Pulmonary Pharmacology & Therapeutics 70 (2021) 102059

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

The inflammatory regulation of TRPA1 expression in human A549 lung epithelial cells☆

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ARTICLE INFO

Keywords:
Transient receptor potential ankyrin 1 (TRPA1)
cation channel
Inflammation
Glucocorticoids
Tumor necrosis factor-alpha (TNF-α)
Interleukin-1beta (IL-1β)
Interferon-gamma (IFN-γ)

ABSTRACT

Transient receptor potential ankyrin-1 (TRPA1) is an ion channel mediating pain and cough signals in sensory neurons. We and others have shown that TRPA1 is also expressed in some non-neuronal cells and supports inflammatory responses. To address the pathogenesis and to uncover potential targets for pharmacotherapy in inflammatory lung diseases, we set out to study the expression of *TRPA1* in human A549 lung epithelial cells under inflammatory conditions. *TRPA1* expression was determined by RT-qPCR and Western blotting at a mRNA and protein level, respectively and its function was studied by Fluo 3-AM intracellular Ca^{2+} measurement in A549 lung epithelial cells. *TRPA1* promoter activity was assessed by reporter gene assay.

TRPA1 expression was very low in A549 cells in the absence of inflammatory stimuli. Tumor necrosis factor-α (TNF-α) significantly increased *TRPA1* expression and a synergy was found between TNF-α, interleukin-1β (IL-1β) and interferon-γ (IFN-γ). Reporter gene experiments indicate that the combination of TNF-α and IL-1β increases *TRPA1* promoter activity while the effect of IFN-γ seems to be non-transcriptional. Interestingly, the glucocorticoid dexamethasone downregulated *TRPA1* expression in A549 cells by reducing *TRPA1* mRNA stability in a transcription-dependent manner. Furthermore, pharmacological blockade of TRPA1 reduced the production of the pro-inflammatory cytokine IL-8.

In conclusion, TRPA1 was found to be expressed and functional in human A549 lung epithelial cells under inflammatory conditions. The anti-inflammatory steroid dexamethasone reduced *TRPA1* expression through post-transcriptional mechanisms. The results reveal TRPA1 as a potential mediator and drug target in inflammatory lung conditions.

1. Introduction

Transient Receptor Potential Ankyrin 1 (TRPA1) is an ion channel on the plasma membrane. It permeates cations, most importantly Na^+ and Ca^{2+}, which are believed to mediate its biological effects. Previously, TRPA1 has been studied mainly in sensory neurons, where it is most abundantly expressed in Aδ- and C-fibers. In these cells, TRPA1 acts as a nociceptor and chemosensor, mediating pain, cough, itch and neurogenic inflammation [1]. In neurogenic inflammation, TRPA1 activation leads to Ca^{2+} influx which is followed by exocytosis of proinflammatory and vasodilatory neuropeptides, such as substance P and calcitonin-gene related peptide [2].

TRPA1 is known to be activated by several environmental pungent compounds but also by some endogenous mediators, particularly reactive oxygen (ROS) and nitrogen (RNS) species [3–7]. Examples of the exogenous activator compounds comprise allyl isothiocyanate [8] and allicin [9], pungent compounds of mustard oil and garlic, respectively. In addition to direct gating by agonists, TRPA1 sensitivity is indirectly modulated by inflammatory mediators acting through G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). For example, prostaglandin E_2 (PGE2), bradykinin and the proteases trypsin and tryptase, via their respective GPCRs, sensitize TRPA1 by activating protein kinase A (PKA) and phospholipase C (PLC) signalling pathways

☆ The study was supported by grants from The Academy of Finland; Tampere Tuberculosis Foundation, Finland; Research Foundation of Rheumatic Diseases, Finland; Competitive Research Funding of Tampere University Hospital; and Finnish Pharmacological Society. The funding bodies had no role in the study design, or in data collection, analysis or interpretation, or in writing the manuscript.

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https://doi.org/10.1016/j.pupt.2021.102059
Received 26 March 2021; Received in revised form 6 June 2021; Accepted 17 July 2021
Available online 21 July 2021

List of nonstandard abbreviations

3′UTR	three prime untranslated region
Act D	actinomycin D
AITC	allyl isothiocyanate
ANOVA	analysis of variance
AUF1	AU-rich element RNA-binding protein 1
CM	cytomix (in the present study refers to a combination of cytokines TNF-α, IL-1β and IFN-γ)
COPD	chronic obstructive pulmonary disease
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GRE	glucocorticoid-response element
HIF-1α	hypoxia-inducible factor 1α
HRE	hypoxia responsive element
HuR	human antigen R
IFN-γ	interferon-γ
IL	interleukin (family of cytokines, further classified by number, e.g. IL-1β)
iNOS	inducible nitric oxide synthase
MAP kinase	mitogen-activated protein kinase
NF-κB	nuclear factor kappa B
PGE_2	prostaglandin E_2
PKA	protein kinase A
PLC	phospholipase C
RNS	reactive nitrogen species
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RT-qPCR	quantitative reverse transcription polymerase chain reaction
Th1	T helper lymphocyte, type 1
TNF-α	tumor necrosis factor-α
TRPA1	transient receptor potential ankyrin-1
TSLP	thymic stromal lymphopoietin
TTP	tristetraprolin

[10–13]. In addition, the pro-allergic cytokine thymic stromal lymphopoietin (TSLP) has been reported to sensitize TRPA1 secondarily via activating its RTK and PLC in the downstream [14].

Recently, reports on *TRPA1* expression in non-neuronal cells have emerged. For example, *TRPA1* expression has been reported in synoviocytes [15], chondrocytes [16] and keratinocytes [17–19], where TRPA1 seems to mediate proinflammatory responses [15–19]. In the lung, *TRPA1*-expressing non-neuronal cells include epithelial cells, fibroblasts and smooth muscle cells [20–23]. Interestingly, some reports indicate that *TRPA1* expression is upregulated by inflammatory factors, such as tumor necrosis factor-α (TNF-α) in synoviocytes [15] and keratinocytes [19], interleukin-1β (IL-1β), interleukin-17 (IL-17), resistin and lipopolysaccharide in chondrocytes [16] and interleukin-13 in mast-cells [24]. However, only very limited knowledge is available on the mechanisms of regulation of TRPA1 expression. In human synoviocytes, TNF-α upregulates *TRPA1* via key inflammatory transcription factors, nuclear factor kappa B (NF-κB) and hypoxia-inducible factor 1α (HIF-1α). HIF-1α seems to act via binding to hypoxia responsive elements (HREs) in the *TRPA1* promoter, increasing *TRPA1* mRNA transcription [15]. In murine sensory neurons, p38 MAP kinase activation was reported to upregulate *TRPA1* expression [25], whereas genetic ablation of gp130, an essential signal transducer of IL-6 family cytokines, seems to downregulate *TRPA1* [26].

In the airways, TRPA1 seems to contribute to pathologies, such as asthma. In experimental models, TRPA1 has been shown to mediate the asthma phenotype, including changes in airway hyperreactivity, cough, extravasation and release of proinflammatory factors [27–32]. Intriguingly, particular *TRPA1* single nucleotide polymorphisms have been associated with the development of childhood asthma [33]. In addition, we have shown that TRPA1 supports proinflammatory responses in murine models such as carrageenan-induced acute inflammation [34], gout [35], osteoarthritis [36] and ovalbumin-induced allergic inflammation [37].

Although TRPA1 expression has been reported in some non-neuronal cells of the lung [20–22], the knowledge on the regulatory mechanisms of *TRPA1* expression in general, and especially in alveolar epithelial cells, remains limited. In the present study, we set out to study the regulation of *TRPA1* expression in human lung epithelial cells (by using A549-cell line) under inflammatory conditions.

2. Methods

2.1. Cell culture

Human A549 lung epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Ham's F–12K (Kaighn's modification) medium supplemented with 5 % heat-inactivated fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 250 ng/ml amphotericin B (all from Gibco/Life Technologies, Carlsbad, CA, USA) at 37 °C in 5 % CO_2. Cells were seeded (0.4×10^6 cells/ml) and grown for 48 h before the experiments were started. During the experiments the cells were cultured with the following compounds or their combinations as indicated: TNF-α, IL-1β and IFN-γ (all from R&D Systems Europe Ltd), actinomycin D (Millipore Sigma, St. Louis, MO, USA), dexamethasone (Orion corp., Espoo, Finland) and the TRPA1 antagonists HC-030031 (Millipore Sigma) and A-967079 (Millipore Sigma).

2.2. RNA extraction and RT-qPCR

At indicated time points, RNA was extracted (GenElute Mammalian Total RNA Miniprep kit, Millipore Sigma) and reverse-transcribed to cDNA (TaqMan® Reverse Transcription Reagents, Applied Biosystems, Foster City, CA, USA). The primer and probe sequences and concentrations for *GAPDH* were optimized according to the manufacturer's instructions and were: 5′-AAGGTCGGAGTCAACGGATTT-3' (*GAPDH*, forward, 300 nM), 5′-GCAACAATATCCACTTTACCAGAGTTAA-3' (*GAPDH*, reverse, 300 nM), and 5′-CGCCTGGTCACCAGGGCTGC-3' (*GAPDH*, probe, 150 nM, containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher) (Metabion, Martinsried, Germany). TaqMan Gene Expression assay for *TRPA1* (Hs00175798_m1) was obtained from Life Technologies (Life Technologies Europe BV, Bleiswijk, the Netherlands). When calculating results, mRNA expression levels were first normalized against *GAPDH* mRNA levels. The $^{\Delta\Delta}$Ct method [38] was used in the calculations.

2.3. Western blot

Protein extraction, immunoprecipitation of TRPA1 and Western blot analysis were carried out as previously described [16,19]. In the Western blot analysis, a TRPA1-specific antibody NB110-40763 (Novus

Biologicals, LCC, Littleton, CO, USA) was used as the primary antibody and goat anti-rabbit HRP-conjugate (sc-2004, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as the secondary antibody.

2.4. Immunoassay

Interleukin-8 (IL-8) concentrations in A549 medium samples were measured by enzyme-linked immunosorbent assay (ELISA) and the reagents were purchased from R&D Systems Europe Ltd (Abingdon, United Kingdom).

2.5. Intracellular Ca^{2+} measurements

TRPA1-mediated increase in intracellular Ca^{2+} was determined by Fluo 3-AM method as previously described [39]. In brief, the A549 cells were first loaded for 30 min in room temperature with 4 μM fluo-3-acetoxymethyl ester (Fluo 3-AM, Millipore Sigma) and 0.08 % Pluronic F-127® in Hanks' balanced salt solution (HBSS, Lonza, Verviers, Belgium) containing 1 mg/ml bovine serum albumin, 2.5 mM probenecid and 25 mM HEPES pH 7.2 (all from Millipore Sigma). The intracellular free Ca^{2+} levels were analyzed with excitation/emission wavelengths of 485/535 nm using Victor3 1420 multilabel counter (PerkinElmer, Waltham, MA, USA). The cells were first preincubated for 30 min at 37 °C with the specific TRPA1 blocker HC-030031 (100 μM, Millipore Sigma) or the vehicle. Thereafter, the cells were activated by adding the TRPA1 agonist allyl isothiocyanate (AITC, 50 μM, Millipore Sigma) and the measurements were continued for 30 s.

2.6. Reporter gene assay

A *TRPA1* reporter plasmid, which includes luciferase gene and pro6130, a *TRPA1* promoter construct from −5847 to +283 at the DNA

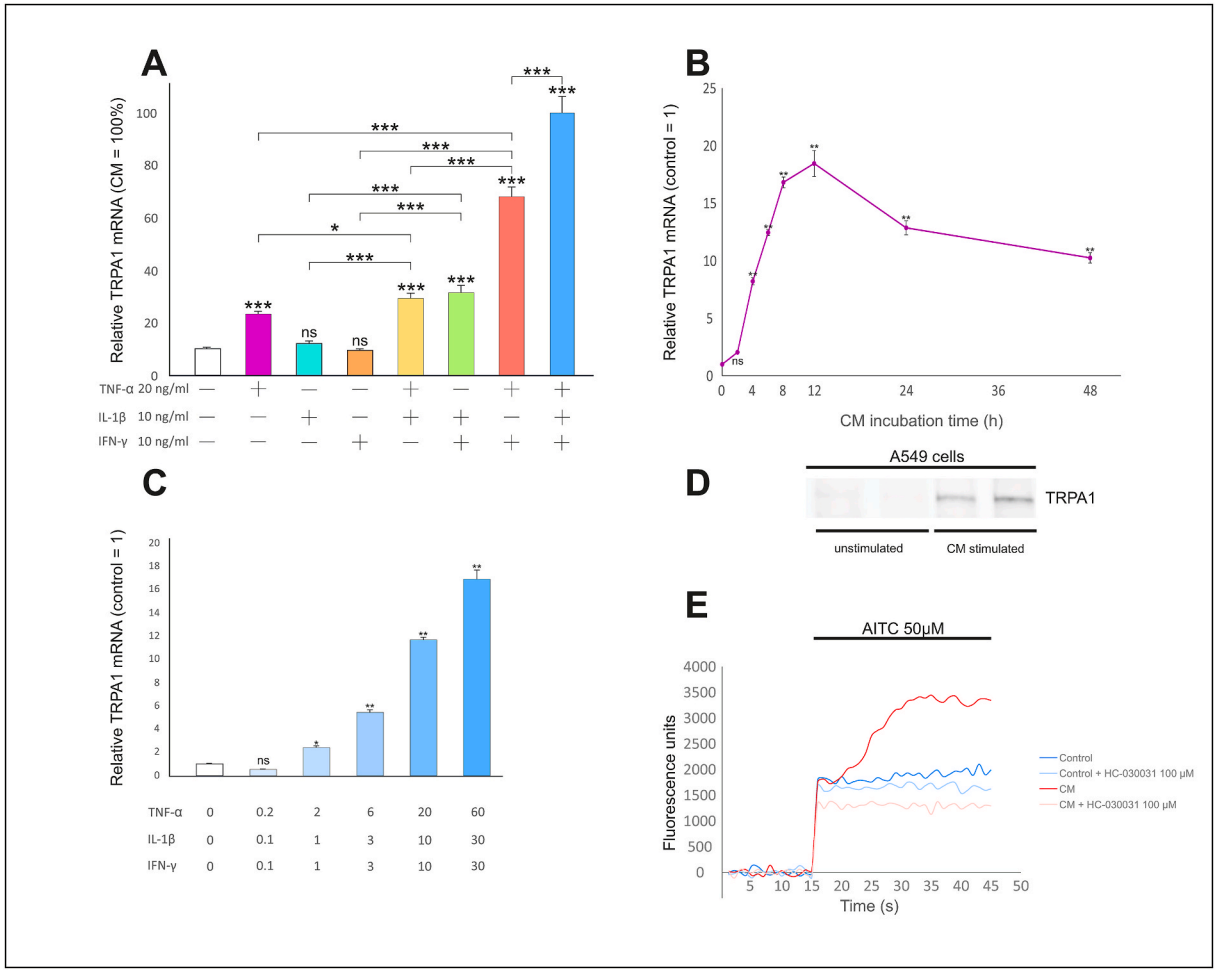

Fig. 1. *TRPA1* expression is enhanced by proinflammatory cytokines in human A549 lung epithelial cells. In **(A)**, the effect of three inflammatory cytokines on *TRPA1* mRNA expression is shown. A549 cells were stimulated with TNF-α (20 ng/ml), IL-1β (10 ng/ml), IFN-γ (10 ng/ml) or their combinations for 24 h. The graph in **(B)** shows that cytomix [CM, i.e. the combination of TNF-α (20 ng/ml), IL-1β (10 ng/ml) and IFN-γ (10 ng/ml)] upregulated *TRPA1* mRNA in a time-dependent manner. **(C)** displays the dose-dependent effect of the cytomix (CM, i.e. the combination of TNF-α, IL-1β and IFN-γ) on *TRPA1* expression during 24-h incubation time. At the end of each experiment in **(A–C)**, total RNA was extracted and *TRPA1* mRNA expression was measured by RT-qPCR. Results were normalized against *GAPDH* mRNA and the results are expressed as mean ± SEM, n = 4. *, ** and *** represent p=<0.05, p=<0.01 and p=<0.001, respectively. Ns = not significant. Asterisks on bars indicate comparison to control. Asterisks on brackets indicate comparison between the bars indicated. Statistical significance was calculated using one-way ANOVA with Bonferroni's **(A)** or Dunnett's **(B, C,** against the control without cytokines**)** post-test.
In **(D)**, TRPA1 protein expression was confirmed. A549 cells were stimulated with cytomix [CM, i.e. the combination of TNF-α (20 ng/ml), IL-1β (10 ng/ml) and IFN-γ (10 ng/ml)] for 24 h. After protein extraction and immunoprecipitation, TRPA1 was detected using Western blotting. The blot is representative of four distinct experiments with similar results.
In **(E)**, the function of the TRPA1 channel is shown. A549 cells were stimulated with CM for 24 h. Thereafter, the cells were loaded with Fluo 3-AM and the TRPA1-mediated Ca^{2+} elevation was measured. The cells were first preincubated with the TRPA1 antagonist HC-030031 (100 μM) or vehicle for 30 min before the TRPA1 agonist AITC (50 μM) was added and the measurements were continued for 30 s.

nucleotides (referred to the distance from transcription start site), and pCMV-βgal plasmid (for normalization of transfection efficiency) were transfected into A549 cells using lipofectamine 3000 (Thermo Fisher Scientific, Yokohama, Japan). After 15 h of transfection, cells were incubated for 24 h with the tested compounds and harvested. Luciferase assays were performed with the luciferase reporter gene assay kit (Sigma-Aldrich Japan K.K., Tokyo, Japan) according to the manufacturer's instructions. The light emission was measured using Spark multimode microplate reader (TECAN, Kawasaki, Japan). Each luciferase activity was expressed after normalization with the expression level of β-galactosidase in the same sample.

2.7. Statistical analysis

Graph-Pad InStat version 3.00 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics version 27.0 (IBM Corp., Armonk, NY, USA) softwares were used in the statistical analysis. The results are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's multiple comparisons test was used in the analysis as indicated.

3. Results

3.1. TRPA1 expression is enhanced by inflammatory cytokines in A549 cells

Human A549 lung epithelial cells expressed *TRPA1* at low levels in the absence of inflammatory stimuli. Applying TNF-α (20 ng/ml) for 24 h significantly upregulated *TRPA1* mRNA whereas interleukin-1β (IL-1β) or interferon-γ (IFN-γ) at 10 ng/ml alone had no significant effect on the expression. These cytokines in combinations further increased the *TRPA1* expression: the combinations of TNF-α and IL-1β, IL-1β and IFN-γ as well as TNF-α and IFN-γ produced a significant upregulation of *TRPA1* mRNA while applying all three cytokines together (cytomix, CM)

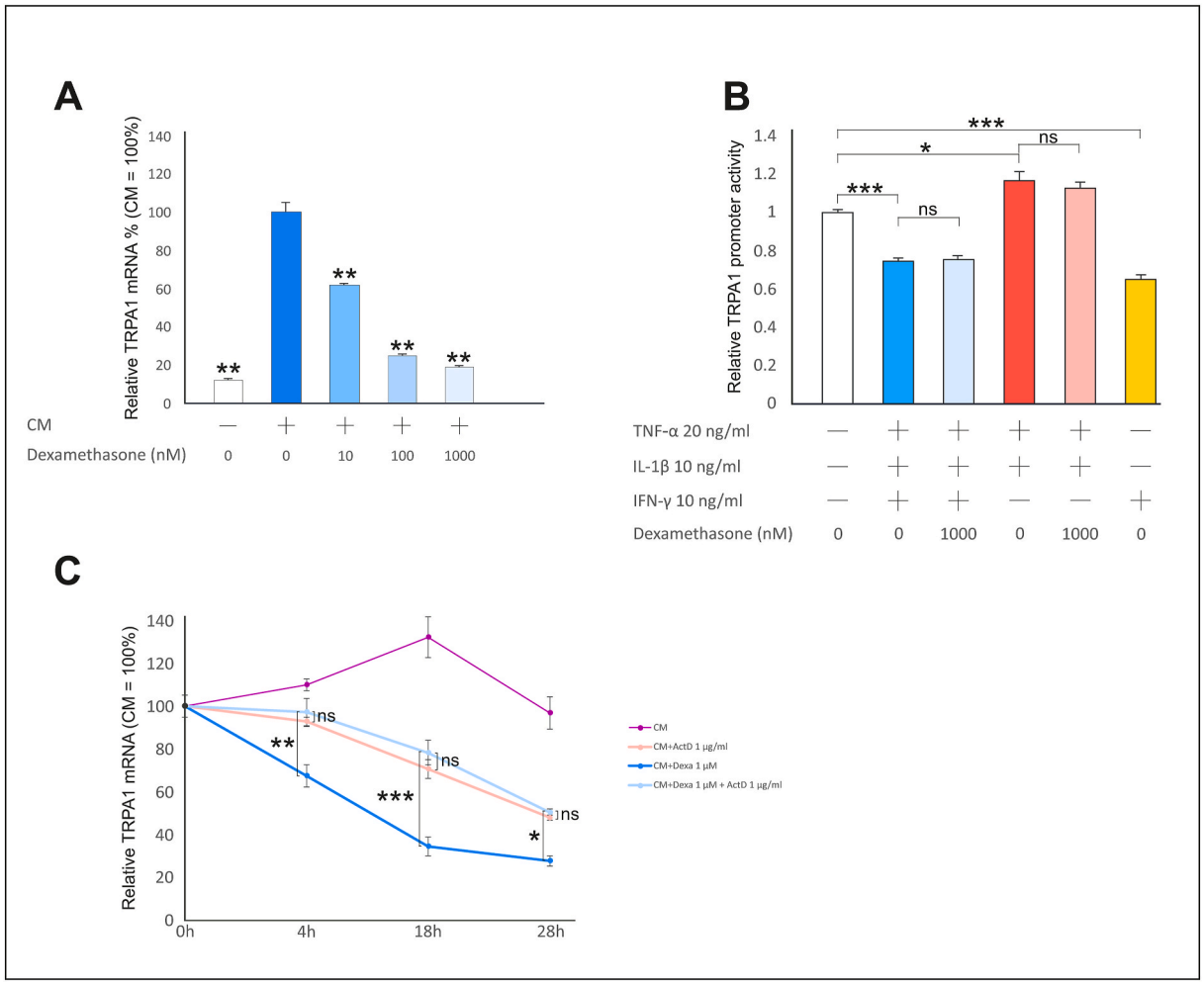

Fig. 2. Dexamethasone inhibits cytokine-induced TRPA1 expression in human A549 lung epithelial cells by increasing *TRPA1* mRNA decay. In **(A)**, the dose-dependent effect of dexamethasone in downregulating *TRPA1* mRNA is shown. A549 cells were stimulated with the combination of cytomix [CM, i.e. combination of TNF-α (20 ng/ml), IL-1β (10 ng/ml) and IFN-γ (10 ng/ml)] in the presence of increasing concentrations of dexamethasone (10–1000 nM) for 24 h. Thereafter, total RNA was extracted and *TRPA1* mRNA expression was measured by RT-qPCR. The results were normalized against *GAPDH* mRNA. In **(B)**, a plasmid with *TRPA1* promoter upstream of luciferase gene was transfected to A549 cells for 15 h. The cells were then cultured in the presence of cytokines and dexamethasone as indicated for 24 h. Thereafter, luciferase activity was measured as an indicator of *TRPA1* promoter activity. **(C)** demonstrates that dexamethasone increases *TRPA1* mRNA decay in a transcription-dependent manner. To upregulate TRPA1, A549 cells were first stimulated with cytomix [CM, i.e. combination of TNF-α (20 ng/ml), IL-1β (10 ng/ml) and IFN-γ (10 ng/ml)] for 6 h. Thereafter (at 0 h in the Figure), a transcription inhibitor actinomycin D (Act D, 1 μg/ml), dexamethasone (1 μM) or their combination was added to the cells. *TRPA1* mRNA expression was measured as in **(A)** at time points indicated. The results are expressed as mean ± SEM, n = 4 **(A,C)** or n = 3 **(B)**. *, ** and *** represent p=<0.05, p=<0.01 and p=<0.001, respectively. Ns = not significant. Asterisks on bars indicate comparison to control. Asterisks on brackets indicate comparison between the bars indicated. Statistical significance was calculated using one-way ANOVA with Dunnett's post-test against CM alone **(A)** or Bonferroni's post-test **(B, C).**

resulted in the strongest increase (Fig. 1 A). CM stimulation enhanced *TRPA1* expression in a time- and dose-dependent manner: *TRPA1* expression increased up to 12 h, and thereafter slightly declined (Fig. 1 B). The lowest CM concentration found to significantly upregulate *TRPA1* mRNA was 2, 1, 1 ng/ml for TNF-α, IL-1β and IFN-γ, respectively and the effect increased up to the highest tested concentration of 60, 30, 30 ng/ml (Fig. 1 C).

TRPA1 protein expression was confirmed by Western blot analysis. When the cells were cultured for 24 h in the presence of TNF-α, IL-1β and IFN-γ, increased TRPA1 protein levels were detected (Fig. 1 D).

To confirm the functionality of the induced-TRPA1 channel, we conducted intracellular Ca^{2+} measurements utilizing the Ca^{2+} indicator Fluo 3-AM. A549 cells were cultured with CM or vehicle for 24 h. Thereafter, the cells were incubated in the presence or absence of the TRPA1 antagonist HC-030031 (100 μM), after which the TRPA1 agonist AITC (50 μM) was applied. We found that CM-treated cells had an enhanced Ca^{2+} increase produced by AITC stimulation, which was effectively inhibited by HC-030031, hence suggesting that the upregulated TRPA1 is functional (Fig. 1 E).

Having found that inflammatory cytokines significantly upregulate *TRPA1* expression, we were further interested in the mechanisms involved in this action. In reporter gene experiments, a plasmid with *TRPA1* promoter (pro6130) upstream of luciferase gene was transfected to A549 cells, which were then cultured with or without cytokines; and luciferase activity was measured to obtain the information on *TRPA1* promoter activity. The combination of TNF-α (20 ng/ml) and IL-1β (10 ng/ml) was found to increase the *TRPA1* promoter activity. In contrast, IFN-γ (10 ng/ml) alone or in combination with TNF-α (20 ng/ml) and IL-1β (10 ng/ml), decreased the *TRPA1* promoter activity. Considering our result that IFN-γ in these cytokine combinations increased *TRPA1* expression (Fig. 1 A), these data suggest that IFN-γ upregulates *TRPA1* expression via non-transcriptional mechanisms (Fig. 2 B).

3.2. The glucocorticoid dexamethasone downregulates TRPA1 expression by increasing TRPA1 mRNA decay

Having found that inflammatory cytokines upregulate TRPA1 expression, we were interested if anti-inflammatory steroids could downregulate *TRPA1*. Indeed, we observed that dexamethasone dose-dependently downregulated *TRPA1* mRNA as assessed by RT-qPCR. A549 cells were cultured with CM in the presence of increasing concentrations of dexamethasone for 24 h. A significant downregulatory effect was detected even at 10 nM concentrations of dexamethasone. This effect increased up to 1 μM drug concentrations (Fig. 2 A).

We were further interested in the mechanism of action of dexamethasone in downregulating *TRPA1* expression. We approached this question by conducting reporter gene assay and mRNA stability studies utilizing actinomycin D (Act D). The reporter gene assay data (Fig. 2 B) indicate that during 24 h incubation, dexamethasone (1 μM) does not affect *TRPA1* promoter activity when added together with CM or the combination of IL-1β (10 ng/ml) and TNF-α (20 ng/ml). These data suggest that dexamethasone downregulates *TRPA1* expression in a promoter independent manner. To further elaborate on this mechanism, we carried out experiments with a transcriptional inhibitor, Act D [40] (Fig. 2 C). To upregulate *TRPA1*, A549 cells were first stimulated with CM for 6 h. Thereafter (0 h of the experiment in Fig. 2 C), Act D (1 μg/ml), dexamethasone (1 μM), or their combination was added to the cells. As expected, in the absence of Act D and dexamethasone CM continued to increase *TRPA1* expression. In contrast, adding Act D disabled the transcription, and *TRPA1* mRNA stably decayed during the 28 h' follow-up. Remarkably, dexamethasone added to the culture rapidly reduced *TRPA1* mRNA levels with a considerably higher mRNA decay rate than Act D, indicating that dexamethasone increases *TRPA1* mRNA degradation rate. Moreover, the effect of dexamethasone was reversed by simultaneous addition of Act D, suggesting that the dexamethasone effect is transcription-mediated. Thus, we suggest that dexamethasone downregulates *TRPA1* expression by increasing its mRNA decay and this effect seems to be transcription-dependent; while independent on the *TRPA1* promoter.

3.3. TRPA1 enhances the production of IL-8 in A549 cells

To preliminarily assess the effects of TRPA1 in A549 cells, we measured IL-8 production in A549 cells using enzyme-linked immunosorbent assay (ELISA). CM strongly increased IL-8 production; while the TRPA1 antagonists HC-030031 (10 μM) and A-967079 (10 μM) added simultaneously with CM reduced IL-8 production in a statistically significant manner, suggesting that TRPA1 is intrinsically activated in these conditions and enhances the production of the chemokine IL-8 (Fig. 3).

4. Discussion

The present study reveals TRPA1 as a factor/effector regulated by inflammation in human lung epithelial cells. We demonstrated that the inflammatory cytokines TNF-α, IL-1β and IFN-γ together produced a strong, synergistic upregulation of *TRPA1* via increasing *TRPA1* promoter activity supplemented with non-transcriptional mechanisms. We also confirmed *TRPA1* expression on protein and functional levels, as assessed by Western blot and Fluo 3-AM Ca^{2+} measurement studies. Further, we showed that dexamethasone effectively downregulates *TRPA1* in these cells and proposed that dexamethasone acts independent of *TRPA1* promoter but increases *TRPA1* mRNA decay in a transcription-dependent manner. Moreover, TRPA1 activation enhances the inflammatory response via increasing IL-8 production.

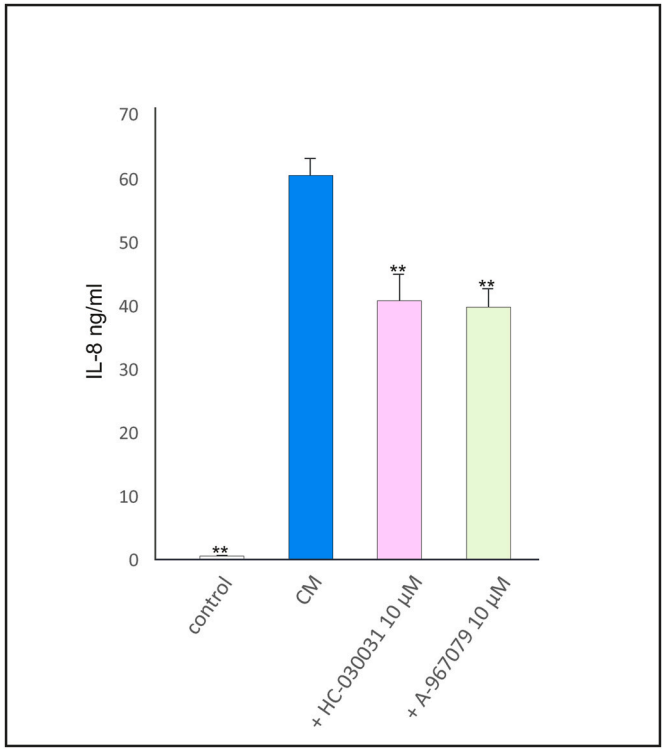

Fig. 3. TRPA1 antagonists HC-030031 and A-967079 inhibit the production of the chemokine IL-8. A549 cells were stimulated with cytomix [CM, i.e. the combination of TNF-α (20 ng/ml), IL-1β (10 ng/ml) and IFN-γ (10 ng/ml)] in the presence and absence of the TRPA1 antagonist HC-030031 (10 μM) or A-967079 (10 μM) for 24 h. Thereafter, IL-8 concentrations in the culture media samples were measured by ELISA. Results are expressed as mean + SEM, n = 4. *, ** and *** represent p=<0.05, p=<0.01 and p=<0.001, respectively. Statistical significance was calculated using one-way ANOVA with Dunnett's post-test against CM.

After being first found in human fibroblasts by Jaquemar et al. [41], TRPA1 has mainly been studied from the perspective of a nociceptor and chemosensor in sensory neurons. *TRPA1* is expressed in the small Aδ- and unmyelinated sensory C-fibers [42–44], where it mediates pain [44,45], itch [14,46,47] and neurogenic inflammation [2]. More recently, reports on *TRPA1* expression also in non-neuronal cells have started to emerge, and *TRPA1* expression has been reported in synoviocytes [15], chondrocytes [16] and keratinocytes [17–19]. In the lung, epithelial cells, fibroblasts and smooth muscle cells have been shown to express *TRPA1* [20–23]. Nevertheless, the physiological functions remain poorly understood.

TRPA1 has been regarded mainly as a constitutively expressed gene. Recently, its character as an inflammation-induced factor has been proposed but understanding on the mechanisms regulating *TRPA1* expression is rather limited. Hatano et al. [15] reported that *TRPA1* promoter with 6130 nucleotides (pro6130) contains at least six and ten putative binding sites for the transcription factors NF-κB and HIF-1α, respectively. Through these factors, TNF-α was shown to upregulate *TRPA1* expression in synoviocytes [15] and in human HaCaT keratinocytes [19]. In addition, we have shown that other inflammatory factors such as IL-1β, IL-17, lipopolysaccharide and resistin upregulate *TRPA1* expression in human chondrocytes [16]. In the current study, we demonstrate that *TRPA1* expression in A549 cells is potently upregulated by combinations of the inflammatory cytokines TNF-α, IL-1β and IFN-γ. We also found that TNF-α alone upregulated *TRPA1* mRNA in A549 cells, which is consistent with our findings in synoviocytes [15] and keratinocytes [19]. On the other hand, in the present study in A549 cells IL-1β alone did not significantly upregulate *TRPA1* expression, whereas in human chondrocytes it was strongly increased by IL-1β [16]. Intriguingly, while IFN-γ alone did not upregulate *TRPA1* expression, we observed a significant synergistic upregulation of the expression when TNF-α and IFN-γ were applied together. In addition, we found that the combination of IL-1β and TNF-α increased *TRPA1* promoter activity, whereas IFN-γ alone had a decreasing effect. As IFN-γ in combination with TNF-α synergistically increased *TRPA1* expression and IFN-γ rather decreased *TRPA1* promoter activity, the effects of IFN-γ are likely mediated by post/non-transcriptional mechanisms. This implicates that *TRPA1* expression is significantly regulated at the post/non-transcriptional levels, which is a novel finding. Indeed, IFN-γ has been shown to regulate gene expression in a post-transcriptional manner, as for example in the case of inducible nitric oxide synthase (iNOS, [48]).

IFN-γ is a cytokine classically associated with Th1-type immunity, which is especially important in cell-mediated immunity e.g. against viral pathogens [49] but also in autoimmunity [50]. Likewise, TNF-α and IL-1β are key proinflammatory cytokines, crucial for the inflammatory response [51]. In the present study, we found that the combination of TNF-α, IL-1β and IFN-γ (CM) potently upregulated *TRPA1* expression in a time-and dose-dependent manner. Such a combination of cytokines in the lung could be encountered by a lung epithelial cell for instance in the context of acute pulmonary infection or persistent inflammation characteristic for asthma and chronic obstructive pulmonary disease (COPD). Therefore, our results indicate that TRPA1 is upregulated by inflammation in human lung epithelial cells and could play a pivotal role in inflammatory pulmonary diseases, such as infections or obstructive lung diseases.

We further demonstrated that TRPA1 activation upregulates IL-8 production in A549 cells, which is supported by the findings by Mukhopadhyay et al. [20]. Our results showed that applying TRPA1 antagonists in combination with CM reduced IL-8 production by A549 cells, suggesting that TRPA1 is intrinsically activated under inflammatory conditions and enhances IL-8 production. IL-8 is a chemokine that primarily promotes neutrophil chemotaxis, playing a critical role in infections [51] but also in obstructive lung diseases. Neutrophil accumulation is a characteristic feature of neutrophilic asthma, which is a major subtype of asthma promoted by cigarette smoking [52]. Another inflammatory pulmonary disorder promoted by smoking, COPD, is also characterized by chronic neutrophilic inflammation with elevated levels of chemotactic factors such as IL-8 [53]. Intriguingly, recent studies have highlighted a role for TRPA1 as a sensor of airway irritants. Indeed, TRPA1 has been shown to mediate cigarette smoke extract-induced inflammation and lung epithelial cell damage *in vitro* [54] and lung inflammation and airway hyperresponsiveness induced by fine particulate matter *in vivo* [55]. Accordingly, in murine models, cigarette smoke has been shown to activate TRPA1 via increasing levels of reactive aldehydes and subsequently to promote neurogenic inflammation [27] and to TRPA1-dependently promote emphysema formation in the lung [56]. Thus, our findings that TRPA1 activation upregulates the production of IL-8 raise the possibility that TRPA1 in the lung epithelium could be a driver of smoking-related inflammatory pulmonary disease such as COPD and neutrophilic asthma. Furthermore, as the epithelial barrier is often the first to encounter with hostile pathogens, TRPA1-mediated IL-8 production could be a significant mechanism in the innate immunity or infection-related exacerbations in obstructive pulmonary diseases. In support to this, TRPA1 has been shown to mediate lipopolysaccharide-induced IL-8 production in human bronchial epithelial cells and acute lung inflammation caused by intraperitoneally administered lipopolysaccharide in mice [57]. However, in a model of inhaled lipopolysaccharide, no proinflammatory role for TRPA1 was found [56]. Further studies are, however, needed to clarify this issue.

Asthma is one of the most common inflammatory lung disorders and causes major disease burden and shortening of life expectancy worldwide [58]. Interestingly, in murine models, TRPA1 has been shown to play an important role in the pathogenesis of asthma. Pharmacological blocking and genetic ablation of TRPA1 function have been reported to alleviate asthmatic responses in different murine models, including allergic [28–32], irritant-induced [27,29] and temperature variation-exacerbated [59] asthma. TRPA1 might also play a role in adaptive immunity in asthma, as TRPA1 is upregulated in $CD4^+$ T-cells of asthmatic mice [31]. In ovalbumin-challenged guinea pigs, inhibition of TRPA1 alleviated cough and airway inflammation by decreasing eosinophilic infiltration [32]. Consistently, TRPA1 blockade alleviated ovalbumin-induced changes in guinea pig lung function and airway narrowing, but in contrast, no difference in eosinophil counts was detected [60]. Taken together, pharmacological inhibition of TRPA1 function or expression provides an interesting novel concept for pharmacotherapy of asthma.

Glucocorticoids are effective and widely used in the treatment of asthma [58]. Intriguingly, here we show that dexamethasone significantly downregulates *TRPA1* expression in A549 cells even at nanomolar concentrations. We have shown this effect also in human chondrocytes at comparable dexamethasone concentrations [61], and in human HaCaT keratinocytes [19]. Based on the present results, we propose that the ability of dexamethasone to downregulate TRPA1 in the pulmonary epithelium and possibly in other cell types could account for at least some of the beneficial effects of glucocorticoids in asthma and lung inflammation.

Glucocorticoids regulate the expression of a number of genes by binding to the glucocorticoid response elements (GREs) in their promoter or by inhibiting the function of key inflammatory transcription factors such as NF-κB [62]. As *TRPA1* promoter contains several binding sites for NF-κB [15], we first hypothesized that dexamethasone could inhibit *TRPA1* expression by repressing its transcription induced by inflammatory factors. However, the reporter assay revealed that dexamethasone had no effect on *TRPA1* promoter activity, underlining the post-transcriptional mechanisms of action, which have previously been observed in the regulation of inflammatory genes such as iNOS by dexamethasone [48]. Indeed, our further studies demonstrated that dexamethasone increased *TRPA1* mRNA decay when added 6 h following the inflammatory stimuli. Furthermore, this effect was reversed by the transcription inhibitor actinomycin D, suggesting that

the action of dexamethasone is dependent on the production of factor(s), which may increase *TRPA1* mRNA degradation. Generally, mRNA stability is regulated by microRNAs and RNA-binding proteins such as tristetraprolin (TTP), AUF1 and HuR [63], but their effects on *TRPA1* mRNA decay remain to be studied. Among them, TTP might be a potential candidate for the factor because it has been shown to be upregulated by dexamethasone in A549 cells [64]. According to the ARE site2 database [65]; http://rna.tbi.univie.ac.at/ARESite2/welcome [Accessed 24 March 2021]), the 3′UTR of *TRPA1* mRNA appears to contain AU-rich elements, which serve as potential consensus binding sites of RNA-binding proteins such as TTP. Downregulation of *TRPA1* expression by dexamethasone, which is due to increased mRNA decay, is a novel finding. This result in combination with the previously discussed findings on the effects of IFN-γ on *TRPA1* expression, further underline the hitherto unknown role of post-transcriptional mechanisms in the regulation of *TRPA1* expression in inflammation. These results also provide the possibility that *TRPA1* belongs to the family of transiently expressed inflammatory genes, which are rapidly called to act upon the inflammatory signal and then effectively downregulated to allow resolving the inflammatory response.

5. Conclusions

In conclusion, *TRPA1* was found to be expressed and functional in human A549 lung epithelial cells under inflammatory conditions; inflammatory cytokines upregulated and the glucocorticoid dexamethasone downregulated *TRPA1* expression. The present findings reveal *TRPA1* expression to be significantly regulated not only at the transcriptional level, but also by post-transcriptional mechanisms, as demonstrated by the novel effects of IFN-γ and dexamethasone on its expression. In addition, TRPA1 was shown to promote the production of the chemokine IL-8 suggesting a proinflammatory role for TRPA1 in lung epithelial cells. The results disclose TRPA1 as a potential mediator and drug target under inflammatory lung conditions.

Author statement

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Acknowledgements

We wish to thank Ms Meiju Kukkonen and Ms Salla Hietakangas for their excellent technical assistance.

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