

TRPA1 Mediates Oxidized Phosphatidylcholine-Induced Inflammatory Mediator Expression in Human Airway Fibroblasts

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Abstract

Background

A role for oxidative stress and lipid peroxidation is emerging for chronic inflammatory airways diseases. Oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine (OxPAPC), a bioactive molecule generated by inflammation-associated oxidative stress, induces transient receptor potential ankyrin 1 (TRPA1)-mediated Ca^{2+} influx and contraction in airway smooth muscle (ASM). Mesenchymal cells like ASM cells and fibroblasts participate in airway inflammation and remodeling. Here, we investigated the role of TRPA1 in OxPAPC-induced inflammatory mediator expression by primary human airway fibroblasts (HAFs) from male and female donors.

Methods

Primary cultured HAFs from lung resection specimens of male and female donors were used to assess OxPAPC (60 $\mu\text{g/mL}$)-induced Ca^{2+} influx with Fluo-4 dye. OxPAPC induced inflammatory responses were evaluated under normal and Ca^{2+} -free or with TRPA1 inhibitor A967079 (1 μM) conditions, using qRT-PCR (IL-6, IL-8, COX2), Luminex assays (IL-6/IL-8 release), and immunoblotting (COX2). Data ($N \geq 5$) were analyzed by one-way ANOVA with Tukey's or Dunnett's post hoc test or two-tailed paired t tests as appropriate, with $p < 0.05$ considered a significant difference.

Results

OxPAPC (80 $\mu\text{g/mL}$) induced a concentration-dependent peak and subsequent sustained plateau in intracellular concentration ($[\text{Ca}^{2+}]_i$). These two $[\text{Ca}^{2+}]_i$ responses could be suppressed by 79.9 ± 0.6 and $77.8 \pm 0.2\%$, respectively, with the TRPA1 inhibitor A967079 (1 μM). OxPAPC also increased IL-6 (5.8 ± 1.0 fold), IL-8 (31.4 ± 7.7 fold), and COX-2 mRNA (20.7 ± 4.5 fold). A967079 suppressed these responses by 35.3 ± 0.8 , 68.8 ± 3.4 , and $89.1 \pm 6.4\%$, respectively. The absence of extracellular Ca^{2+} also inhibited OxPAPC-induced IL-6, IL-8, and COX-2 mRNA by 68.9 ± 1.4 , 69.9 ± 1.6 , and $85.4 \pm 3.7\%$. Selective TRPA1 activation (AITC, 0.5 μM) was sufficient to induce IL-6, but not IL-8 nor COX-2 mRNA. We also confirmed that OxPAPC elevated IL-6 and IL-8 release, and increased COX-2 protein abundance, but this was unaffected by TRPA1 blockade.

Conclusion

Our data reveal that TRPA1 mediates, in part, OxPAPC-induced Ca^{2+} influx and inflammatory gene expression in primary cultured HAFs. This response likely requires additional pathways as it is not strictly dependent on TRPA1. This work provides new insight about how oxidized phospholipids may amplify airway inflammation via effects on airway structural cells.

INTRODUCTION

Environmental pollutants, allergens, viral infections, and cigarette smoke increase the reactive oxygen species (ROS) in the airways and can overwhelm endogenous antioxidant defenses, leading to oxidative stress [1, 2]. Excess ROS, including superoxide and hydrogen peroxide, chemically modify extracellular and intracellular biomolecules, including phospholipids in cell membranes, and extracellular fluid, including surfactant. A spectrum of bioactive oxidized phosphatidylcholines (OxPCs) are produced, including oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (OxPAPC), a damage-associated molecular pattern [3–6]. In cardiovascular disease, OxPAPC drives inflammatory cytokine and chemokine production, impairs efferocytosis, and sustains chronic inflammation [7, 8].

In the lungs, OxPAPC is associated with acute and chronic inflammatory responses, endothelial barrier dysfunction, immune cell activation, altered cytokine profiles, and airway remodeling [9, 10]. Our work revealed that OxPCs are elevated in the airways of atopic asthmatic participants after allergen challenge, and their levels correlate with lung dysfunction [4]. We also report that selectively masking OxPCs using inhaled antibody therapy in allergic mice, is sufficient to suppress inflammation and airway hyperreactivity [11]. OxPAPC also disrupts barrier integrity of human airway epithelial cells in culture, suppresses wound repair, and potentiates ROS-mediated mitochondrial dysfunction to perpetuate oxidative stress [12]. In airway smooth muscle (ASM) cells, OxPAPC activates protein kinase C, leading to increased expression of cyclooxygenase 2 (COX-2), release of pro-inflammatory cytokines and chemokines such as Interleukin-6 (IL-6) and IL-8, the generation of asthma-associated oxylipins, and inhibition of bronchodilation by suppressing β 2-adrenergic signalling [4, 13]

OxPAPC also induces human and murine ASM contraction via activation of transient receptor potential ankyrin 1 (TRPA1), a cell membrane non-selective cation channel [14]. TRPA1, initially identified as a neuronal sensor of noxious stimuli, has emerged as an effector of oxidative stress and inflammation in non-neuronal cells, including airway epithelium, smooth muscle, and immune cells [15–19]. Activated by ROS and lipid peroxidation products, TRPA1 is ideally positioned to function as a molecular sensor of oxidized phospholipids within the inflamed airway environment [14, 17].

Fibroblasts represent a major structural cell in the airway, and they are critically involved in the pathobiology of airway inflammatory diseases and airway remodeling. Beyond their roles in extracellular matrix production and tissue repair, airway fibroblasts actively communicate with a diverse range of immune cells and can orchestrate local immune regulation by secreting cytokines, chemokines, and lipid mediators that amplify innate immune responses to promote leukocyte recruitment [20–23]. To explore the possible link between oxidative stress, fibroblast function, and inflammation, we investigated OxPAPC-induced inflammatory mediator mRNA and protein expression in primary human airway fibroblasts (HAFs) and evaluated the functional involvement of TRPA1.

MATERIALS AND METHODS

TRPA1 mouse monoclonal antibody (SC-376495), GAPDH (sc-47724) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), rabbit polyclonal antibodies COX-2 (4842) and COX-1 (4841) were obtained from Cell Signaling Technology (Beverly, MA), and rabbit polyclonal antibody β -actin (ab8227) from Abcam (Abcam, Waltham, MA, US). Secondary IgG peroxidase conjugate antibodies anti-rabbit (A6154) and anti-mouse (A9044), TRPA1 agonist allyl isothiocyanate (AITC), and TRPA1 inhibitor A967079 were obtained from Sigma (Oakville, ON, Canada). Fluo-4 calcium assay kit (F10471), Trizol (15596018), high-capacity cDNA reverse transcription kit (4368814), SYBR green real-time PCR master mixes (A46109), SYBR safe DNA gel stain, all primer sets and instrument NanoDrop 2000c spectrophotometry (ND-2000) were purchased from Thermo Fisher Scientific (IL, USA). Immuno-blot PVDF membranes, 30% acrylamide/bis solution, and agarose powder were from Bio-Rad. ECL substrate were used from Cytiva Amersham (RPN 2235 and RPN 2109) and DMEM culture media, insulin-transferrin-selenium (ITS), and fetal bovine serum (FBS) were obtained from Gibco, Thermo Fisher Scientific. Black, clear flat-bottom 96-well plates were purchased from Costar®, Corning Incorporated (3603).

Lipid Oxidation

Commercially available PAPC (1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, 35418-58-7) was oxidized as previously reported by exposure to ambient air under a fume hood for 4–5 days until PAPC noticeably changed from white to pale yellowish color [4]. Oxidized-PAPC was reconstituted to a stock concentration of 1 mg/mL in a 2:1 chloroform-methanol solution. Prior to each experiment, OxPAPC stock solution was evaporated by sterile nitrogen gas and residual lipid was resuspended in DMEM starvation medium (containing 1% ITS and 1% penicillin-streptomycin), depending on the experimental conditions.

Primary Human Airway Fibroblast Culture

HAFs were isolated from second to fourth generation bronchi of histologically normal lung tissue obtained from donors undergoing surgical resection (donor characteristics provided in Table 1). Tissue procurement was performed in accordance with institutional ethical guidelines. Fibroblasts were isolated by enzymatic dissociation, and cellular phenotype was confirmed using established airway fibroblast markers as previously described [24]. HAFs were cultured in DMEM supplemented with 5% FBS and 1% penicillin–streptomycin at 37°C in 5% CO₂. For experiments, cells were serum-starved for 24 h in DMEM containing 1% ITS and 1% penicillin–streptomycin. In selected experiments, cells were maintained in serum- and calcium-free (0 mM Ca²⁺) DMEM for no longer than 2 h to avoid cell death or cellular stress associated with prolonged Ca²⁺ deprivation. All experiments were performed using passages 3–6, with equal representation of male and female donor-derived cell lines.

Intracellular Calcium Fluorescence Assay

HAFs were seeded in black, clear flat-bottom 96-well plates and grown to 80–90% confluence, followed by serum starvation for 24 h. Cells were then maintained in serum-free DMEM containing either 1.26 or 0 mM Ca^{2+} , as specified for each experiment. Where indicated, cells were pretreated with the TRPA1 inhibitor A967079 (1 μM) and TRPV1 inhibitor AMG-517 (1 μM), for 30 min and loaded with Fluo-4 Direct Calcium Assay reagent (final concentration 1x), for a total of 60 min (30 min at 37°C in 5% CO_2 , followed by 30 min at room temperature), according to the manufacturer's instructions. Baseline intracellular Ca^{2+} level ($[\text{Ca}^{2+}]_i$) was recorded for 30 s before stimulation with allyl isothiocyanate (AITC; 0.5 μM) or OxPAPC (10–80 $\mu\text{g/mL}$). Changes in fluorescence (F/F_0) were monitored in real time for 180 s using a Cytation 5 plate reader (BioTek) at excitation/emission of 484 ± 20 nm and 525 ± 20 nm, with the gain set to 100 at normal reading speed. Analysis of the peak and plateau fluorescence intensities, respectively corresponding to Ca^{2+} mobilization from internal stores and sustained influx of extracellular Ca^{2+} in mesenchymal cells [14, 25], were quantified at fixed time points (indicated in the Figure legends) using the top five peak fluorescence ratios ($\Delta F/F_0$). All experimental conditions were performed in technical triplicates.

Quantitative Real-Time PCR

Total RNA was isolated from cultured cells using TRIzol reagent according to the manufacturer's protocol. Total RNA concentration and purity were assessed by NanoDrop spectrophotometer and equal amounts of RNA (1 μg) were reverse-transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) under standard reaction conditions. Quantitative real-time PCR was performed using PowerTrack SYBR Green Master Mix (Applied Biosystems) with gene-specific primers (Table 2) on a C1000 Touch CFX thermal cycler (Bio-Rad). Amplification specificity was verified by melt-curve analysis. TRPA1 amplicon identity was further confirmed by electrophoresis on a 2% agarose gel and PCR products were visualized using SYBR Safe staining and blue light. Relative mRNA abundance was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method and normalized with RPS16 as the internal reference gene. All reactions were performed in technical triplicates.

Immunoblotting

Total cellular protein was extracted using Radio Immuno Precipitation Assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF, 1 mM) and protease inhibitor cocktails (1X). Protein concentration was determined using the DC Protein Assay (Bio-Rad). Total protein (50 μg for TRPA1 and 20 μg for COX-1 and COX-2) were resolved on 8% SDS–PAGE gels and transferred to methanol-activated PVDF membranes by wet transfer. Membranes were blocked in 5% non-fat milk in TBST for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against TRPA1 (1:500), COX-2 (1:1000), COX-1 (1:1000), β -actin (1:10000), or GAPDH (1:5000). After 3X washing (10 min each wash), membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Immunoreactive bands were detected using enhanced chemiluminescence (Amersham

ECL) and imaged with a ChemiDoc system (Bio-Rad). Densitometric analyses were performed using AlphaEase FC software version 6.0.0 (Alpha Innotech). Protein abundance was normalized to β -actin or GAPDH.

Cytokine Quantification

Secreted cytokines, chemokines, and growth factors in cell culture supernatants were quantified using a 15-plex Luminex xMAP assay (Eve Technologies, Alberta, Canada). Samples were processed according to company instructions and analyzed in technical duplicates using cells from five independent donors.

Statistical Analysis

Data were analyzed using GraphPad Prism version v10.1.1 and are presented as mean $\pm$ SEM unless otherwise indicated. Statistical significance was assessed using one-way ANOVA followed by Tukey's or Dunnett's post hoc tests, or two-tailed paired t tests for two-group comparisons, as appropriate. Differences were considered statistically significant when $p < 0.05$. Specific statistical tests and number of replicate experiments are indicated in the Figure Legends.

RESULTS

OxPAPC Increases Cytosolic Ca^{2+} and Inflammatory Gene Abundance

OxPAPC induced a dose-dependent increase in $[\text{Ca}^{2+}]_i$, which was significant at a concentration of 20 $\mu\text{g/mL}$ and reached a maximum fluorescence peak response of 2.37 ± 0.3 ($\Delta F/F_0$; relative change from the baseline) at 80 $\mu\text{g/mL}$. The peak signal in response to each concentration OXPAPC was measured between 69–73 seconds post-stimulation (Fig. 1A-B). Next, we assessed the effects of 80 $\mu\text{g/mL}$ OxPAPC on mRNA abundance of the inflammatory genes IL-6, IL-8 and COX-2. A time-course analysis revealed IL-6 mRNA induction was rapid and peaked at 2 hours (5.8 ± 1.0 -fold, $p = 0.002$) before gradually decreasing to basal levels over 24h (Fig. 2A). In contrast, mRNA for IL-8 significantly increased within 2h but peaked at 24h (31.4 ± 7.7 -fold, $p = 0.003$; Fig. 2B), whereas COX-2 was maximally induced within 4h (20.7 ± 4.5 -fold; $p = 0.002$) and remained elevated up to 24h (17.8 ± 4.5 -fold, $p = 0.006$; Fig. 2C).

Analysis of cell culture supernatants using ELISA revealed that out of 15 tested cyto- and chemokines, IL-6 and IL-8 could be consistently detected in response to OxPAPC (80 $\mu\text{g/mL}$). Secretion was significant at 24h and near maximal for IL-6 (38.6 ± 10.8 ; $p = 0.03$) but further increased for IL-8 after 48h (15.0 ± 4.3 , $p = 0.02$, 48h) (Fig. 2D-E). Similarly, COX-2 protein abundance in whole cell lysates (COX-2 is not secreted) was substantially increased at 24h and remained elevated after 48h treatment with OxPAPC (Fig. 2F).

Extracellular Ca^{2+} Requirement for OxPAPC-induced Inflammatory Gene Transcripts

To establish a functional link between OxPAPC-induced cytosolic Ca^{2+} accumulation, and inflammatory gene expression we evaluated the requirement of extracellular Ca^{2+} for OxPAPC-mediated mRNA induction. Cells were maintained in Ca^{2+} -free DMEM for 2 h, a duration optimized to prevent cell death and exposed to OxPAPC. In the presence of physiological concentrations of extracellular Ca^{2+} (1.26 mM), OxPAPC significantly increased the expression of IL-6, IL-8, and COX-2, by 5.8 ± 1.3 - (p = 0.007), 7.3 ± 1.6 - (p = 0.01), and 19.2 ± 3.5 -fold (p = 0.0004), respectively. Complete removal of extracellular Ca^{2+} strongly prevented OxPAPC-induced gene expression, resulting in significant reductions of $68.9 \pm 1.4\%$ (IL-6), $69.9 \pm 1.6\%$ (IL-8), and $85.4 \pm 3.7\%$ (COX-2) (Fig. 3A-C).

We next assessed the importance of extracellular Ca^{2+} in OxPAPC-induced increases in $[\text{Ca}^{2+}]_i$, by treating cells in the presence and absence of extracellular Ca^{2+} . OxPAPC-induced peak $[\text{Ca}^{2+}]_i$ was significantly reduced (by $28 \pm 0.1\%$), while the sustained plateau phase (driven by extracellular Ca^{2+} influx) was absent under Ca^{2+} -free conditions (Suppl. Fig. S1A-C). These findings indicate that extracellular Ca^{2+} (influx) is involved in OxPAPC-induced sustained increases in $[\text{Ca}^{2+}]_i$ and inflammatory gene transcription.

Primary HAFs Express Functional TRPA1 Channels

We showed that the TRPA1 mediates sustained $[\text{Ca}^{2+}]_i$ elevation induced by OxPAPC in human ASM cells [14]. Agarose gel electrophoresis of qRT-PCR products and immunoblot analysis confirmed specific TRPA1 transcript and protein abundance in primary HAF cultures created from bronchial specimens of nine donors, which included four male and five female participants (Fig. 4A-B; donor characteristics in Table 1). To evaluate channel function, HAFs were stimulated with the selective TRPA1 agonist allyl isothiocyanate (AITC; 0.5 μM). In all cultures this resulted in a rapid and sustained increase in $[\text{Ca}^{2+}]_i$. This response was TRPA1-specific, as pre-treatment with the selective TRPA1 antagonist A967079 (1.0 μM) attenuated AITC-evoked rises in peak and plateau $[\text{Ca}^{2+}]_i$ by $68.9 \pm 0.4\%$ and $74.8 \pm 0.3\%$, respectively (Fig. 4C-E). Comparative analyses revealed no sex-dependent differences in TRPA1 mRNA and protein abundance or in AITC-induced Ca^{2+} responses (Suppl. Fig. S2A-C).

TRPA1 Inhibition Strongly Prevents OxPAPC-Induced Elevations in Cytosolic Calcium

To assess the functional contribution of TRPA1 to OxPAPC-induced Ca^{2+} flux, we stimulated HAFs with a submaximal concentration of OxPAPC (60 $\mu\text{g}/\text{mL}$) and measured $[\text{Ca}^{2+}]_i$ in the presence and absence of A967079. Stimulation with OxPAPC elicited a rapid peak (3.0 ± 0.6) and sustained (2.2 ± 0.3) increase in $[\text{Ca}^{2+}]_i$. TRPA1 inhibition significantly suppressed OxPAPC-induced peak and plateau $[\text{Ca}^{2+}]_i$ by $79.9 \pm 0.6\%$ (assessed at t = 59–63s; p = 0.0002) and $77.9 \pm 0.3\%$ (assessed at t = 170–174s; p = 0.0002), respectively (Fig. 5A-C).

Involvement of TRPA1 in the OxPAPC-Induced Inflammatory Response in HAF

To determine whether the effects of OxPAPC on inflammatory gene expression are mediated through TRPA1 signaling, the mRNA abundance of IL-6, IL-8 and COX-2 was assessed at peak expression (IL-6 at 2 h; IL-8 and COX-2 at 24 h; Fig. 2A-C) in HAFs stimulated with OxPAPC (80 μ g/mL) in the presence and absence of A967079 (1.0 μ M). Pharmacological inhibition of TRPA1 significantly attenuated OxPAPC-induced mRNA for IL-6 ($-35.3 \pm 0.8\%$; $p = 0.012$), IL-8 ($-68.8 \pm 3.4\%$; $p = 0.003$), and COX-2 ($-89.2 \pm 6.4\%$; $p = 0.02$; Fig. 6A-C). Notably, treatment with OxPAPC for up to 48h did not alter TRPA1 mRNA or protein content, indicating that OxPAPC exposure leads to activation of pre-existing TRPA1 channels rather than upregulating TRPA1 expression (Suppl. Fig. S3A-C). We next examined whether transcriptional suppression translated into changes at the protein level. Pre-treatment with A967079 did not significantly reduce OxPAPC-induced IL-6 and IL-8 release, or COX-2 protein abundance (Fig. 6D-F). Collectively, these data establish TRPA1 as an effector of OxPAPC-induced inflammatory genes in HAFs. The lack of effect on protein expression suggests that, while TRPA1-dependent Ca^{2+} signaling is essential for inflammatory gene induction, additional TRPA1-independent pathways contribute to the OxPAPC-driven inflammatory phenotype in these cells.

Direct TRPA1 Activation is Insufficient to Fully Recapitulate the OxPAPC-Induced Inflammatory Response

We next asked if direct activation of TRPA1 alone is sufficient to induce an inflammatory response in HAFs. Timepoints for mRNA analysis were based on findings in Fig. 2A-C. Stimulation with the selective TRPA1 agonist AITC (0.5 μ M) resulted in a modest but significant increase in IL-6 mRNA (3.0 ± 0.5 -fold induction; $p = 0.014$) (Fig. 7A). In contrast, AITC did not alter mRNA abundance of IL-8 or COX-2, albeit a trend for an increase was evident (Fig. 7B-C). In line with the limited effects on mRNA, AITC did not elevate IL-6 or IL-8 release in supernatant or COX-2 abundance in cell lysates (Fig. 7D-F).

DISCUSSION

Our prior work demonstrates that OxPAPC induces mitochondrial dysfunction and DNA damage in airway epithelial cells, also triggering protein kinase C, inflammatory signaling, and Ca^{2+} -dependent contraction of human ASM cells via TRPA1 and ryanodine receptor activation [4, 14, 26]. This establishes OxPAPC as a mediator that modulates function of different airway cells. The current study extends this paradigm to include HAFs, a functionally distinct mesenchymal cell population that contributes to airway inflammation, extracellular matrix remodeling, and airway fibrosis in association with pathobiology of asthma and other lung disorders [23, 27, 28]. In contrast to alveolar lung fibroblasts, airway fibroblasts reside in close proximity to both the airway epithelium and ASM where they can produce cytokines, chemokines, and oxylipins that modulate local immune response [23, 29]. Prior to our study, the direct impact of OxPAPC on airway fibroblast function was undefined. Our data here for primary cultured HAFs demonstrate that pathophysiological concentrations of OxPAPC [4] induce cytosolic Ca^{2+} accumulation, in part via Ca^{2+} influx involving TRPA1, which acts as an early and essential event in an OxPAPC-induced inflammatory response. These findings are consistent with evidence that oxidized lipids are potent Ca^{2+} mobilizing agents in non-excitable cells, and that this links oxidative stress to induction of inflammatory

gene induction [30, 31]. This work positions airway fibroblasts as a target of OxPAPC signaling and provides a mechanistic framework for understanding how OxPCs contribute to airway pathobiology in disease such as asthma or COPD.

We show that OxPAPC-induced intracellular Ca^{2+} is functionally coupled to the induction of inflammatory mediators, including IL-6, IL-8, and COX-2, at both the mRNA and protein levels. These mediators are well-established contributors to asthma pathogenesis. IL-6 promotes Th17 differentiation and airway remodeling, IL-8 drives neutrophilic inflammation, and COX-2 regulates prostaglandin synthesis that modulates airway tone and inflammation [32–34]. Our demonstration of the requirement of extracellular Ca^{2+} for OxPAPC-induced inflammatory gene expression provides direct evidence that calcium influx is a regulatory component of the fibroblast inflammatory response. This aligns with studies showing that Ca^{2+} -dependent signaling pathways, such as NF- κ B, NFAT and MAPKs, are central regulators of cytokine gene transcription in skin and cardiac fibroblasts, and airway epithelial cells [35–38]

Because we previously identified TRPA1 as a OxPAPC-responsive Ca^{2+} channel in human ASM, here we explored whether a concomitant role exists in HAFs. TRPA1 is a redox-sensitive, non-selective cation channel that is well characterized in sensory neurons and is being increasingly recognized in non-neuronal cells as a molecular sensor of oxidative and electrophilic stress [39]. Although TRPA1 expression and functional relevance have been reported in distal lung fibroblasts [40–43], its role in airway fibroblasts, particularly in the context of inflammatory signaling, is unexplored. In the present study, we demonstrate that TRPA1 is expressed and functional in HAFs, independent of biological sex, and that OxPAPC activates TRPA1 channels to induce pronounced Ca^{2+} influx. This response occurs without changes in TRPA1 transcript and protein expression and is attenuated by the pharmacologic inhibition of TRPA1. This reveals a functional role for TRPA1 in effecting OxPAPC-induced Ca^{2+} entry in airway fibroblasts and extend the emerging paradigm for TRPA1-dependent redox sensing in the lung.

Despite effective suppression of OxPAPC-induced IL-6, IL-8, and COX-2 mRNA, corresponding protein levels were not significantly reduced by TRPA1 inhibition. This dissociation between gene induction and/or transcript half-life with protein synthesis demonstrates that OxPAPC-driven inflammatory signaling is not exclusively dependent on TRPA1-mediated Ca^{2+} entry. Thus, additional parallel or compensatory pathways are likely involved, and await identification in future work. A functional interaction between TRPA1 and TRPV1 in inflammatory signaling has been reported [44, 45]. However, pharmacological inhibition of TRPV1 with AMG517 did not attenuate OxPAPC-induced Ca^{2+} influx, indicating a negligible role for TRPV1 in HAFs (Fig. S4A-C). OxPAPC can activate multiple signaling cascades, including PKC, MAPKs, and Toll-like receptor-associated pathways that can sustain inflammatory protein expression independently of Ca^{2+} -dependent transcriptional events [46, 47]. Additionally, post-transcriptional regulatory mechanisms, such as mRNA stability, microRNA regulation, and feedback autocrine signaling, can contribute to the persistence of inflammatory protein expression despite partial upstream inhibition [48, 49].

To delineate the impact of direct TRPA1 activation on the inflammatory response, we stimulated HAFs with the canonical TRPA1 agonist, AITC. Notably, this induced IL-6 mRNA without significantly affecting IL-8 or COX-2, and AITC failed to increase the abundance of any of the mediators studied at the protein level. This confirms that TRPA1 activation alone is insufficient to fully recapitulate the inflammatory HAF phenotype induced by OxPAPC. Therefore, OxPAPC likely engages additional receptors and/or intracellular targets that cooperate with TRPA1 to drive a broader inflammatory response. This is consistent with the multifunctional nature of OxPCs, which can simultaneously act as ion channel agonists, kinase activators, and transcriptional modulators [50, 51]

We performed our studies using primary airway fibroblasts isolated from healthy donors. Future studies will be aimed at studying OxPAPC responsiveness and TRPA1 sensitivity in cells isolated from donors suffering from chronic airway diseases, including asthma and COPD. Gaining insight in potential disease specific differences could have important implications for our understanding of mechanisms that underpin and perpetuate (chronic) airway inflammation and remodeling. In addition, although pharmacological inhibition with A967079 provides strong evidence for TRPA1 involvement, complementary genetic approaches such as siRNA-mediated knockdown or CRISPR-Cas9 based deletion would further strengthen causal inference and help exclude potential off-target effects. Notably, the persistence of IL-6, IL-8 and COX-2 protein expression despite TRPA1 inhibition suggests the existence of TRPA1-independent signaling pathways downstream of OxPAPC signaling. Elucidating these mechanisms will be essential for a more complete understanding of OxPAPC-driven inflammatory responses in airway fibroblasts. In addition, *in vivo* validation of TRPA1-dependent and -independent pathways in relevant models of airways disease are necessary to confirm the physiological relevance of our *in vitro* findings and assess the therapeutic potential of targeting OxPCs or TRPA1.

Collectively, our results suggest a model for OxPAPC to function as a danger-associated molecular pattern mediator in the airway microenvironment, triggering Ca^{2+} -dependent inflammatory activation of airway fibroblasts via TRPA1-dependent and -independent mechanisms. The selective sensitivity of IL-6 induction by TRPA1 activation highlights the possibility that cytokine-specific regulatory pathways exist and suggests that fibroblast-derived IL-6 may be particularly responsive to oxidative stress-induced effects. Given the role of airway fibroblasts in coordinating inflammation and remodeling, our findings enhance understanding of how oxidative stress perpetuates chronic airway disease and identifies TRPA1 and OxPCs as partial therapeutic targets.

In conclusion, we report that OxPAPC induces a Ca^{2+} -dependent inflammatory phenotype in primary HAFs, and identify TRPA1 as a key effector, including the induction of inflammatory genes. The incomplete suppression of inflammatory protein expression by TRPA1 inhibition in our studies indicates that OxPAPC engages multiple signaling pathways to promote inflammation. This is new mechanistic insight for how oxidative stress-derived lipids amplify airway inflammation and reveal complexity of Ca^{2+} -dependent signaling networks in airway structural cells. Targeting OxPAPC-induced signaling pathways may represent a strategy to mitigate effects of oxidative stress associated airway inflammation and remodeling.

Declarations

Conflict of Interest:

None of the authors have nothing to disclose.

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Author Contribution

D.P. designed and coordinated the study, including data collection and analysis, and prepared the manuscript. A.D. provided technical assistance and prepared OxPAPC stocks. A.Y. developed and characterized the HAF model used in this study. D.S. was involved in data interpretation and edited the MS. A.J.H. conceived the project, its design and the data plan, with support from D.S and D.P., and performed the final edit of the manuscript.

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Tables

Table 1: Clinical Characteristics of Human Airway Fibroblast Donors

Cell Type	History	Sex	Age (Yrs.)	PFT	
Primary Human Airway Fibroblast	Non-COPD and Ex-Smoker	Male		FEV1	FEV1/FVC
			64	3.2=103%	3.2/4.28=75%
			65	2.78=86%	2.78/3.86=72%
			77	2.69=90%	2.69/3.67=73%
			76	3.32=88%	3.32/4.55=73%
		Female	56	2.63=111%	2.63/3.49=93%
			57	2.67=108%	2.67/3.18=84%
			59	2.49=98%	2.49/3.14=79%
			61	2.25=91%	2.25/2.91=77%
			65	2.18=96%	2.18/2.67=82%

Table 2: *Primer Sets Used for qRT-PCR Studies*

Sr. #	Gene	Forward	Reverse
1	RPS16	CAATGGTCTCATCAAGGTGAACGG	CTGGATAGCATAAATCTGGGC
2	GAPDH	ATGGAAATCCCATCACCATCT	CAGCATCGCCCCACTTG
3	TRPA1	CCCCTCTGCATTGTGCTGTA	TGGCTCCTCTCCACATAGCT
4	GM-CSF	CACTGCTGCTGAGATGAATGAAA	GTCTGTAGGCAGGTCGGCTC
5	IL-8 (CXCL8)	GCAGCTCTGTGTGAAGGTGC	AAAGGTTTGGAGTATGTCTTTATGCA
6	IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTTAC
7	COX-2	CAAATTGCTGGCAGGGTTG	GGTCAATGGAAGCCTGTGATA

Figures

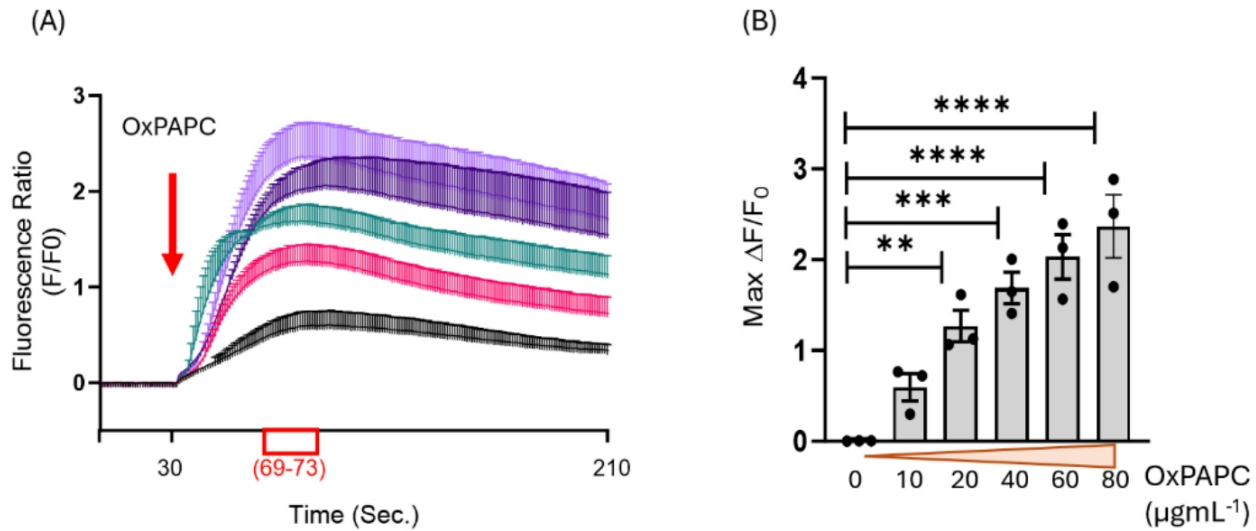


Figure 1

OxPAPC induces a concentration-dependent increase in intracellular Ca^{2+} in primary HAFs. (A) Time-course curves showing OxPAPC (10–80 $\mu\text{g/mL}$)-induced changes in intracellular fluorescence intensity (correlating with $[\text{Ca}^{2+}]_i$). The top five peak fluorescence values were recorded between 69–73 s after stimulation. (B) Bar graph summarizing the concentration-dependent effects of OxPAPC on peak $[\text{Ca}^{2+}]_i$, expressed as the mean of the top five fluorescence values between 69–73 s after stimulation for each concentration. Data represent means $\pm$ SEM from 3 different primary cell lines and were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

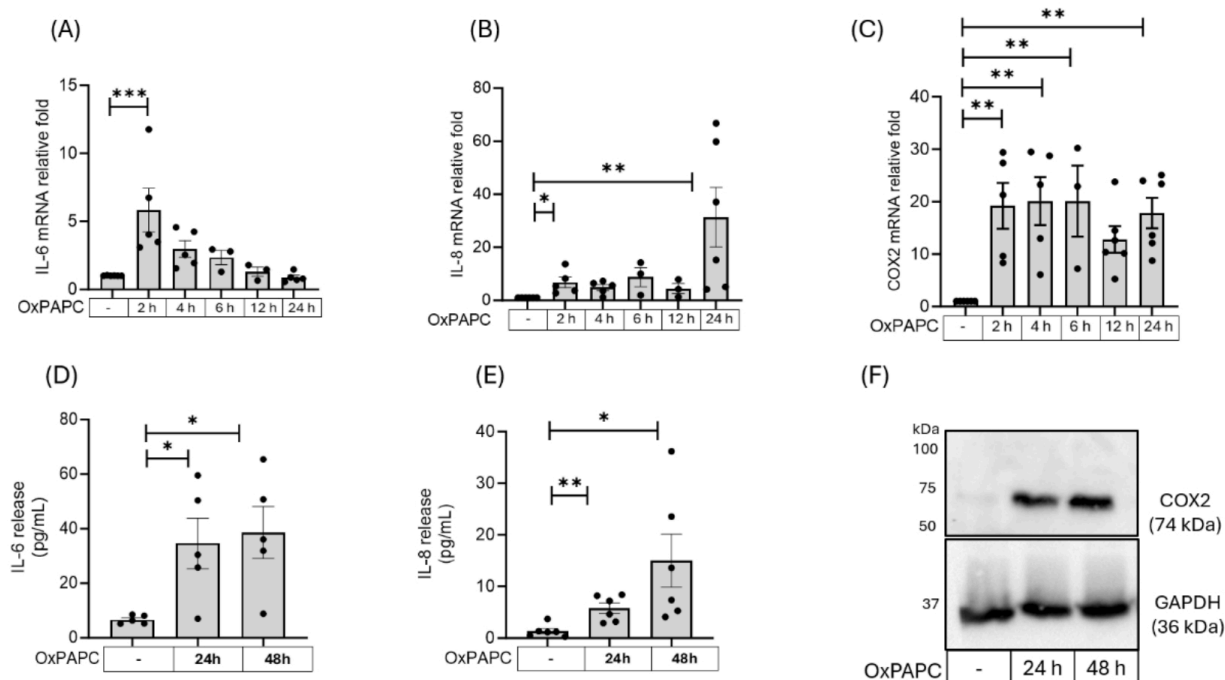


Figure 2

OxPAPC induces inflammatory mediator mRNA and protein expression in HAFs. (A-C) Bar graphs depicting the temporal effects (0-24h) of OxPAPC (80 μ g/mL) on the mRNA abundance of the inflammatory genes IL-6, IL-8, and COX-2 in primary HAFs. (D-E) Bar graphs showing IL-6 and IL-8 protein levels in supernatant following OxPAPC (80 μ g/mL) exposure for 24 and 48 h. (F) Immunoblot analysis of COX-2 protein (74 kDa) expression in HAFs treated with OxPAPC for 24 and 48 h. Whole cell lysates were prepared from the same HAF cultures used for IL-6 and IL-8 secretion analyses; GAPDH was used as a loading control. Data represent means $\pm$ SEM (N=5-6 donors) and were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison test *P < 0.05, **P < 0.01.

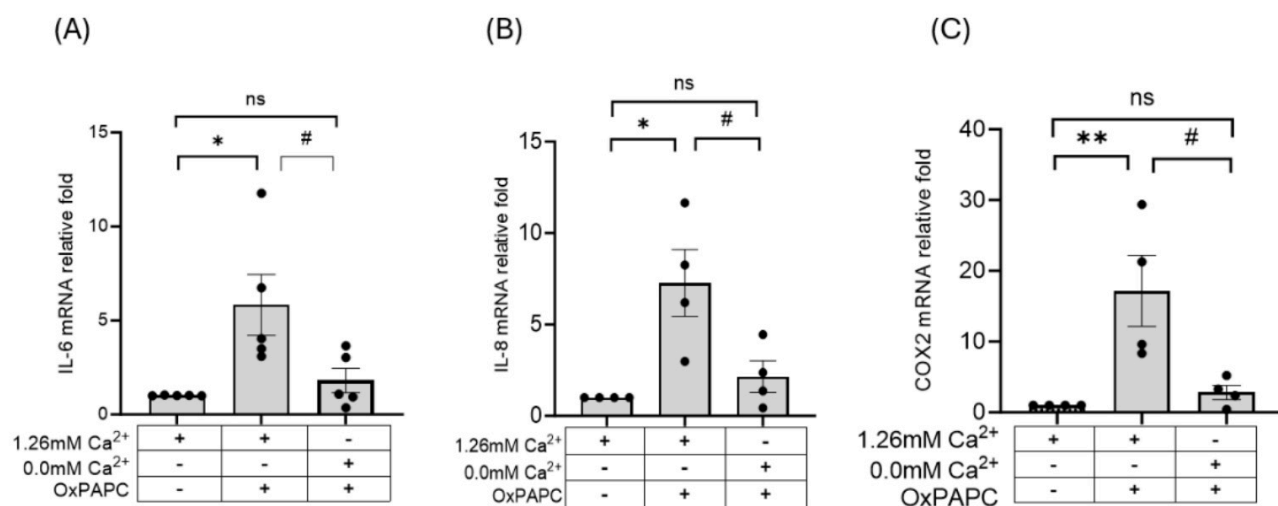


Figure 3

OxPAPC-triggered extracellular Ca^{2+} influx is required for inflammatory gene expression in HAFs. (A-C) Bar graphs showing the mRNA abundance of the inflammatory genes IL-6, IL-8 and COX-2 following exposure to OxPAPC (80 $\mu\text{g}/\text{mL}$) under Ca^{2+} -free (0 mM Ca^{2+}) and Ca^{2+} -replete (1.26 mM Ca^{2+}) conditions. Data represent means $\pm$ SEM (N=4-5 donors) and were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

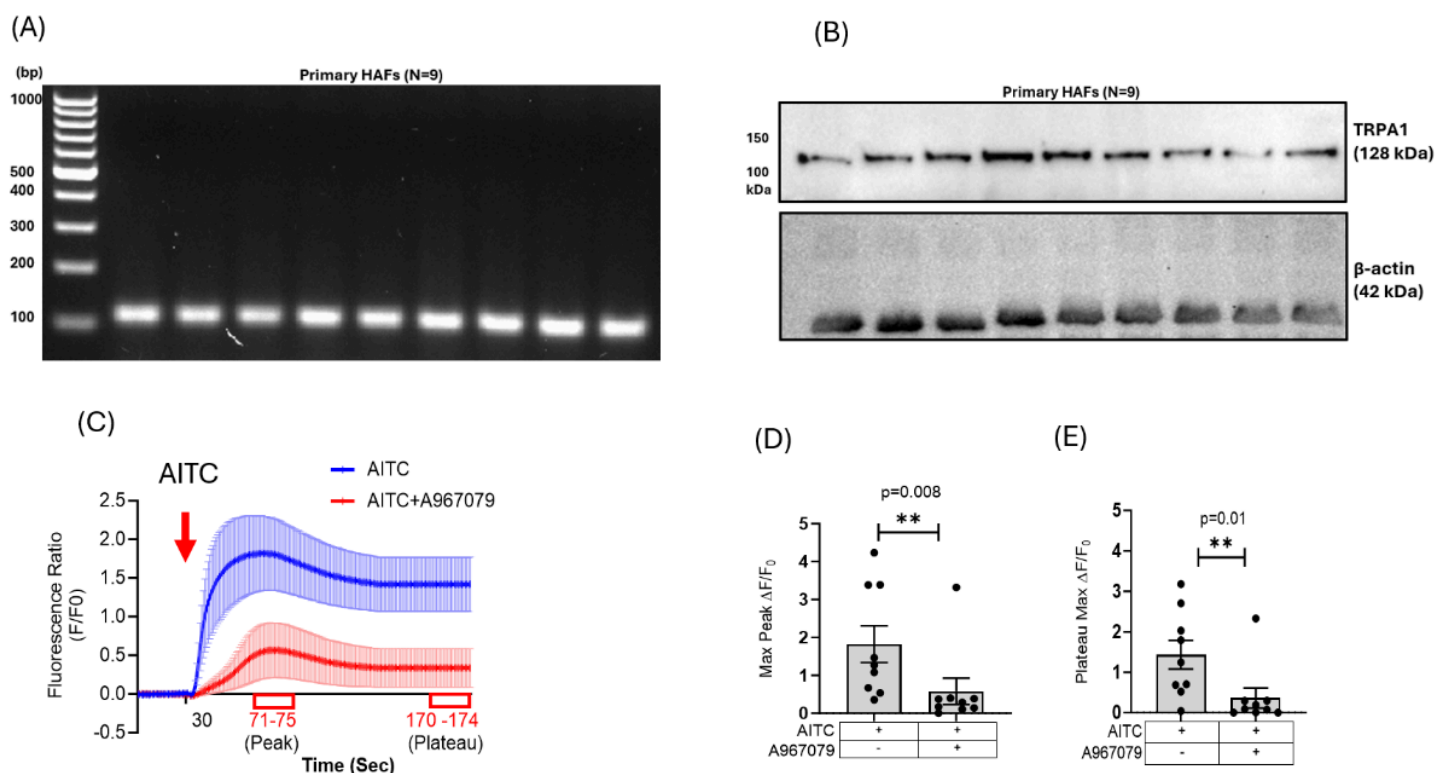


Figure 4

Primary HAFs express functional TRPA1 channels. (A) Agarose gel image showing TRPA1 mRNA abundance in HAFs obtained from all 9 donors (4 males, 5 females). (B) Immunoblot confirming TRPA1 protein expression (~128 kDa) in whole HAF lysates from the same 9 donors; β -actin was used as a loading control. (C) Temporal Ca^{2+} tracings showing a rapid and transient increase in $[\text{Ca}^{2+}]_i$ in response to AITC (blue), which was abrogated by preincubation with the selective TRPA1 inhibitor A967079 (1 μM ; red) for 30 min (N=9 donors) (D) Bar graph summarizing the mean peak (measured between 71–75s; left panel) and plateau (measured between 170–174s; right panel) values in response to AITC in the absence and presence of A967079. Data represent means $\pm$ SEM (N=9 donors) and were analyzed by a paired two-tailed t-test. *P < 0.05, **P < 0.01.

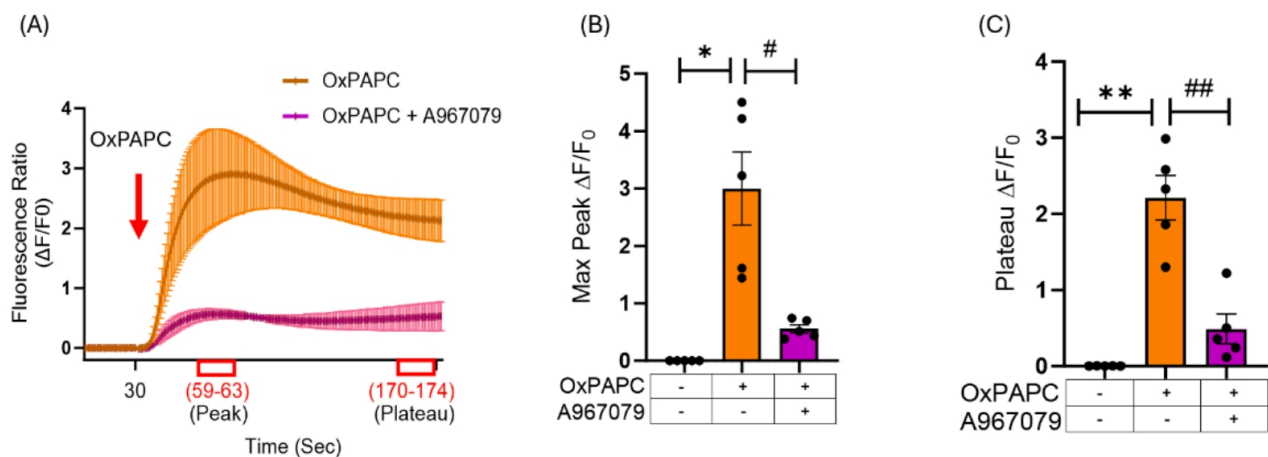


Figure 5

TRPA1 channel blockade abrogates OxPAPC-induced rises in intracellular Ca^{2+} in HAFs. (A) Time-course curve showing $[\text{Ca}^{2+}]_i$ peak (measured at 59–63s) and plateau (measured at 170–174s) levels in response to OxPAPC alone (60 $\mu\text{g}/\text{mL}$; yellow) or in the presence of the selective TRPA1 inhibitor A967079 (1 μM ; pink) (N = 5). (B-C) Bar graphs comparing OxPAPC-induced peak and plateau Ca^{2+} responses in the absence and presence of A967079. Data represent means $\pm$ SEM (N=5 donors) and were analyzed by a paired two-tailed t-test. *P < 0.05, **P < 0.01.

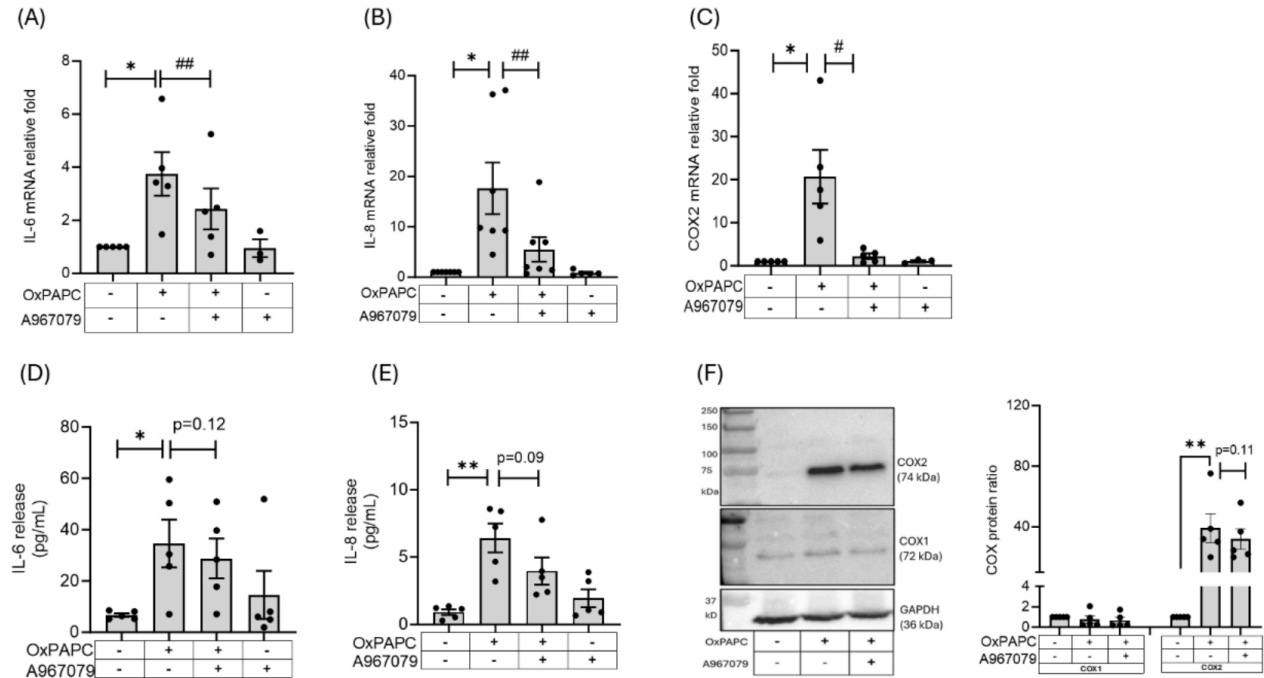


Figure 6

TRPA1 inhibition prevents OxPAPC-induced inflammatory mediator mRNA but not protein abundance in HAFs. (A-C) Bar graphs showing IL-6, IL-8, and COX-2 mRNA abundance following OxPAPC (80 µg/mL) stimulation in the absence and presence of the TRPA1 inhibitor A967079 (1 µM, 60 min pre-incubation) for 24 h. (D-F) Bar graphs depicting IL-6 and IL-8 protein levels in supernatants as well as COX-1 and -2 abundance in cell lysates under the same treatment conditions. GAPDH was used as a loading control for both COX-1 and COX-2. Data were presented as means ± SEM (N=5 donors) and were analyzed using a paired two-tailed t-test comparing OxPAPC versus OxPAPC + A967079 treatment groups and one-way ANOVA followed Sidak's multiple comparison test for cytokines release. *P < 0.05, **P < 0.01.

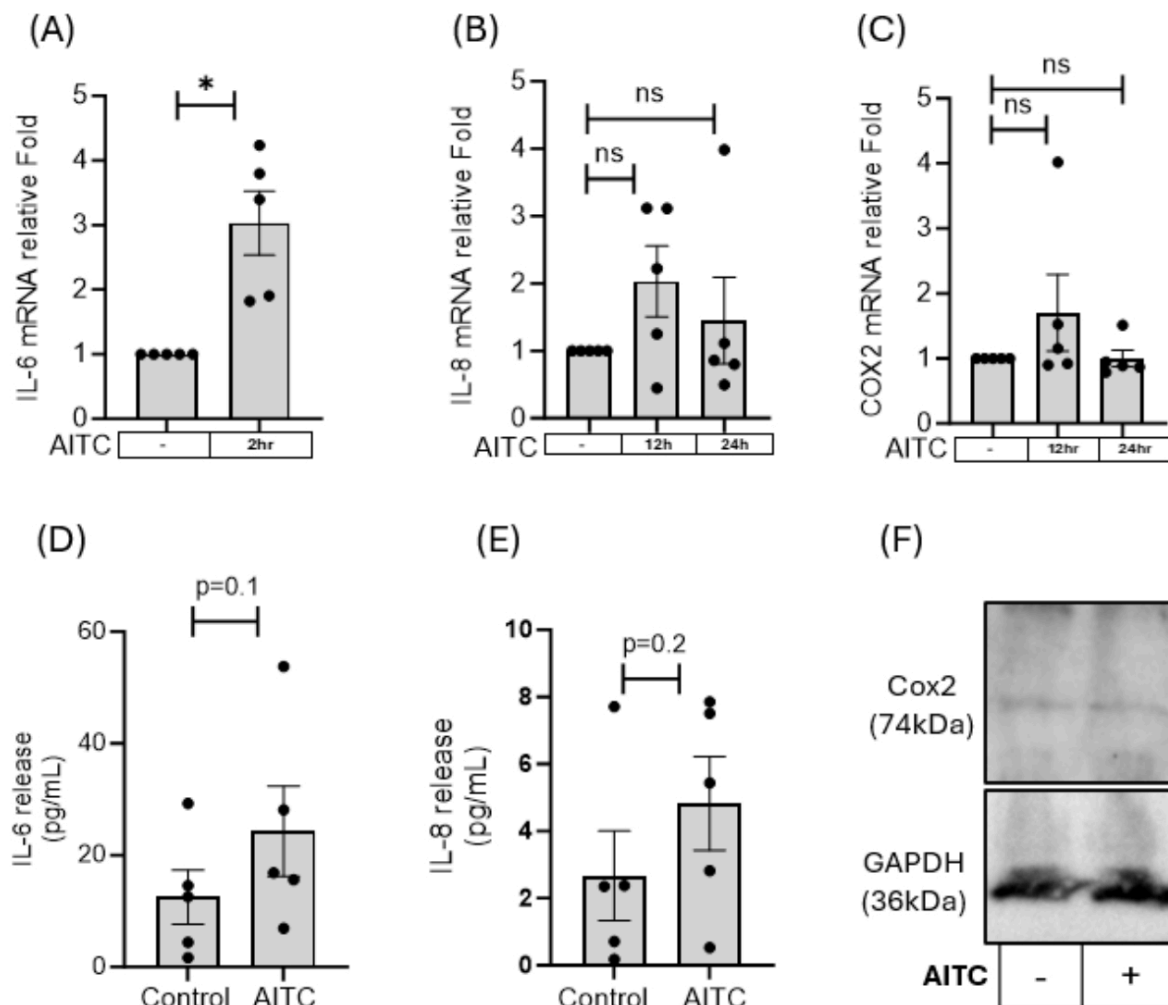


Figure 7

Direct TRPA1 activation by AITC is insufficient to induce inflammatory mediator expression. (A–C)

Analysis of IL-6, IL-8, and COX-2 mRNA abundance following time point exposure of AITC (0.5 M) stimulation. **(D–E)** Bar charts showing IL-6 and IL-8 protein supernatant levels following 24h exposure to AITC. **(F)** Immunoblot of COX-2 protein abundance in HAF lysates after AITC incubation for 24h; GAPDH was used as a loading control. Bar graphs represent means ± SEM (N=5 donors). Statistical significance was determined using a paired two-tailed t-test. *P < 0.05.

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