TGFβ1 induces resistance of human lung myofibroblasts to cell death via down-regulation of TRPA1 channels

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Background and Purpose: TGFβ1-mediated myofibroblast activation contributes to pathological fibrosis in many diseases including idiopathic pulmonary fibrosis (IPF), where myofibroblast resistance to oxidant-mediated apoptosis is also evident. We therefore investigated the involvement of redox-sensitive TRPA1 ion channels on human lung myofibroblasts (HLMFs) cell death and TGFβ1-mediated pro-fibrotic responses.

Experimental approach: The effects of TGFβ1 stimulation on TRPA1 expression and cell viability was studied in HLMFs derived from IPF patients and non-fibrotic patients. We also examined a model of TGFβ1-dependent fibrogenesis in human lung. We used qRT-PCR, immunofluorescent assays, overexpression with lentiviral vectors and electrophysiological methods.

Key Results: TRPA1 mRNA, protein and ion currents were expressed in HLMFs derived from both non-fibrotic patient controls and IPF patients, and expression was reduced by TGFβ1. TRPA1 mRNA was also down-regulated by TGFβ1 in a model of lung fibrogenesis in human lung. TRPA1 over-expression or activation induced HLMF apoptosis, and activation of TRPA1 channel activation by H2O2 induced necrosis. TRPA1 inhibition following TGFβ1 down-regulation or pharmacological inhibition, protected HLMFs from both apoptosis and necrosis. Lentiviral vector mediated TRPA1 expression was also found to induce sensitivity to H2O2 induced cell death in a TRPA1-negative HEK293T cell line.

Conclusion and Implications: TGFβ1 induces resistance of HLMFs to TRPA1 agonist- and H2O2-mediated cell death via down-regulation of TRPA1 channels. Our data suggest that therapeutic strategies which prevent TGFβ1-dependent down-regulation of TRPA1 may reduce myofibroblast survival in IPF and therefore improve clinical outcomes.

Abbreviations: AITC, allyl-isothiocyanate; HLMF, human lung myofibroblast; IPF, idiopathic pulmonary fibrosis; NFC, non-fibrotic patient control; TRPA1 OE, HLMFs treated with TRPA1 overexpression lentiviral construct.

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1 | INTRODUCTION

Diseases characterised by pathological fibrosis are a major cause of morbidity and premature mortality (Wynn, 2004). Idiopathic pulmonary fibrosis (IPF) is a common interstitial lung disease and affects over 5 million people worldwide (Meltzer & Noble, 2008). The median survival rate for IPF patients is less than 3 years (American Thoracic Society, 2000). The currently approved treatments for IPF, pirfenidone and nintedanib, are of limited efficacy and have significant and unpleasant side effects (Noble et al., 2011; Richeldi et al., 2014). The present paradigm outlines the pathogenesis of IPF as a dysregulated wound healing response to alveolar epithelial injury with the critical involvement of TGFβ1 (Antoniou et al., 2013; Border & Noble, 1994; Margaritopoulos et al., 2012) and myofibroblasts (Scotton & Chambers, 2007).

The termination of adaptive wound healing is associated with the apoptosis of recruited myofibroblasts (Tettamanti et al., 2004). IPF and other fibrotic disorders are characterised by the persistence of activated myofibroblasts and, in IPF, foci of myofibroblasts are a pathological hallmark of the disease (Scotton & Chambers, 2007). Myofibroblasts may transdifferentiate from resident fibroblasts, result from expansion of resident cells, or result from recruitment of circulating progenitors (Blasin et al., 2020; Gharaee-Kermani et al., 2009; Gomperts & Strieter, 2007). There is evidence that this persistence represents a failure of appropriate myofibroblast apoptosis (Hecker et al., 2014; Horowitz & Thannickal, 2018). Therapies that target this may have potential for preventing disease progression and perhaps even reversing established fibrosis (Hecker et al., 2014; Horowitz & Thannickal, 2018).

In fibrosis, including IPF, there is evidence of oxidative stress and persistent increased production of H2O2 (Kuwano et al., 1996; Kuwano et al., 2002; Kuwano et al., 2003; Lenz et al., 1996; Maier et al., 1991). An important source of this H2O2 may be myofibroblasts under the influence of TGFβ1 (Amara et al., 2010; Hecker et al., 2009). These myofibroblasts are also resistant to H2O2-induced death (Thannickal & Horowitz, 2006; Waghray et al., 2005). The mechanism for myofibroblast resistance to oxidant-induced cell death in IPF has not been elucidated to-date.

Ca2+ signalling is necessary for diverse cellular activities including myofibroblast activation (Roach et al., 2016) and cell death (Orrenius et al., 2003). Redox-sensitive Ca2+-permeable transient receptor potential (TRP) channels ankyrin 1 (TRPA1) and melastatin 2 (TRPM2) have been implicated in the induction of cell death (Chen et al., 2013; Deveci et al., 2019; Kang et al., 2018; Lowin et al., 2018; Yin et al., 2018; Zhang et al., 2003; Zhang et al., 2006), including oxidant-mediated death (Kang et al., 2018). TGFβ1 induces fibroblast resistance to cell death via many mechanisms (Ajayi et al., 2013; Bai et al., 2019; Horowitz et al., 2004; Horowitz et al., 2007), but whether this involves the redox-sensitive TRP ion channels has not been investigated.

TRPA1 channels have been recognized as a drug target with channel antagonists in Phase I and II clinical trials for cough and some specific pain disorders (Carr, 2009; Gilron & Dickenson, 2014). These channels provide a potential target for a range of pain conditions, chronic cough, airway inflammation, cancer, and fibrosis (Belvisi et al., 2011; Gilron & Dickenson, 2014; Hox et al., 2013; Kremeyer et al., 2010; Kurahara et al., 2018; Okada et al., 2014; Takahashi et al., 2018). Work with synovial fibroblasts suggests that TRPA1 channels may be involved in fibroblast cell death (Lowin et al., 2018), but in cancer cell lines there is evidence that these channels are co-opted to contribute to resistance to H2O2-induced cell death (Takahashi et al., 2018). Animal models of corneal (Okada et al., 2014), cardiac (Oguri et al., 2014) and intestinal fibrosis (Kurahara et al., 2018) have implicated TRPA1 channels, although with contradictory results. A bleomycin-induced model of lung fibrosis in guinea pigs suggested increased expression of lung tissue TRPA1 mRNA at 14 days post injury (Guo et al., 2019). Whether TRPA1 channels play any part in driving the fibrosis observed was not explored, although its involvement in cough sensitivity has been suggested.

The contribution of TRPA1 channels to myofibroblast sensitivity to H2O2-induced cell death in IPF and to human lung fibrosis is not known. Here, we have sought to investigate such involvement using human lung myofibroblasts (HLMFs) derived from non-fibrotic patient...
control (NFC) and IPF patients and a model of TGFβ1-dependent fibrogenesis in human lung tissue (Roach et al., 2018).

2 METHODS

2.1 Experimental fibrosis model

Healthy pieces of human lung parenchyma were acquired from lung removed during routine surgery for lung carcinoma at Glenfield Hospital Leicester, with written informed consent and ethical approval from the National Research Ethics Service (references 10/H0402/12and 17/EM/0231). Tissue was cultured for 7 days with or without TGFβ1 (10 ng ml\(^{-1}\)) as described previously (Roach et al., 2013) with either the TRPA1 channel agonist JT-010 (50 nM), the TRPA1 channel antagonist A967079 (670 nM), or vehicle control (0.1% DMSO). Drugs and TGFβ1 were added simultaneously. The mRNA expression of 84 fibrosis-associated genes was assessed from this tissue using a Qiagen RT2 profiling array as described previously (Roach et al., 2013).

2.2 HLMF cell culture and characterisation

Non-fibrotic patient control (NFC) myofibroblasts were derived from healthy pieces of lung tissue obtained from patients undergoing lung resection for carcinoma at Glenfield Hospital (see above). IPF-derived and NFC myofibroblasts were a gift from Prof Carol Feghali-Bostwick (Medical University of South Carolina). IPF-derived HLMFs were shown to have usual interstitial pneumonia (UIP) on histological examination. HLMF cultures were grown in T75 flasks in Dulbecco’s modiﬁed Eagle’s medium containing Glutamax™ and 4.5 g L\(^{-1}\) glucose (Invitrogen, Paisely, UK) supplemented with 10% foetal bovine serum (Gibco, Paisely, UK), 1% antibiotic-antimycotic (containing streptomycin, amphotericin B and penicillin) and 100-μM non-essential amino acids (Invitrogen, Paisely, UK). All cell cultures were maintained at 37°C with 5% CO2 and 95% air.

All HLMF cultures were characterised in detail for myofibroblast features as described previously (Roach et al., 2013). Briefly, immunofluorescent staining was used to ensure HLMFs expressed fibroblast surface protein, α-smooth muscle actin and CD90 at passage 2 or 3. All preparations contained >99% HLMFs. All experiments are carried out on cells between passage 2 and 3.

2.3 RNA extraction and TRP channel qRT-PCR

For cell culture experiments, RNA extraction using Qiagen RNeasy mini prep kits was performed as described previously (Roach et al., 2013). cDNA was first synthesised based on a standard quantity of mRNA using the SuperScript™ VILO cDNA synthesis kit. Taqman® gene expression assays were then used according to the manufacturer’s instructions with primer/probes for TRPA1-FAM (Hs00929065_M1), other TRP channel family members and β2-microglobulin-VIC with the QuantStudio 5 Real-Time PCR system. Relative expression was calculated using the 2\(^{-\Delta\Delta C_T}\) method, where ΔCT is the difference between the CT value for the gene of interest and the housekeeping gene β2 microglobulin (B2M).

2.4 Patch clamp electrophysiology

Single cell recordings from individual HLMFs were performed using the whole cell variant of the patch clamp technique as described previously (Roach et al., 2013). Recordings were performed in a zero Ca\(^{2+}\) bath solution in the presence of the Ca\(^{2+}\) chelator EGTA (5 mM) to prevent rapid Ca\(^{2+}\)-dependent inactivation of TRPA1 channel currents (Nilius et al., 2012). The internal patch pipette solution included Cs\(^{+}\) and tetraethylammonium (TEA) to eliminate K\(^{+}\) currents. The composition of the internal patch pipette and external bath solutions were identical to those as described previously by others (Kurahara et al., 2018). The extracellular solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 5 mM EGTA, 10 mM HEPES, and 10 mM glucose. Patch pipettes were filled with 130 mM Cs-aspartate, 10 mM TEA-Cl, 5 mM BAPTA, 1.374 mM Ca-gluconate, 1 mM MgCl\(_2\), 2 mM MgSO\(_4\), 2 mM Na\(_2\)ATP, and 10 M HEPES. The potent and selective TRPA1 agonist JT-010 or the less selective agonist allyl isothiocyanate (AITC) were used to activate TRPA1 channels, and the selective TRPA1 channel antagonist A967079 was used to further confirm the specificity of any agonist-induced currents. A voltage step command protocol of 10 mV steps from −100 to 100 mV and a holding potential of −20 mV was used to elicít ion currents.

2.5 Immunofluorescent TRPA1 staining

The antibody-based procedures used in this study comply with the recommendations made by the British Journal of Pharmacology. HLMFs or negative control HEK293T cells were stained with a primary conjugated anti-TRPA1 antibody from Santa Cruz (monoclonal mouse anti-ANKTM1 [C-5]: sc-37,649, RRID_AB_11149377) as described previously (Virk et al., 2019).

2.6 Assay of expression of TRPA1 channels by flow cytometry

An antibody directed at the intracellular C-terminus of TRPA1 channels (Santa-Cruz, monoclonal mouse anti-ANKTM1 [C-5]: sc-37,649, RRID_AB_11149377) conjugated to AlexaFluor™ 647 was validated by overexpression and knockdown (Virk et al., 2019). HLMFs were stained with Zombie Aqua fixable viability dye, then fixed and permeabilised using the Intracellular Fixation and Permeabilization Buffer set from eBioscience according to protocol B from the manufacturer. HLMFs were then stained with the primary antibody for 20 min at room temperature and then analysed using the Attune Nxt Flow Cytometer.
2.7 | Lentiviral overexpression of TRPA1 channels

Oligonucleotide primers were designed in order to generate a PCR product with the coding sequence of TRPA1 protein, bound by restriction sites appropriate for directional cloning into the multiple cloning site of pCDH-CMV-MCS-EF1-copGFP. The TOPO™ TA Cloning™ Kit method was used to sub-clone the PCR product into the pCR™4-TOPO™ vector. Traditional restriction enzyme-based cloning was then used to generate the TRPA1 overexpression vector.

The hTRPA1 insert was fully sequenced in all vectors and the functionality of the viral particles was confirmed by transducing HeLa cells (ATCC Cat# CRM-CCL-2, RRID:CVCL_0030) and HLMFs and analysing by flow cytometry. Co-transfection of these plasmids with 2nd generation lentiviral packaging vectors in HEK293(T) cells (ATCC Cat# CRL-11268, RRID:CVCL_1926) was used to produce viral particles. Viral supernatant was concentrated using PEGit™ viral precipitation solution. Transduction of 5x10⁴ HeLa cells was used to determine viral titre in HeLa TU/ml by measuring %GFP positive by flow cytometry. Co-transfection of these plasmids with concentrated viral supernatant, with equal viral titre for comparison, was then used to generate the TRPA1 overexpression vector.

2.8 | Cell viability/apoptosis assay by Annexin-V-APC and Sytox Orange staining by flow cytometry or plate reader

HLMFs were plated at 1 x 10⁵ per well of a six-well plate on day 0. They were serum starved on day 1 and then stimulated with TGFβ1 (10 ng ml⁻¹) or control on day 2. Annexin-V-APC and Sytox Orange staining from Invitrogen was performed according to the manufacturer’s instructions on day 4. The cells were then analysed by flow cytometry using the Attune NxT Flow Cytometer as illustrated (see Figure S3). Where data was normalised, this was with reference to wells treated with 0.2% saponin to induce complete cell permeabilization to the dye as confirmed by fluorescent microscopy. This was to adjust for variability between HLMFs derived from different patients in terms of cell death at baseline.

Alternatively, TRPA1-transduced or control HLMFs were stained with 0.5% Sytox Orange solution in PBS in the 96 well black plates. Intensity of Sytox Orange staining was determined using an Envision Multimode plate reader (PerkinElmer, Waltham, MA, USA). Readings were performed in triplicate per condition. Where data is normalised this was with reference to wells treated with 0.2% saponin to induce complete cell permeabilization to the dye as confirmed by fluorescent microscopy. This was to adjust for variability in cell numbers.

2.9 | Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology. Statistical significance for the purpose of this study was defined as P < .05. When comparing the effects of TGFβ1 and the effect of IPF, two-way ANOVAs, or the non-parametric equivalents were used. In specific experiments, the effects of IPF or TGFβ1 were studied in isolation; in these cases, Student’s t tests or non-parametric equivalents were used. Where indicated paired tests were used, if HLMFs derived from the same patient were studied with or without TGFβ1 stimulation. The specific statistical tests used are explained in the figure legends. Statistical analyses were carried out using GraphPad Prism® software version 7.04 (GraphPad Prism, RRID: SCR_002798). Where any normalisation or transformation of data was applied this is explained in the figure legends and specific methods section. The sample sizes used for specific experiments are shown in the Figures, and statistical tests were only applied when at least five independent observations were available.

2.10 | Materials

JT010, AITC, DMSO, and H₂O₂, were purchased from Sigma Aldrich (Gillingham, Dorset, UK). Sytox Orange, annexin binding buffer and Annexin-APC were purchased from Invitrogen. Zombie Aqua was purchased from Invitrogen Molecular Probes (Paisley, UK). Anti TRPA1-AF647 antibody was purchased from Santa Cruz (Heidelberg, Germany, catalogue no. 376495C-5, RRID AB_11149377). Plasmids were prepared using the EndoFree Plasmid Maxi kit from Qiagen (Crawley, UK). Taqman gene expression assays (Applied Biosystems, Warrington, UK) were used for quantification of TRP channel mRNA by qRT-PCR. TGFβ1 was purchased from R&D Systems (Abingdon, UK).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Human lung myofibroblasts express TRPA1 mRNA, protein, and channels, and are all down-regulated by TGFβ1

Initially, we screened HLMFs derived from non-fibrotic patient controls (NFC) and IPF patients for the expression of mRNA for the Ca²⁺ permeable TRP ion channel family, using qRT-PCR. TRPA1 was the most highly expressed family member (Figure 1a). The other redox-sensitive TRP ion channels (TRPM2, TRPV1, and TRPC5) showed little or no expression. There was heterogeneity in TRPA1 mRNA expression between HLMFs derived from different patients, and no significant difference between IPF and NFC cell samples (Figure 1b). Expression of TRPA1 mRNA was dose-dependently down-regulated by TGFβ1 treatment in vitro (0.1 to 10 ng ml⁻¹ for 24 h; Figure 1c).
HLMF TRPA1 expression and its down-regulation by TGFβ1. (a) RT-qPCR for members of the TRP ion channel family in HLMFs derived from non-fibrotic patient controls (NFC, n = 5) and patients with idiopathic pulmonary fibrosis (IPF, n = 5). TRPA1 mRNA was the most highly expressed mRNA. (b) There was no significant difference in the relative expression of TRPA1 mRNA between IPF (n = 6) and NFC patient HLMFs (n = 9). (c) mRNA expression for TRPA1 (mean, SEM of n = 5) was dose-dependently down-regulated by 24-h incubation with TGFβ1 (0.1-10 ng ml⁻¹). * P < .05, significant effect of TGFβ1; one-way ANOVA (Friedman's test) with Dunn's multiple comparison test. (d) Representative flow cytometry staining for TRPA1 in single live HLMFs derived from an NFC patient using an assay validated previously using TRPA1 artificially expressed in a control cell line. TRPA1 was down-regulated by TGFβ1 (10 ng ml⁻¹). (e) Display expression of TRPA1 in HLMFs from n = 6 NFC and n = 6 IPF patients demonstrating consistent down-regulation by TGFβ1 (significant main effect of TGFβ1 by two-way ANOVA), but no significant difference between IPF and NFC. (f) Representative immunofluorescent staining of NFC-derived HLMFs treated with TGFβ1 and immunofluorescent staining of a negative control cell line (HEK293T cells) for TRPA1 (red) and nuclei (DAPI, blue). (g and h) The current–voltage relationships derived from whole cell patch clamp single cell recordings of HLMFs derived from IPF and NFC patients; recorded at baseline, following addition of AITC (g) or JT-010 (h), and then following the addition of A967079 (mean current density ± SEM). Observed currents had the characteristic TRPA1 current–voltage relationship and were abolished by the selective antagonist A967079. In (g), 12 HLMFs from n = 8 IPF patients and eight HLMFs from n = 4 NFC patients were recorded (pooled NFC and IPF data shown). Three groups comparisons of current amplitude at +80 mV between baseline, AITC and AITC + A967079; Kruskal–Wallis test with post hoc Dunn's multiple comparison test. In (h), 8 HLMFs from n = 3 NFC patients were recorded. Three groups comparisons of current amplitude at +80 mV showed significant differences between baseline and JT-010, and between JT-010 and JT-010 + A967079; Kruskal–Wallis test with post hoc Dunn's multiple comparison test. (i) The current–voltage relationships derived as above from HLMFs treated with TGFβ1 (10 ng ml⁻¹) for 24 h (n = 16 cells from three NFC patients), or unstimulated controls (n = 15 cells, from the same 3 NFC), recorded at baseline and following addition of JT-010 as above (baseline subtracted currents displayed). (j) The current density of JT-010 stimulated currents (as in i) at +80 mV was decreased by TGFβ1 pretreatment. * P < .05, significantly different as indicated; Mann–Whitney test. NFC: non-fibrotic patient controls, IPF: Idiopathic pulmonary fibrosis, RQ: Relative quantity, HLMF: Human lung myofibroblasts, HEK: Human embryonic kidney 293 T cell line and SSC-A: Side scatter area.
A flow cytometry assay and immunofluorescent staining using a validated antibody (Virk et al., 2019) confirmed the expression of TRPA1 protein in HLMFs (Figure 1d–f). Consistent with the mRNA expression, TRPA1 protein expression was similar between NFC and IPF HLMFs and down-regulated by 48-h treatment with 10 ng ml⁻¹ TGFβ1, relative to untreated controls (Figure 1d–f). The expression of functional ion currents with the characteristic current–voltage relationship of TRPA1 channels was observed in greater than 95% of both NFC and IPF-derived HLMFs when activated with allyl-isothiocynate (150 μM; AITC) or the highly potent and selective TRPA1 channel agonist, JT-010 (50 nM, Takaya et al., 2015). These currents were sensitive to the specific TRPA1 channel antagonist A967079 (670 nM; McGaraughty et al., 2010) (Figure 1g,h, also see Figure S1). There was no significant difference in the size of the currents in NFC compared to IPF-derived HLMFs (Figure S2). Currents induced by JT-010 (50 nM) were significantly smaller in HLMFs pretreated with TGFβ1 (10 ng ml⁻¹; Figure 1i,j).

### 3.2 TRPA1 expression is down-regulated in a TGFβ1-dependent model of fibrogenesis in human lung tissue

TRPA1 mRNA was expressed in human lung parenchymal tissue, ex vivo. Culturing human lung parenchyma for 7 days with TGFβ1 (10 ng ml⁻¹) drives early fibrotic changes in the tissue that are sensitive to drug intervention (Roach et al., 2018). Using this model, we found TRPA1 mRNA to be down-regulated in human lung tissue after 7 days exposure to TGFβ1 (see Figure S3).

This ex vivo model was also used to study the effect of the TRPA1 channel antagonist A967079 and the TRPA1 channel agonist JT-010 on the TGFβ1-dependent pro-fibrotic changes in mRNA expression. As previously shown (Roach et al., 2018), exposure to TGFβ1 (10 ng ml⁻¹) for 7 days significantly up-regulated the mRNA expression for a range of pro-fibrotic molecules associated with IPF pathophysiology (Figure 2, left panel). A967079 at a concentration that completely suppresses TRPA1 functional ion channel currents (670 nM) did not significantly modify the mRNA expression of any of the pro-fibrotic genes modulated by TGFβ1 (Figure 2, right panel). JT-010 (50 nM) induced activation of TRPA1 ion currents but also did not modify fibrosis relevant gene expression in the presence of TGFβ1 (Figure 2, middle panel) (higher doses were not used because of the reduced cell viability described below). Incubation with JT-010 (50 nM) alone did not alter the expression of any fibrosis-associated genes (Figure S4). In cultured HLMFs, A967079 did not affect TGFβ1-induced αSMA mRNA or protein expression, wound healing or their ability to contract collagen gels (see Figures S5–S7).

### 3.3 Activation of TRPA1 channels in HLMFs causes cell death that is reduced by TGFβ1

HLMFs stimulated with the TRPA1 channel agonists AITC and JT010 displayed dose-dependent loss of cell integrity and viability, and the induction of apoptosis (Figure 3, measured by light scattering assays, staining with viability dyes and apoptosis markers, see Figure S8). HLMFs stimulated with the TRPA1 channel agonists AITC or JT010 for 1 h, exhibited predominantly late apoptotic cell death detected using staining for AnnexinV-APC and Sytox Orange by flow cytometry (Figure 4a,b). This response to TRPA1 channel agonists was markedly reduced by prior treatment with TGFβ1 (10 ng ml⁻¹ for 48 h), which down-regulates TRPA1 expression, as described above (Figure 4a–c). The specificity of the cell death response to TRPA1 channel activation was confirmed using the selective antagonist A967079 (Figure 4d). When using the highly selective TRPA1 agonist JT010, HLMF cell death was significantly inhibited by A967079, and this inhibition was not significant in cells pretreated with TGFβ1. The proportion of viable cells following exposure to the TRPA1 channel agonist JT-010 was negatively correlated significantly with the baseline expression of TRPA1 protein expression assessed by flow cytometry (Figure 4e), suggesting that baseline differences in TRPA1 expression mediate differential sensitivity to agonist-induced apoptosis.

### 3.4 H₂O₂-dependent HLMF necrosis is inhibited by TGFβ1 pretreatment through down-regulating TRPA1 channels

Given the evidence that many oxidant species may be important endogenous TRPA1 channel agonists, and that H₂O₂ activity is increased in fibrotic tissues, in part due to increased TGFβ1 activity, we assessed if H₂O₂ caused HLMF death and if this involved TRPA1 channel function. H₂O₂ (100 μM) activated ion currents in HLMFs with a current–voltage relationship characteristic for TRPA1 channels, and these currents were inhibited by the selective TRPA1 channel antagonist A967079 (670 nM, Figure 5a). We assayed HLMF death, using H₂O₂ at different concentrations, in the absence of serum or other nutrients (in PBS), in order to specifically look at the direct contribution of H₂O₂ rather than that of oxidised serum proteins. H₂O₂ induced dose-dependent necrotic cell death (Figure 5b,c) and TGFβ1 pretreatment (which down-regulated TRPA1 channels) or the selective TRPA1 channel antagonist A967079 induced relative resistance to H₂O₂-mediated cell death (Figure 5c).

### 3.5 Overexpression of TRPA1 channels in HLMFs causes cell death, and expression of these channels in a HEK293T cell line enhances sensitivity to cell death induced by TRPA1 channel agonist or H₂O₂

The specificity of these observations linking TRPA1 channel activity to cell death were confirmed using lentiviral-mediated overexpression of TRPA1 channels in primary HLMFs (Figure 6a). We have previously demonstrated the validity of these lentiviral overexpression constructs for inducing functional TRPA1 channels (Virk et al., 2019). When a multiplicity of infection of 0.5 transducing units ml⁻¹ was used to...
FIGURE 2
Antagonism or agonism of TRPA1 channels in a model of fibrosis in human lung tissue does not affect TGFβ1-dependent expression of fibrosis-associated genes. (left panel) Out of 84 genes, 29 were statistically significantly up-regulated and 1 down-regulated by TGFβ1 (10 ng ml⁻¹) after 7 days (in the presence of drug vehicle control 0.05% DMSO), following a 5% false discovery analysis with Benjamin, Krieger and Yekutieli, two-stage step-up method adjusted \( P < .05 \) and absolute value of log₂(FC) ≥ 0.5 (results are mean of \( n = 10 \) individual tissue donors). The log₂ fold regulation of the 84 genes in individual donors is depicted in this heatmap, with mean log₂(FC), and statistical significance indicated. Significance was calculated using a Student's \( t \) test on the \( \Delta \Delta CT \) for each gene. (middle and right panels) Both the TRPA1 agonist JT010 (50 nM in 0.05% DMSO) and the TRPA1 antagonist A967079 (670 nM in 0.05% DMSO) failed to disrupt TGFβ1-induced changes in fibrosis-associated gene expression within human lung tissue over 7 days when compared to TGFβ1 in 0.05% DMSO (results are mean of \( n = 5 \) individual donors).
transduce HLMFs for TRPA1 overexpression (TRPA1 OE), this overexpression enhanced sensitivity to agonist-induced HLMF cell death and a trend towards constitutive cell death, compared with the corresponding controls (Figure 6b). Overexpression of TRPA1 channels in HLMFs induced by lentiviral transduction with an MOI greater than 0.5 transducing units per ml caused constitutive cell death (Figure 6c,d). The cell death following TRPA1 overexpression was partly inhibited by the TRPA1 channel antagonist A967079 (Figure 6d), indicating that it is mediated at least in part by TRPA1 channels, even in the absence of any exogenously added TRPA1 channel agonists. Staining of HLMFs transduced for TRPA1 channel overexpression, relative to vector control is shown in Figure S9.

We have also generated HEK293T cells that express TRPA1 channels following lentiviral-mediated transduction, while control HEK293T cells do not express protein or functional TRPA1 ion currents (Virk et al., 2019). We used this model system to demonstrate further that expression of these channels is associated with sensitivity to cell death induced by TRPA1 channel agonist- or H2O2 (Figure 6e,f). These data confirm that TRPA1 expression can enhance cell death responses to the TRPA1 channel agonist JT-010 or H2O2.

Taken together with our data demonstrating that TGFβ1 down-regulates HLMF TRPA1 and reduces sensitivity to TRPA1 channel agonist- and H2O2 induced cell death, we have robust evidence that TRPA1 channel activity increased sensitivity to cell death, at least in HLMFs and TRPA1-expressing HEK293T cells. Fibrotic tissues are characterised by high levels of H2O2 and oxidative stress which would usually cause cell death. Down-regulation of TRPA1 channels by TGFβ1 in fibrotic tissue may account for the persistence of pathogenic myofibroblasts in fibrotic tissues. Preventing TGFβ1-induced down-regulation of TRPA1 channels in IPF may therefore constitute a means of restoring cell death sensitivity in pathogenic myofibroblasts.

4 | DISCUSSION

Our work demonstrated that cultured primary NFC- and IPF-derived HLMFs express functional TRPA1 ion channels, with no significant difference in the levels of expression between healthy lung and IPF lung. TRPA1 channels were strongly and consistently down-regulated by TGFβ1, a growth factor that is critical to the development of pathological fibrosis, including IPF (Antoniou et al., 2013; Border & Noble, 1994; Margaritopoulos et al., 2012). Inhibition or activation of TRPA1 channels did not prevent any of the TGFβ1-dependent pro-fibrotic responses in either HLMFs or ex vivo human lung parenchyma. However, activation of TRPA1 channels mediated both channel agonist- and H2O2-dependent cell death in HLMFs, which was confirmed through several experimental approaches.

The persistence of myofibroblasts in the lung, and their resistance to apoptotic death in particular, are characteristic features of IPF (Hecker et al., 2014; Horowitz & Thannickal, 2018). Our work suggests this may, at least in part, be due to the suppressive effects of TGFβ1 on TRPA1 channel expression, and consistent with this, we found that TRPA1 channels were down-regulated in a human lung tissue model of TGFβ1-driven fibrosis. While this TGFβ1-driven model does not allow for the recruitment of cells such as fibrocytes from the circulation (Gomperts & Strieter, 2007), and the lung tissue in the model is not subject to ventilatory cycles, it nevertheless reproduces many of the changes in gene expression present in IPF tissue (DePlianto et al., 2015; Roach et al., 2018), accompanied by
FIGURE 4 Activation of TRPA1 channels causes HLMF death that is inhibited by TGFβ1 pretreatment or TRPA1 channel blockade. (a) The top panel demonstrates the gating strategy used with HLMFs stained with Sytox Orange Dead Cell Stain and Annexin V-APC. HLMFs were either heat treated (90°C for 4 min), treated with 100 μM H2O2 (1 h), or harvested without treatment. These treatments were used to define live, early apoptotic, late apoptotic and necrotic cell populations. Further details are provided in the supplementary information. The lower panel shows representative dot-plots of HLMF death after treatment with AITC (20 μM). This shows a higher proportion of cell death in untreated controls compared to HLMFs pretreated with TGFβ1 (10 ng ml−1), and this is predominantly in the late apoptotic gate. (b–d) Summary of experiments comparing HLMF death (using both Sytox Orange and Annexin V) or viability (using Sytox Orange only) with 20 μM AITC (left), 260 nM JT-010 (middle) or 6.5 μM JT-010 (right). (b) HLMFs treated with 20-μM AITC (n = 10), 260 nM JT-010 (n = 3) or 6.5 μM JT-010 (n = 5) undergo cell death predominantly in the late apoptotic gate. Pretreatment with TGFβ1 (10 ng ml−1) inhibits AITC- and JT-010 induced HLMF death. Statistical analyses were performed on total cell death data as presented below. (c) Experiments examining the viability of HLMFs pretreated with TGFβ1 (10 ng ml−1) compared with unstimulated controls. TRPA1 channel agonists (20 μM AITC n = 9, 260 nM JT-010 n = 9, or 6.5 μM JT-010 n = 7) decreased cell viability but TGFβ1 pretreated HLMFs were more resistant to this loss of cell viability. * P < .05, significantly different as indicated, paired Student’s t-test. (d) Experiments comparing the viability of HLMFs treated with TRPA1 channel agonists in the presence of the selective TRPA1 channel antagonist A967079 (670 nM) or vehicle control (0.05% DMSO). TRPA1 channel agonists (20 μM AITC n = 6, 260 nM JT-010 n = 8 or 6.5 μM JT-010 n = 5) induced loss of cell viability, which was attenuated in the presence of A967079. The effect of A967079 appears to be decreased when TRPA1 channel expression was down-regulated by pretreatment with TGFβ1 (10 ng ml−1). A967079 reversed the effects of JT-010 and TGFβ1 reversed the effects of AITC. A967079 was only effective at preventing cell death in the unstimulated cells, but not TGFβ1 pretreated cells, where TRPA1 expression is down-regulated. * P < .05, significantly different as indicated; two-way ANOVA with Sidak’s multiple comparison test. (e) Basal expression of TRPA1 protein (geometric mean intensity of TRPA1 fluorescence measured by flow cytometry, GMI TRPA1) in HLMF donors predicts the magnitude of JT-010-induced cell death at 260 nM (n = 9) or 6.5 μM (n = 7). Pearson’s correlation coefficients (r) shown were statistically significant. Necrot: Necrotic, AITC: Allyl isothiocyanate, HLMF: Human lung myofibroblast. n numbers signify the number of independent experiments using HLMFs from different donors.
proliferation of resident fibroblasts and deposition of tissue collagens (Roach et al., 2013). We therefore hypothesise that activation of TRPA1 channels during normal wound healing, may be important for the resolution of the wound healing response, by induction of fibroblast cell death. In contrast, in pathological conditions, such as IPF, where there is an excessive and or sustained exposure to TGFβ1, then down-regulation of TRPA1 channels would facilitate HLMF survival and persistence through suppression of TRPA1 channel-dependent cell death (driven by elevated concentrations of channel agonists, including ROS).

From our data on cultured cells, it might seem logical that an agonist of TRPA1 channels would attenuate the pro-fibrotic effects of TGFβ1 in the ex vivo lung model. However, with the rapid down-regulation of TRPA1 channels following TGFβ1 exposure and the sustained exposure to TGFβ1 in the model which is likely to mimic the disease setting (Broekelmann et al., 1991; Kapanci et al., 1995; Khalil et al., 1991; Salez et al., 1998), relatively few TRPA1 channels are likely to be available for activation by an agonist. This would suggest that agonists of these channels are unlikely to be effective treatments. Preventing TGFβ1-dependent down-regulation of TRPA1

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**Figure 5** H2O2 activates TRPA1 channels in HLMFs which results in necrotic cell death. (a) Current–voltage relationship of whole cell recordings from single HLMFs by patch clamp electrophysiology (11 cells from n = 3 donors). Mean and SEM current density for each command potential shows a current–voltage relationship characteristic of TRPA1, and the current is inhibited by the selective TRPA1 antagonist A967079. (b) Example of 100-μM H2O2-induced HLMF cell death measured by Sytox Orange Dead Cell Stain and Annexin V-APC staining by flow cytometry. (c) Left panel—a dose response to H2O2 comparing HLMF viability in cells treated with TGFβ1 (10 ng ml-1) and unstimulated controls (n = 4). TGFβ1-pretreatment prevented loss of cell viability. Right panel—H2O2 dose–response examining viability in the presence of the TRPA1 channel antagonist A967079, compared with vehicle control (DMSO) (n = 6). * P < .05, significant effects of H2O2 dose and of A967079; two-way ANOVA. Cell viability was normalised to the viability of each donor in PBS only (no H2O2 vehicle control) to control for variability of baseline viability of cells in PBS. HLMF: Human lung myofibroblast. n numbers signify the number of independent experiments using HLMFs from different donors, experiments with n < 5 are not subjected to statistical analysis.
Overexpression of TRPA1 channels in HLMFs causes cell death, and TRPA1 expression in HEK293T cells confers sensitivity to TRPA1-mediated cell death. (a) A representative example of immunofluorescent staining for TRPA1 in HLMFs transduced with control or TRPA1 vectors using lentiviral particles. (b) Sytox Orange™ and AnnexinV staining of HLMFs analysed as described above. HLMFs were either untransduced, transduced with a control vector or transduced with a TRPA1 overexpression vector (TRPA1 OE). A multiplicity of infection (MOI) of 0.5 transducing units per cell was used for both viral vectors because higher viral titres induced constitutive cell death (see below). 48-h following transduction cells were stimulated with 20 μM AITC or left unstimulated. TRPA1 overexpression induced HLMF sensitivity to AITC-induced death (n = 3 HLMF donors). Analysis of total cell death showed more cell death in TRPA1 OE HLMFs than in vector controls, stimulated with AITC. (c and d) Sytox Orange™ dead cell staining intensity quantified by plate reader. (c) HLMFs transduced with different viral volumes of TRPA1 overexpression construct or vector control (n = 2) show that the effect of TRPA1 expression was not due to non-specific toxicity of the viral supernatants. (d) HLMFs transduced with viral vectors (MOI = 2) in the presence or absence of the TRPA1 channel antagonist A967079 (670 nM). TRPA1 overexpression results in higher levels of cell death that is prevented by A967079. Dead cell staining intensity was normalised to cells killed in a separate well with 0.2% saponin to account for cell number variation between donors. Pooled analysis (n = 5) of the effect of TRPA1 overexpression in HLMFs in (c) and (d) gives significant effect of TRPA1 overexpression on cell death for (MOI = 2) (e and f) HEK293T cells that do not constitutively express TRPA1 channels were transduced to produce TRPA1-positive (TRPA1) and TRPA1-negative (vector) cell lines. In (e), HEK293T cells were stimulated with the TRPA1 channel agonist JT-010 (260 nM) for 1 h, then analysed for viability by flow cytometry. Only TRPA1-positive HEK293T cells were sensitive to 260 nM JT-010 induced cell death (n = 4 independent experiments). In (f), HEK293T cells were stimulated with H₂O₂ (25-300 μM) for 1 h and viability assessed by flow cytometry. TRPA1 expression in HEK293T cells enhances sensitivity to cell death induced by H₂O₂ (n = 3 independent dose–response experiments). Viability of vector control cells was unchanged over the dose range of H₂O₂, whereas the TRPA1 HEK293T cells lost viability at 100 and 300 μM H₂O₂. Viability was normalised to PBS treated controls. Experiments with n < 5 were not subjected to statistical analysis, although the effects observed were large and consistent. AITC: Allyl isothiocyanate, HLMF: Human lung myofibroblast and TRPA1 OE: TRPA1 overexpressing human lung myofibroblasts.
channels is likely to be more fruitful, allowing the cells to respond to relevant physiological death signals acting on these channels. However, targeting TGFβ1 signalling directly is problematic because of its pleiotropic activities.

More broadly, our work shows that TRPA1 ion channels mediate cell death in response to oxidant stress and analogous findings have been reported for the full length splice variant of TRPM2 channels (Kang et al., 2018). Altered expression of a redox sensitive TRP channel may constitute an important aspect of TGFβ1 signalling. TGFβ1, which induces H2O2 production in myofibroblasts, reduces TRPA1 expression and thereby facilitates myofibroblast survival in environments with high levels of H2O2. This suggests that TGFβ1 acting on myofibroblasts simultaneously induces cellular changes that favour both pro-oxidant production and resistance to oxidant-induced cell death. Restoring sensitivity to oxidant-induced cell death by preventing TGFβ1-induced down-regulation of TRPA1 channels may therefore represent a strategy for limiting the pathogenic activity of myofibroblasts driven by TGFβ1.

Although TRPA1 mRNA is readily detectable in human lung, and reduced by TGFβ1, a limitation of our study is that it has not been possible to assess TRPA1 protein expression in IPF lung using immunohistochemistry as we have been unable find specific TRPA1 antibodies that work for immunohistochemistry in human lung, in spite of extensive efforts (Virk et al., 2019). The availability of TRPA1-specific antibodies that work for flow cytometry means that it may be able to use this technique and/or single cell sequencing to examine TRPA1 expression in IPF myofibroblasts in freshly isolated cells, but the lack of specific surface markers to differentiate between structural cells such as fibroblasts, myofibroblasts and smooth muscle, and their subtypes, is a significant complication in understanding the results (Singh & Hall, 2008).

In summary, we have been able to show that activation or over-expression of redox-sensitive TRPA1 channels can promote myofibroblast cell death through the induction of both necrosis and apoptosis. TGFβ1 reduces the sensitivity of myofibroblasts to oxidative death signals via the down-regulation of TRPA1 channels, promoting myofibroblast survival in the presence of oxidative stress-dependent signalling. This may be a central aspect of TGFβ1-driven fibrosis in IPF and in other organs. We hypothesise that preventing TGFβ1-induced TRPA1 down-regulation may constitute a therapeutic approach in IPF, while activation or inhibition of TRPA1 channels by itself would not be effective.

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AUTHOR CONTRIBUTIONS
H.S.V contributed to conception, design, acquisition and interpretation of data and drafting of the manuscript. M.S.B. and D.T.S. contributed to design and data analysis strategy for HEK293T experiments and revision of manuscript. E.C. and V.W.B. contributed to electrophysiology experimental design and data acquisition. J.M. contributed to design of flow cytometry experiments and data analysis. C.A.W. contributed to production and validation of overexpression constructs and lentiviral transduction strategy. S.M.D contributed to electrophysiology experimental design and data acquisition. K.M.R. contributed to conception, design, acquisition and analysis of data and revision of the manuscript. P.B. and Y.A. contributed to conception, design, review and interpretation of the data, and drafting of the manuscript. All authors approved the manuscript before submission.

CONFLICT OF INTEREST
None declared.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Immunoblotting and Immunohemistry, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT
All data generated during this study are either published in the main text or the supplementary information. The plasmids used to over-express human TRPA1 channels for this work are available upon request from the corresponding author.

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**SUPPORTING INFORMATION**

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