapsigargin (Ca2+-store depletion; Tocris), GM1489 and Z-PDLDA-NHOH (pan-MMP inhibitors; Endogen, Rockford, IL), and W-7 (calmodulin blocker at 10 μM; Sigma).

**Cell culture.** BEAS-2B cells were obtained from ATCC (Rockville, MD), maintained as previously described (Li et al. 2009), and are a non smoking adult volunteer. We obtained tracheo bronchial cells derived from healthy, human bronchial epithelial (HBE) cells were obtained from ATCC (Rockville, MD), maintained as previously described (Li et al. 2009), and from ATCC (Rockville, MD), maintained (L. Birnbaumer and S. Muallem. These cDNAs were driven by CMV promoters in eukaryotic expression plasmids, the coding region fused to C-terminal to 10 amino-acids N-terminal to the homology domain of Bruton’s tyrosine kinase, phosphatidyl inositol (3,4,5)-trisphosphate (PIP3) levels, the mCherry is cytoplasmic; and TRPV4.

**Results**

The phosphorylated ERK (phospho-ERK) trafficking assay was performed as described previously (Li et al. 2009). Briefly, BEAS-2B cells were stimulated either with DEP or with OE and fixed (4% paraformaldehyde) at 5-, 10-, 20-, and 30-min time points. Phospho-ERK1/2 was verified by fluorescent immunodetection and quantified densitometrically (≥75 cells/time point), corrected for background, in the nuclear area using ImageJ software (version 1.42q; Rasband 2009).

Confocal imaging was conducted after immunocytochemical staining for acetylated α-tubulin, PLCβ3, TRPV4, and PAR-2. Fluorescently labeled sections were visualized using a Zeiss LSM710 confocal imaging suite with lasers tuned to the emission spectra of the secondary fluorescent antibodies.

**Results**

**Electrophysiological recordings.** Extracellular Ca2+ was precipitated by addition of EGTA. We conducted PI3-K Förster resonance energy transfer (FRET) imaging based on FRET of membrane-targeted enhanced green fluorescent protein (eGFP; donor) and the pleckstrin membrane-targeted enhanced green fluorescent protein (acceptor). With low phosphatidylinositol (3,4,5)-trisphosphate (PIP3) levels, the mCherry is cytoplasmic; with increased PIP3 levels, it translocates to the membrane leading to FRET, which we quantified using two-photon fluorescence lifetime imaging. Additional details for electrophysiological recordings are given in Supplemental Material (doi:10.1289/ehp.1002807). Our findings suggest that the carbonaceous core of DEP, by size a carbon nanoparticle, acts as a vehicle carrier for delivering a highly active, water-insoluble organic fraction to the plasma membrane of human airway epithelia to elicit MMP-1 activation.

**Extracellular Ca2+ influx is necessary for activation of MMP-1.** Previous studies in lung cells and neurons have shown that particulate matter evokes Ca2+ transients (Agopyan et al. 2004); other studies have shown that Ca2+ influx is necessary for activation of MMP-1 (Lee et al. 2009). Therefore, we examined whether DEP and/or OE causes Ca2+ influx and whether this can activate MMP-1.
We found that DEP and OE evoke extracellular Ca\(^{2+}\) influx (Figure 1A–D), as indicated by curtailment of the response by addition of EGTA (Figure 1C,D). P90 control carbon nanoparticles had no effect on Ca\(^{2+}\) (Figure 1B), whereas DEP activated a uniquely protracted and monotonically increasing response with a peak at approximately 60 min that gradually declined (data not shown). In comparison, the response to OE increased more rapidly, reaching a maximum at approximately 20 min and decreasing to baseline in the next 10 min (Figure 1B), indicating that the particle core retarded Ca\(^{2+}\) influx by slowing delivery of the organic fraction to the plasma membrane.

To determine whether DEP-induced Ca\(^{2+}\) influx was necessary for transcriptional activation of MMP-1, we exposed cells to DEP in the presence and absence of extracellular Ca\(^{2+}\) and measured MMP-1 transcriptional activation at 2 and 24 hr and the appearance of nuclear phospho-ERK at 30 min (Figure 1E–I). These experiments indicated that extracellular Ca\(^{2+}\) was necessary for both nuclear translocation of phospho-ERK and MMP-1 activation in response to DEP or OE. In primary HBE cells, EGTA eliminated MMP-1 secretion in response to DEP or OE (Figure 1H), thus confirming the validity of this mechanism.

We were unable to demonstrate functionality of SOCE in DEP/OE–evoked Ca\(^{2+}\) influx [see Supplemental Material, Figure 2A–C (doi:10.1289/ehp.1002807)]. Preincubation with 5 \(\mu\)M thapsigargin or 5 \(\mu\)M GdCl\(_{3}\) (Bird et al. 2008) did not markedly change DEP-evoked Ca\(^{2+}\) increase. Furthermore, cotransfection of STIM1-DN and ORAI1-3–DN, both known to function in SOCE, did not significantly alter Ca\(^{2+}\) responses evoked by DEP or OE.

Finally, we found that DEP and OE also caused the Ca\(^{2+}\)-dependent secretion of pro-inflammatory mediators RANTES (Jeffery 2004) and IP-10 (Torvinen et al. 2007) [see Supplemental Material, Figure 2D,E (doi:10.1289/ehp.1002807)]. Thus, in human lung cells, Ca\(^{2+}\) influx is necessary for DEP/OE–evoked activation of MMP-1, IP-10, and RANTES.

PAR-2 is a DEP-sensitive G-protein–coupled receptor (GPCR) that activates G\(_{\alpha i/o}\), PLC\(_{3}\), and PI3-K. We focused on PAR-2 because an earlier low-throughput proteomics screen revealed that, compared with DEP alone, DEP plus PAR-2–activating peptide (PAR-2-AP) increased MMP-1 secretion (data not shown). We first conducted experiments in BEAS-2B cells by siRNA-mediated knockdown of PAR-2. PAR-2 mRNA was significantly reduced by PAR-2 siRNA but not by the scrambled control [see Supplemental Material, Figure 3A (doi:10.1289/ehp.1002807)]. Cells treated with PAR-2 siRNA exhibited significantly reduced Ca\(^{2+}\) influx, MMP-1 reporter gene activation, and MMP-1 secretion (Figure 2A; see also Supplemental Material, Figure 3B,C). This demonstrates that PAR-2 functions upstream of Ca\(^{2+}\)-mediated MMP-1 activation. In addition to scrambled siRNA controls, PAR-1–specific siRNA had no effect on MMP-1 activation (data not shown).

Costimulation of BEAS-2B and primary HBE cells with DEP or OE and PAR-2-AP potentiated MMP-1 activation [Figure 2B; see also Supplemental Material, Figure 3D,E (doi:10.1289/ehp.1002807)]. To boost its moderate expression level in BEAS-2B cells, we overexpressed PAR-2. This led to increased baseline and DEP-evoked MMP-1 activation, indicating that PAR-2 overexpression is sufficient to increase MMP-1 expression and to render the cell more responsive to DEP (see Supplemental Material, Figure 3F). Thus, specific activation and inhibition of PAR-2 imply that this receptor is critical in DEP-mediated Ca\(^{2+}\) influx that leads to MMP-1 activation.

We next investigated whether secreted MMP-1 activates PAR-2 proteolytically, as
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it does for PAR-1 (Boire et al. 2005), which might explain the protracted Ca\(^{2+}\) influx in response to DEP. This was not the case, because MMP inhibitors accelerated MMP-1 reporter gene activity in response to DEP [see Supplemental Material, Figure 3G (doi:10.1289/ehp.1002807)].

We addressed whether β-arrestins are necessary for PAR-2-mediated Ca\(^{2+}\) influx in response to DEP or OE (Cottrell et al. 2003). This was not the case in view of Ca\(^{2+}\) increase in the absence of β-arrestins 1 and 2 [siRNA-mediated knockdown; see Supplemental Material, Figure 3H (doi:10.1289/ehp.1002807)]. We previously verified elimination of MMP-1 activation by siRNA-mediated β-arrestin knockdown (Li et al. 2009). Together, these results implicate β-arrestins as MAPK scaffolds necessary for the DEP–MMP-1 response yet dispensable for PAR-2–mediated Ca\(^{2+}\) influx in response to DEP or OE.

We examined G\(_{i/o}\) signaling because of PAR-2’s known signal transduction mechanisms via this G-protein (Ollanas et al. 2007). We found that the DEP–MMP-1 response, namely, Ca\(^{2+}\) influx, MMP-1 transcription, and MMP-1 secretion, depends on G\(_{i/o}\), which we targeted specifically with pertussis toxin in both BEAS-2B and primary HBE cells [Figure 2A,B; see also Supplemental Material, Figure 4A (doi:10.1289/ehp.1002807)]. Because G\(_{i/o}\) is known to activate PLC (Exton 1996), we next treated cells with PLC-selective inhibitor, U73122, which led to a marked DEP–MMP-1 response (Figure 2A,B; see also Supplemental Material,
Figure 4B). PLC has several isoforms; we investigated the β-isomers because of PLCβ's link to GPCRs, specifically PLCβ3, in view of its previously established link to G_{i/o} (Senyshyn et al. 1998). When we immuno-labeled for PLCβ1–4, we found the most robust expression for PLCβ3 in primary HBE cells (data not shown). Interestingly, using a phospho-specific antibody against PLCβ3, we documented phospho-PLCβ3 up-regulation within 30 min after DEP application (Figure 2C). This finding can help explain the protracted Ca^{2+} influx because PLCβ3, being upstream of extracellular Ca^{2+} influx, was previously demonstrated to be attenuated by phosphorylation (Yue et al. 2000).

Another phospholipid-metabolizing enzyme that signals downstream of G_{i/o} and upstream of TRP channel Ca^{2+} conductances is PI3-K (Zhuang et al. 2004). We identified its critical role in response to DEP or OE using the PI3-K inhibitor LY294002 by documenting significant reduction of Ca^{2+} influx and subsequent MMP-1 activation in both BEAS-2B and primary HBE cells (Figure 2D; see also Supplemental Material, Figure 4C (doi:10.1289/ehp.1002807)), suggesting the signaling position of PI3-K upstream of Ca^{2+} influx. Moreover, using a novel FRET-based assay, we could visualize the enzymatic activity of PI3-K (change in PI3P) in BEAS-2B cells in response to DEP or OE, which indicated PI3-K activity as an early signaling event (Figure 2D). Furthermore, in addition to time-scale resolution after DEP or OE exposure, this method illustrates the confinement of PI3-K signaling to the plasma membrane.

**TRPV4 forms a DEP-sensitive Ca^{2+} pathway downstream of PI3-K/PLC-β3.** PAR-2 has been shown to sensitize TRP channels, including TRPV1, TRPV4, and TRPA1 (Amadesi et al. 2006; Garti et al. 2006; Grant et al. 2007). Because TRPV4 is expressed in tracheobronchial epithelia (Lorenzo et al. 2008), we addressed whether it functions downstream of the above signaling cascade, initially by inhibiting its function in BEAS-2B cells expressing TRPV4-DN [see Supplemental Material, Figure 5A–C (doi:10.1289/ehp.1002807)], which produced strong reduction of Ca^{2+} influx in response to DEP yet no reduction for TRPV3-DN (Figure 3A). We also knocked down TRPV4 using specific siRNA, which effectively down-regulated TRPV4 mRNA and protein (see Supplemental Material, Figure 5B,C). Compared with the scrambled control, the siRNA-TRPV4 knockdown reduced Ca^{2+} influx in response to DEP or OE (Figure 3B). Thus, TRPV4 is necessary for DEP-evoked Ca^{2+} influx.

Next we addressed whether DEP-evoked, TRPV4-mediated Ca^{2+} influx activates MMP-1. Using MMP-1 reporter assays, we found that TRPV4-specific siRNA decreased MMP-1 transcriptional activation, thus implying that TRPV4 is critical for DEP/OE-evoked Ca^{2+} influx, which then activates MMP-1 (see Supplemental Material, Figure 5D (doi:10.1289/ehp.1002807)). Based on these results, we used the MMP-1 reporter platform to determine that TRPV4 functions downstream of PAR-2 because siRNA-mediated TRPV4 knockdown virtually eliminated potentiated MMP-1 activation by DEP or OE plus PAR-2-AP (see Supplemental Material, Figure 5E). This effect of TRPV4-specific siRNA was recapitulated for DEP-evoked MMP-1 secretion in BEAS-2B cells (Figure 3C). Finally, we found that ruthenium red, an unspecific TRP(V) blocker, decreased MMP-1 activation (see Supplemental Material, Figure 5F).

TRPV4 activation by 4α-PDD or hypertonicity strongly increased MMP-1 secretion, indicating that in airway epithelia, TRPV4 activation is sufficient to up-regulate MMP-1 (Figure 3D). Furthermore, TRPV4 transfection in BEAS-2B cells increased MMP-1...
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Because these findings were obtained in the BEAS-2B cell line, we also tested TRPV4 function in primary HBE cells. First, we were able to significantly attenuate the DEP-evoked Ca\textsuperscript{2+} response by GSK205, a specific small-molecule TRPV4 inhibitor (Phan et al. 2009), in a dose-dependent manner (Figure 3E). In addition, secreted MMP-1 in response to DEP was significantly reduced by two concentrations of GSK205 (Figure 3F). Thus, the cornerstones of TRPV4’s involvement in the DEP–MMP-1 response, namely, dependence of the Ca\textsuperscript{2+} response and MMP-1 secretion on TRPV4, were recapitulated in primary HBE cells. Whenever possible, we performed the same experiment in human primary HBE cells as in permanent human BEAS-2B cells.

Taken together, these findings point toward critical functioning of TRPV4 in Ca\textsuperscript{2+} influx into human airway epithelia evoked by DEP, a globally relevant air pollutant.

TRPV4 signaling complex is located on motile cilia of primary human airway epithelia. Because TRPV4 channels have been found in primary motile cilia of mouse tracheal epithelia (Lorenzo et al. 2008), we determined TRPV4’s subcellular location in human ciliated airway epithelia. Primary HBE cells were differentiated in culture until they became ciliated. They showed ciliary location of TRPV4 channels in primary HBE cells.

Figure 4. Colocalization of ciliary marker acetylated (Ac) α-tubulin with PLCβ3 (A) or TRPV4 (B), and colocalization of PAR-2 with PLCβ3 (C) or TRPV4 (D) in cilia of primary HBE cells. Columns are as follows: green channel, anti-mouse; red channel, anti-rabbit; the merged image; and the XZ-series reconstruction. Confocal micrographs are top view for the first three columns, and the XZ-series (fourth column) depicts a schematic rendering of an enlarged lateralized section. Bars = 10 μm. Primary HBE cells in D were not fully differentiated, showing “budding” cilia at the time of immunolabeling. More elongated cilia were present in C, PAR-2 colabeled for PLCβ3, and in A, Ac α-tubulin colabeled for PLCβ3. Nevertheless, A–C suggest that PAR-2 and TRPV4 colocalize to cilia of primary HBE cells.
In BEAS-2 cells, protein–β- and calmodulin; after exposure to DEP, the signal remained high at least during the course showing increased Ca^{2+} influx. (A) Representative Western blots of immunoprecipitation (IP) experiments performed pre-exposure (Pre) and 30 and 120 min after DEP exposure; for controls, a control antibody was used for IP. With PLCβ exposure, for MMP-1 secretion by transfected BEAS-2B cells. 1First, transfection of TRPV4 wt increased MMP-1 secretion by transfected BEAS-2B cells. TRPV4wt and TRPV4P19S were present and appreciable. With TRPV4 IP, complexes formed containing calmodulin; after DEP exposure both interactions clearly increase. PAR-2 IP shows that PAR-2 forms a protein–protein complex with calmodulin and that this interaction increased after DEP exposure. Potentiating effect of the specific calmodulin inhibitor W-7 on DEP-evoked Ca^{2+} influx (B) and MMP-1 secretion (C). The arrow in B indicates the time of DEP exposure.

Figure 5. In BEAS-2 cells, protein–protein complex formation after exposure to DEP involves PAR-2, PLCβ3, TRPV4, and calmodulin (which is functional in DEP-evoked Ca^{2+} influx). (A) Representative Western blots of immunoprecipitation (IP) experiments performed pre-exposure (Pre) and 30 and 120 min after DEP exposure; for controls, a control antibody was used for IP. With PLCβ3 IP (top three panels), complexes formed containing TRPV4, PAR-2, and calmodulin; after exposure to DEP, the protein–protein interaction increased for PAR-2 and calmodulin. Under Pre conditions, the PLCβ3–calmodulin and PLCβ3–TRPV4 complexes were present and appreciable. With TRPV4 IP, complexes formed containing calmodulin; after DEP exposure both interactions clearly increase. PAR-2 IP shows that PAR-2 forms a protein–protein complex with calmodulin and that this interaction increased after DEP exposure. Potentiating effect of the specific calmodulin inhibitor W-7 on DEP-evoked Ca^{2+} influx (B) and MMP-1 secretion (C). The arrow in B indicates the time of DEP exposure.

*p < 0.001 for W-7 DEP compared with control DEP and for W-7 control compared with control control.

Figure 6. In BEAS-2 cells, TRPV4P19S functions as a gain-of-function channel in response to DEP, causing increased MMP-1 activation via influx of Ca^{2+}. (A) Time course showing increased Ca^{2+}-facilitated activation of TRPV4P19S compared with TRPV4wt (n ≥ 24 cells per group). (B) Time course showing DEP-evoked Ca^{2+} influx in Ca^{2+}-free buffer (note the absence of Ca^{2+} in OE) followed by the addition of external Ca^{2+} (2 mM), which leads to accelerated Ca^{2+} influx for TRPV4P19S versus TRPV4wt. The signal of TRPV4wt declined after the peak, whereas TRPV4P19S-transfected cells did not desensitize; the signal remained high at least during the observation period. Arrows in A and B indicate the time of Ca^{2+} or OE exposure. (C) Internal [Ca^{2+}], was significantly elevated in TRPV4P19S-expressing BEAS-2B cells cultured in external media containing Ca^{2+}. (D) In keeping with Ca^{2+} responses shown in B, TRPV4P19S increased MMP-1 transcriptional activation. First, transfection of TRPV4wt increased MMP-1 reporter gene activation in response to OE by approximately 2-fold. Second, transfection of TRPV4P19S strikingly increased baseline MMP-1 reporter gene activation in response to OE by a factor of approximately 4-5 versus control-transfected cells and by a factor of >2 versus TRPV4wt. TRPV4wt and TRPV4P19S-mediated increases were virtually eliminated with a second point mutation, M680K (selectivity-filter block). This finding indicates that the effects of TRPV4wt and TRPV4P19S are mediated by an influx of external Ca^{2+} through the channel’s pore. (E) Validity of results and conclusions for MMP-1 secretion by transfected BEAS-2B cells.

*p < 0.05, and **p < 0.01.
all localize to motile cilia of primary human HBE cells.

**DEP-facilitated recruitment to a membrane-associated receptor-signaling multicomplex.** Given the ciliary localization of the DEP-evoked transduction cascade, we asked whether these membrane-associated signaling molecules coaggregate in response to DEP or OE (Figure 5). In aggregate, DEP-responsive membrane-bound signaling is characterized by a nonintercemenal interaction between PLCβ3 and TRPV4, with subsequent recruitment of PAR-2 and calmodulin caused by DEP exposure. Calmodulin’s response to DEP is inhibitory, because specific inhibition of calmodulin with W-7 increased Ca2+ signaling and MMP-1 activation (Figure 5B,C). This suggests as explanatory mechanisms an increase in intracellular Ca2+ concentration ([Ca2+]i) via disinhibited TRPV4 and/or activation of PLCβ3, both of which have previously been shown to bind calmodulin.

**COPD-associated TRPV4P19S increases MMP-1 activation in response to DEP or OE.** For human TRPV4, a number of genetic polymorphisms enhance susceptibility for COPD; one of them, P19S, is located in the coding region (Zhu et al. 2009). In another study, TRPV4P19S was reported as a DN channel in transfected HEK cells in response to weak but not strong hypotonicity (Tian et al. 2009). Because our finding that DEP-evoked Ca2+ influx via TRPV4 causes MMP-1 activation rationalizes airway injury by MMP-1 as caused by TRPV4 channel activity, not by DN channels, we attempted to resolve these seemingly contradictory concepts.

Compared with wild-type TRPV4 (TRPV4wt), TRPV4P19S exhibited gain-of-function effects in Ca2+ influx, patch clamp, MMP-1 reporter gene activation, and MMP-1 secretion [Figure 6; see also Supplemental Material, Figure 6 (doi:10.1289/ehp.1002807)]. For MMP-1 transcriptional activation, TRPV4P19S gain-of-function effects were strictly dependent on Ca2+ influx. This was evidenced by inhibitory effects of TRPV4P19S/M680K, where the selectivity-filter–blocking M680K mutation causes Ca2+ impermeability, leading to elimination of gain of function (Figure 6D). Furthermore, Ca2+ influx in response to changes in Ca2+ concentration and to DEP or OE were significantly increased in TRPV4P19S versus TRPV4wt, as was nonstimulated [Ca2+], extracellular Ca2+ concentration, 2 mM (Figure 6A–C; see also Supplemental Material, Figure 6). Thus, in a human airway epithelium–derived cell line with robust similarity to primary HBE cells, TRPV4P19S functions as Ca2+-permeable gain-of-function channel to hyperactivate the pathogenic mediator gene MMP-1 in response to the common air pollutant DEP.

**Figure 7.** Schematic overview showing effects of DEP on signaling in human ciliated airway epithelia leading to TRPV4-mediated Ca2+ influx. Abbreviations: β, G-protein β; γ, G-protein γ; C, C-terminus of TRPV4; N, N-terminus of PAR-2; P, position 19 of TRPV4 ion channel protein proline (wild-type); S, position 19 of TRPV4 ion channel protein proline serine (P19S polymorphism). (A) Overview of respiratory epithelia exposed to airborne DEP. (Left) Apical DEP with attached OE approaching the ciliary brush, basement membrane, and innervating nerve endings (blue). (Right) Detailed view of cilia showing DEP core particles contacting cilia and delivering organic chemicals (OE; light green circles) to the plasma membrane (ciliary colocalization is shown in Figure 4). (B) The signaling cascade begins with activation of PAR-2 (green); this ultimately leads to the influx of Ca2+ (dark green circles) via TRPV4 (blue) by GPCR signaling encompassing Gαq, which in turn leads to activation of PLCβ3 and PI3-K. PLCβ3 is phosphorylated (Phos) in response to DEP, partially accounting for the protracted Ca2+ response. PLCβ3 and PI3-K then regulate Ca2+ influx through TRPV4, which binds calmodulin (CaM), which is enhanced by DEP exposure; the increased CaM also protracts Ca2+ influx. (C) TRPV4-mediated Ca2+ entry activates RAS-RAF-MEK MAPK signaling (Li et al. 2009), resulting in reprogramming of transcriptional mechanisms that orchestrate remodeling of the extracellular matrix via activation of MMP-1. The COPD-susceptibility polymorphism TRPV4P19S functions as a gain-of-function channel for additional Ca2+ influx and MMP-1 activation, thus being relevant to human health.
Discussion

We identified a novel DEP-activated signaling pathway in human airway epithelia that consists of a GPCR (PAR-2) signaling to a TRP channel (TRPV4). Specific activation of PAR-2 by DEP leads to Ca²⁺ influx via two mechanisms in human airway epithelia. First, PAR-2 enhances Ca²⁺ influx by activating TRPV4. Second, Ca²⁺ influx is mediated by TRPV4 and PAR-2 in response to DEP or OE leads to maladaptive, proinflammatory reprogramming of gene-regulatory mechanisms in human airway epithelia.

In the novel signaling mechanism in human airway epithelia described here, Ca²⁺ influx is characterized by uniquely slow kinetics. Two possible causes for the slow kinetics are a) PAR-2-dependent PLCβ phosphorylation, and b) Ca²⁺-enhanced calmodulin binding to a receptor-signaling multiplex containing TRPV4 and PLCβ3 (Figures 5, 7), both of which attenuate signaling via known properties of the modified phospholipase or channel. The attractive hypothesis of pro-inflammatory activation of PAR-2 by MMP-1 has not been corroborated.

This study is relevant for global human health because of the global presence of DEP. However, we also discovered a possible novel mechanism of airway injury that is caused by PAR-2 yet enhanced by the human COPD-susceptibility polymorphism TRPV4R195P. Our identification of TRPV4R195P as a gain-of-function Ca²⁺-permeable channel in a human respiratory epithelial cell line, in response to DEP, links COPD pathogenesis to pathologically increased Ca²⁺ influx into human airway epithelia elicited by a globally relevant air pollutant. Furthermore, our results imply that two human genetic polymorphisms are linked to respiratory health, TRPV4R195P and MMP-1 (−1607G/G), thus highlighting the concept of disease susceptibility as a function of genetic “makeup” combined with environmental insults. Finally, we note yet another translational medical implication: the novel pathway described here can be targeted by inhalation of compounds that can specifically inhibit critical signaling molecules. In other words, although DEP injures respiratory epithelia via a luminal-apical unloading mechanism of DEP organics delivered by carbonaceous nanoparticles, this very same route could become the avenue for safe and effective therapy now that key participants are known.



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