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RESEARCH ARTICLE

TRPA1 up-regulation mediates oxidative stress in a pulpitis model in vitro

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Background and Purpose: Pulpitis is associated with tooth hypersensitivity and results in pulpal damage. Thermosensitive transient receptor potential (TRP) ion channels expressed in the dental pulp may be key transducers of inflammation and nociception. We aimed at investigating the expression and role of thermo-TRPs in primary human dental pulp cells (hDPCs) in normal and inflammatory conditions.

Experimental Approach: Inflammatory conditions were induced in hDPC cultures by applying polyinosinic:polycytidylic acid (poly(I:C)). Gene expression and pro-inflammatory cytokine release were measured by RT-qPCR and ELISA. Functions of TRPA1 channels were investigated by monitoring changes in intracellular Ca^{2+} concentration. Mitochondrial superoxide production was measured using a fluorescent substrate. Cellular viability was assessed by measuring the activity of mitochondrial dehydrogenases and cytoplasmic esterases. TRPA1 activity was modified by agonists, antagonists, and gene silencing.

Key Results: Transcripts of TRPV1, TRPV2, TRPV4, TRPC5, and TRPA1 were highly expressed in control hDPCs, whereas TRPV3, TRPM2, and TRPM3 expressions were much lower, and TRPM8 was not detected. Poly(I:C) markedly up-regulated TRPA1 but not other thermo-TRPs. TRPA1 agonist-induced Ca^{2+} signals were highly potentiated in inflammatory conditions. Poly(I:C)-treated cells displayed increased Ca^{2+} responses to H_2O_2, which was abolished by TRPA1 antagonists. Inflammatory conditions induced oxidative stress, stimulated mitochondrial superoxide production, resulted in mitochondrial damage, and decreased cellular viability of hDPCs. This inflammatory cellular damage was partly prevented by the co-application of TRPA1 antagonist or TRPA1 silencing.

Abbreviations: hDPCs, human dental pulp cells; Poly(I:C), polyinosinic:polycytidylic acid.

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RM and BIT contributed equally to the work as senior authors.

Conclusion and Implications: Pharmacological blockade of TRPA1 channels may be a promising therapeutic approach to alleviate pulpitis and inflammation-associated pulpal damage.

KEYWORDS

dental pulp, endodontics, immunopharmacology, inflammation, reactive oxygen species, transient receptor potential channels, TRPA1

1 | INTRODUCTION

Transient receptor potential (TRP) ion channels represent a heterogeneous group of non-specific cationic channels activated by a variety of stimuli. Many members of the TRP family are sensitive to changes in physical and chemical parameters of the extracellular environment including among others temperature, redox signals, pH, osmolarity, mechanical stimuli, and various ligands. Therefore, they are generally considered cellular sensors of environmental challenges (Voets et al., 2005). Expressed in sensory fibres, especially the thermosensitive members play an indispensable role in transduction of temperature, pain, and itch (Nilius & Szallasi, 2014; Vriens et al., 2014). Moreover, their expression and function can be regulated by inflammatory signals and altered in pathological conditions resulting, for example, in various forms of inflammatory hyperalgesia (Silverman et al., 2020).

The expression of the thermosensitive TRP channels is not restricted to the sensory neurons; they are abundantly expressed by non-neuronal cells surrounding the somatosensory nerve endings, for example, in the skin or in mucosal membranes. These epithelial thermo-TRP channels can also shape sensory functions in a local intercellular crosstalk (Nilius & Szallasi, 2014; Szöllősi et al., 2022). Moreover, cutaneous TRPs can regulate local inflammation influencing, among others, the release of inflammatory mediators (Szántó et al., 2019; Szöllősi et al., 2018; Tóth et al., 2014).

Recently, thermosensitive TRP channels were also described in the dental pulp and are expressed by both innervating trigeminal fibres and non-neuronal pulpal cells (Hossain et al., 2019). Trigeminal TRP channels are linked to various forms of orofacial pain, (Luo et al., 2021), and emerging data suggest that TRP channels expressed by non-neuronal pulp cells may also contribute to sensory functions (Bernal et al., 2021; Sato et al., 2018; Shibukawa et al., 2015). Here, we hypothesized that thermosensitive TRP channels in non-neuronal cells of the human pulp can be involved in inflammatory signal transduction and contribute to the symptoms of pulpitis.

Pathogen-associated molecular patterns (PAMPs) related to the invading pathogenic microbes and damage-associated molecular patterns (DAMPs) released from the injured and inflamed tissue are primary initiators of pulpitis. PAMPs and DAMPs activate pattern recognition **Toll-like receptors (TLRs)** in the pulpal cells. The initiated inflammatory signalling results in serious consequences including the release of inflammatory cytokines, production of reactive oxygen species (ROS), and invasion of inflammatory immune cells. These processes are typically associated with the damage of the pulpal tissue (Cooper et al., 2014).

What is already known

- Thermosensitive TRP channels are expressed in the dental pulp.
- Expression of TRPA1 channels can be up-regulated by inflammatory signals.

What does this study add

- Poly(I:C)-induced inflammation up-regulated TRPA1 channels and induced oxidative stress in human dental pulp cells.
- TRPA1 up-regulation increases sensitivity towards reactive oxygen species and partly contributes to diminished viability.

What is the clinical significance

- Pulpal TRPA1 channels are a promising target to alleviate oxidative damage in pulpitis.

In our study, we investigated the potential effects of thermosensitive TRP channels in inflammation-related signalling pathways in primary human dental pulp cells (hDPCs). Therefore, we established inflammatory conditions in vitro by treating hDPCs with **polyinosinic: polycytidylic acid (poly(I:C))**, a powerful inducer of inflammation. Poly(I:C) activates pattern recognition receptors, especially **toll-like receptor 3 (TLR3)**, a target of double-stranded RNA either from viral origin or released from own, damaged tissue during inflammation. The subsequent signalling results in the activation of the NF-κB pathway and the release of pro-inflammatory cytokines initiating and further exacerbating inflammation (Alexopoulou et al., 2001; Cavassani et al., 2008; Karikó et al., 2004). Indeed, experimentally applied poly(I:C) was shown to induce strong inflammatory responses in pulp fibroblasts and odontoblasts (Staquet et al., 2008). We investigated the expression of thermosensitive TRP channels, and we found that **TRPA1** was markedly up-regulated in inflammatory conditions,

which was associated with an increased sensitivity towards ROS. Our data suggest that up-regulated TRPA1 channels can contribute to inflammation-associated cellular damage and may, therefore, provide a novel target to combat pulpitis.

2 | METHODS

2.1 | Establishment of primary hDPC cultures

Primary hDPCs were isolated from sound third molars extracted under local anaesthesia from otherwise healthy subjects for orthodontic reason at the Faculty of Dentistry, University of Debrecen. Patients provided written informed consent, and the procedure was approved by the Regional and Institutional Ethics Committee of the University of Debrecen under the ID Number: ETT TUKEB 49849-3/2016/EKU. Following extraction, teeth were disinfected with 3% sodium hypochlorite solution for 2 min and rinsed with phosphate-buffered saline (PBS, 115 mM NaCl, 20 mM Na_2PO_4, pH 7.4; all from Sigma-Aldrich/Merck, St. Louis, MO, USA) and dried using cotton gauze. A corono-apical cut was made using a sterilized dental diamond fissure bur (Hager & Meisinger GmbH, Neuss, Germany) along with a high-speed handpiece (W&H Dentalwerk Bürmoos GmbH, Bürmoos, Austria) under copious water supply until a thin but still continuous dentin wall remained around the pulp chamber.

Sectioned teeth were placed into the transport medium containing Dulbecco's modified essential medium-F12 (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Waltham, MA, USA), and penicillin 500 $U \cdot ml^{-1}$, streptomycin 500 $\mu g \cdot ml^{-1}$, and amphotericin B 1.25 $\mu g \cdot ml^{-1}$ (all from Sigma-Aldrich). Samples placed on ice were transferred to the cell culture laboratory for subsequent processing. Under sterilized laminar flow cabinet, the teeth were split with a sterile Bein dental root elevator (MEDICOR Kéziműszer Zrt, Debrecen, Hungary), and the coronal and radicular pulp tissues were removed with a sterile endodontic H-file # 30 (Dentsply Sirona, Charlotte, USA). Dental pulp tissue was transferred into a new petri plate containing the same medium and minced into 1–2 mm pieces using surgical blade # 20 (Feather, WPI, Sarasota, FL, USA). The pulp tissue was then digested in a solution of collagenase type I (3 $mg \cdot ml^{-1}$, Sigma Aldrich, St Louis, MO, USA) and dispase (4 $mg \cdot ml^{-1}$, Gibco, Waltham, MA, USA) for 1 h at 37°C. The cell suspension was seeded and cultured in 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA) in the same medium at 37°C in a humidified incubator supplemented with 5% CO_2. Cultures were observed daily under an inverted microscope (Olympus Corp., Center Valley, PA, USA) for any contamination and cell growth.

When cultures reached 70%–80% confluency, they were either subcultured and used for a subsequent assay or cryopreserved in liquid nitrogen for later use. During subculturing, cells were thoroughly washed with PBS twice, trypsinized with 0.05% Trypsin-EDTA (Sigma Aldrich) for 10 min, neutralized by culturing medium, centrifuged (125 x *g*, 8 min, at room temperature), and resuspended in cell culture medium (DMEM-F12 containing 10% FBS). Only hDPCs subcultured ≤5 times were used in experiments.

2.2 | Gene expression studies

The expression of mRNA transcripts was determined by quantitative PCR following reverse transcription (RT-QPCR). Briefly, total RNA was separated utilizing TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and the isolated RNA was quality-checked by Nanodrop-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA); 2 μg of total RNA was then reverse-transcribed into cDNA by using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. PCR amplification was performed by using the TaqMan universal PCR master mix protocol (Thermo Fisher Scientific) and TaqMan gene expression assays (assay ID-s: Hs00175798_m1 for TRPA1, Hs00202960_m1 for **TRPC5**, Hs01066091_m1 for **TRPM2**, Hs00257553_m1 for **TRPM3**, Hs01066596_m1 for **TRPM8**, Hs00218912_m1 for **TRPV1**, Hs00275032_m1 for **TRPV2**, Hs00376854_m1 for **TRPV3**, Hs00222101_m1 for **TRPV4**, Hs00985639_m1 for **IL-6**, Hs00174103_m1 for **IL-8/CXCL8**, Hs01551078-m1 for TLR3, Hs00533490_m1 for SOD1 [superoxide dismutase 1], Hs00167309_m1 for SOD2, Hs00156308_m1 for **CAT [catalase]**, and Hs00829989_gH for GPX1 [glutathione peroxidase 1]). As internal controls, transcripts of peptidyl-prolyl isomerase A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) were determined (assay ID-s: Hs99999904_m1, Hs99999905_m1, Hs99999903_m1). Three technical replicates were used to ensure the reliability of single gene expression (CT) values. The amount of the transcripts was normalized to those of the housekeeping genes using the ΔCT method. The geometrical mean CT of the housekeeping genes served for reference.

2.3 | Measurement of inflammatory cytokine secretion

Supernatants were collected from hDPC cultures (100,000 cells per culture) exposed to various treatments for 24 h and analysed for human IL-6 and IL-8 using a commercially available ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocols. In brief, plates were coated with capture antibody diluted in coating buffer (0.1-M Na_2CO_3, pH 9.5 with 10-N NaOH) and incubated overnight at 4°C. Then plates were incubated with assay diluent (10% fetal bovine serum in PBS) at room temperature for 1 h, while standard and sample dilutions were prepared in assay diluent. Concentration standards and samples were added into appropriate wells and incubated for 2 h at room temperature. After 2 h, working detector (detection antibody + SAv-HRP reagent) was added to each well and incubated for 1 h at room temperature. Between steps, plates were washed with wash buffer (0.05%

Tween-20 in PBS). After washing, substrate solution (tetramethylbenzidine and hydrogen peroxide in citrate-buffer, pH 5.0) was added to every well for 30 min in the dark, followed by stop solution (2 N H_2SO_4). Absorbance was read at 405 nm within 30 min of stopping the reaction. The amount of cytokines, as pg·ml^{-1}, was calculated from a calibration curve created by serial dilution of IL-6 and IL-8 standards.

2.4 | Fluorescent measurements of intracellular Ca^{2+} concentration

Changes in the cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_{IC}$ were measured using the fluorescent Ca^{2+}-sensitive dye Fura-2. Measurements were performed according to our previously optimized protocol (Kelemen et al., 2020). Briefly, hDPCs were seeded in 96-well black-well/clear-bottom plates (Greiner Bio-One, Kremsmünster, Austria) at a density of 20,000 cells per well in DMEM-F12 medium and cultured for 1 day. The next day, cells were treated either by poly(I:C) or by vehicle for additional 24 h. Following the treatment, cells were loaded with 2-μM Fura-2 AM (Thermo Fisher Scientific) dissolved in culture medium at 37°C for 30 min. The cells were washed three times and incubated in Ca^{2+}-buffer (150-mM NaCl, 5-mM KCl, 1-mM $MgCl_2 \times 6H_2O$, 2-mM $CaCl_2 \times 2H_2O$, 10-mM glucose xH_2O, 10-mM HEPES, pH 7.4, all from Sigma-Aldrich) (100 μl per well). The plates were then placed into a FlexStation 3 multimodal microplate reader (Molecular Devices, Sunnyvale, CA, USA), and $[Ca^{2+}]_{IC}$ was monitored during the application of compounds in various concentrations. In one well, cells were exposed to only one given concentration of the agents during the measurements. Experiments were performed in multiple wells, and cells in different wells were seeded, cultured, and treated individually and independently. Data are presented as F_{340}/F_{380}, where F_{340} is the fluorescence measured at 340 nm excitation and 510 nm emission wavelengths whereas F_{380} is the fluorescence measured at 380 nm excitation and 510 nm emission wavelengths. During data analysis, N represents individual wells containing independently seeded, cultured, treated, and measured cells. By experimental design, n = 6, however a few samples were excluded from the analysis due to obvious pipetting error and/or lack of response to the positive control ionomycin applied at the end of each experiment.

To assess the distribution of the responsive cells, $[Ca^{2+}]_{IC}$ was measured in individual cells using a fluorescent microscope. For these measurements, cells were seeded in glass bottom petri dishes (ibidi, Gräfelfing, Germany) suitable for fluorescent microscopic observation and treated with poly(I:C) or vehicle control for 24 h. Then, cells were loaded with 2-μM Fluo-4 AM (Thermo Fisher Scientific) and subjected to the stage of a Zeiss LSM 5 Live confocal fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany) to measure fluorescence. Compounds dissolved in Ca^{2+} buffer were applied via a gravity-driven local perfusion system near to the investigated cells, and the buffer was continuously drained via a perfusion pump-driven suction system ensuring a continuous flow during the measurements. Fluorescence was monitored at 490-nm excitation and 520-nm emission wavelengths. Changes in $[Ca^{2+}]_{IC}$ were characterized by F1/F0, where F1 represents the actual fluorescence and F0 represents the average of the basal fluorescence at the beginning of the measurements before applying any compounds.

2.5 | Measurement of mitochondrial superoxide production

Mitochondrial superoxide production was measured using the MitoSox Red assay kit (Thermo Fisher Scientific); 2×10^4 cells per well were seeded and cultured in 200 μl DMEM-F12 medium in 96-well black-well/clear-bottom plates (Greiner Bio-One) for 24 h. The next day, 50 μl MitoSox Red fluorogenic dye solution was added to each well, and incubated for 20 min at 37°C, then the cells were treated by polyinosinic:polycytidylic acid (poly(I:C), InvivoGen, San Diego, CA, USA), and TRPA1 channel agonists and antagonists in various concentrations; 10 μg·ml^{-1} Antimycin A was applied as a positive control. The fluorescence was measured by a FlexStation 3 microplate reader (Molecular Devices), using 510 nm excitation and 580 nm emission wavelengths after 60 min. During data analysis, n represents individual wells containing independently seeded, cultured, treated, and measured cells.

2.6 | Assessment of cellular viability

Cellular viability was assessed by two methods measuring mitochondrial and cytoplasmic enzyme activity. Mitochondrial fitness was investigated via the conversion of a non-toxic tetrazolium compound into a soluble formazan dye by mitochondrial dehydrogenases using EZ4U (Biomedica, Wien, Austria) assay following the manufacturer's protocol. Briefly, cells were plated in 96-well cell culture plates, cultured for 24 h, and treated with various compounds for an additional 24 h. Following the treatment, 20 μl of the tetrazolium substrate containing working solution was added to 180 μl phenol red-free culturing medium in each well and incubated for an additional hour. After incubation, the absorbance of the formazan product was measured at 450 nm and reference was measured at 620 nm using a FlexStation 3 microplate reader (Molecular Devices). Absorbances were normalized to the mean optical density of the control group. Cytoplasmic enzyme activity was assessed by the activity of cytoplasmic esterases converting membrane-permeable calcein acetoxymethyl ester (calcein-AM) into fluorescent, non-membrane-permeable calcein accumulating in the cytoplasm. Cells were plated and treated as for EZ4U assay then loaded with 2 μM calcein-AM for 30 min. Following incubation and washing, the fluorescence of calcein was measured at 490 nm excitation and 520 nm emission wavelengths using a FlexStation 3 microplate reader. Experiments were performed in multiple wells, and cells in different wells were seeded, cultured, and treated individually and independently.

2.7 | Gene silencing

To silence TRPA1 expression in hDPCs, we applied RNA interference techniques, using small interfering RNAs (siRNA) targeting TRPA1 coding mRNA transcripts. Cells were seeded in either six-well or 96-well plates and 24 h later, they were transfected with siRNA probes using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) following the manufacturer's protocol. RNAiMAX and siRNA probes were gently mixed in Opti-MEM medium (Thermo Fisher Scientific), incubated for 5 min, and gently added to the cultures. In final concentration, 100 pmol·ml^{-1} siRNA was added to the cells and Lipofectamine RNAiMAX was applied in 3:1000. Cells were treated with poly(I:C), TRPA1 antagonists, or vehicle controls 24 h after the transfection and were assayed in additional 24 h (i.e., 48 h after the transfection). The efficacy of three TRPA1 targeting sequence (Silencer® Select siRNA, Thermo Fisher Scientific, Assay IDs: s17147, s17148, s17149) was tested in preliminary experiments 48 h after transfection (Figure S1), and the most effective one (Assay ID: s17148) was used in the experiments. Non-targeting negative control siRNA (Thermo Fisher Scientific) was used to control non-specific effects of the transfection.

2.8 | Data and statistical analysis

During the experiments, treated and non-treated samples originated from the same primary cultures were compared; therefore, no randomization was applied. The person carrying out the statistical analysis was blinded for the experimental conditions. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). Origin 2018 (OriginLab Corporation, Northampton, MA, USA) was used for analysis and data display. If not mentioned otherwise, individual data are presented in scatterplots marking mean ± SD. Logistic dose–response curves were fitted using the equation $y = A2 + (A1 - A2)/(1 + (x/x0)^p)$ where the calculated parameters are as follows: A1: initial value (y_{min}), A2: final value (y_{max}), x0: centre (EC50), and p is the calculated power. Statistical analysis was performed using IBM SPSS Statistics 22 software (IBM, Armonk, NY, USA). In gene expression studies, expression values were log10 transformed then subjected for statistical analysis. Prior to the application of parametric statistical tests, suitability of the data was investigated. Normality of the data was checked by Shapiro–Wilk test, and homogeneity of variances was tested by Levene's test. To compare two groups, Student's *t* test for independent samples was applied. To compare gene expression of two samples of the same donors, paired sample *t* tests were carried out. To compare multiple groups, one-way ANOVA was used. If *F* value was significant, pairwise comparison was carried out with Tukey HSD post-hoc tests. If multiple groups were compared with a single control group, Dunnett post hoc test was applied for pairwise comparison. To investigate interactions between two treatments, two-way ANOVA was applied. In every case, $^*P < 0.01$ was considered as significant.

2.9 | Materials

To establish inflammatory conditions, polyinosinic:polycytidylic acid (poly(I:C), InvivoGen, San Diego, CA, USA) was applied for 24 h. The TRPA1 agonists **allyl isothiocyanate (AITC)** and **cinnamaldehyde** were obtained from Sigma-Aldrich and Santa Cruz (Santa Cruz CA, USA), respectively. The TRPA1 antagonist **A967079** was purchased from MedChemExpress, whereas **HC030031** was from Sigma-Aldrich, as well as **hydrogen peroxide (H_2O_2)** and **glutathione**. In certain measurements, ionomycin (Abcam, Cambridge, UK) or antimycin A (Sigma-Aldrich) were used as positive controls.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | Thermosensitive TRP channels are expressed in hDPCs

To study the expression of thermosensitive TRP ion channels in primary hDPCs, we isolated total RNA from hDPC cultures from several donors and subjected them to Q-PCR following reverse transcription. As shown in Figure 1, hDPCs expressed a marked amount of the warm-sensitive TRPV2 and TRPV4 channels. The expression of TRPV1 transcripts was approximately one magnitude lower, and TRPV3, TRPM2, and TRPM3 were detected at low levels and only in some of the donors. Investigating the cold-sensitive TRPs, we detected a high expression level of TRPA1 in most of the donors, and TRPC5 transcripts showed slightly lower expression. Importantly, TRPM8, which is the main cold sensor in somatosensory neurons (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Paricio-Montesinos et al., 2020), was not detected in any samples.

3.2 | Poly(I:C) induces inflammatory responses and up-regulates TRPA1 channels

To question whether the expression of the above TRP channels changes in inflammatory conditions, we also investigated their expression in a poly(I:C)-induced inflammatory model. Therefore, we treated hDPCs with poly(I:C) for 24 h to establish inflammatory conditions in our primary cultures. As illustrated in Figure 2, poly(I:C) induced a marked and concentration-dependent up-regulation of proinflammatory cytokine transcripts IL-6 and IL-8/CXCL8 (Figure 2a) and also increased the release of these cytokines (Figure 2c). Poly(I:C) also up-regulated TLR3 expression, which is a known marker of its activation

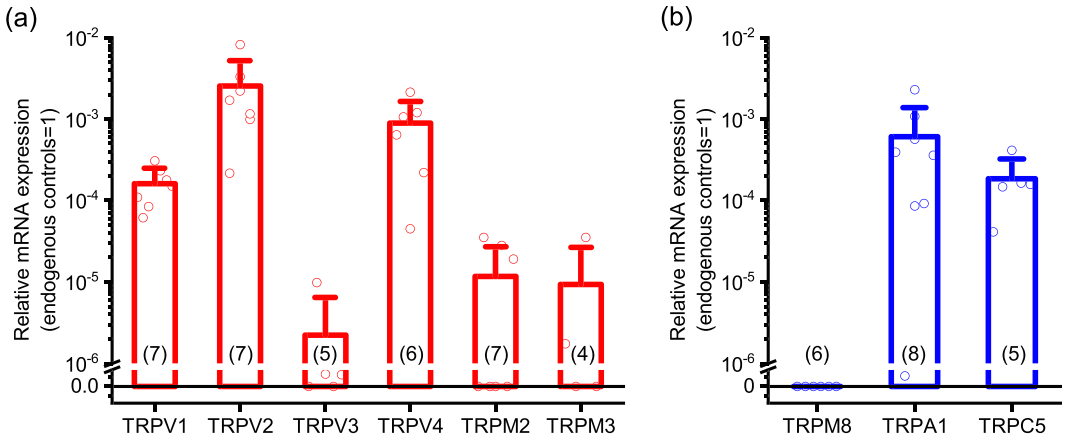


FIGURE 1 Thermosensitive TRP channels are expressed in human dental pulp cells. The relative expression of (a) warm and (b) cold sensitive TRP ion channel transcripts determined by Q-PCR in hDPC cultures from several donors. PPIA, ACTB, and GAPDH were determined as endogenous controls and their average was used for normalization (details of the analysis are provided in Section 2). Data shown are individual (for each donor) values with means ± SD. The numbers of the donors are indicated in parentheses.

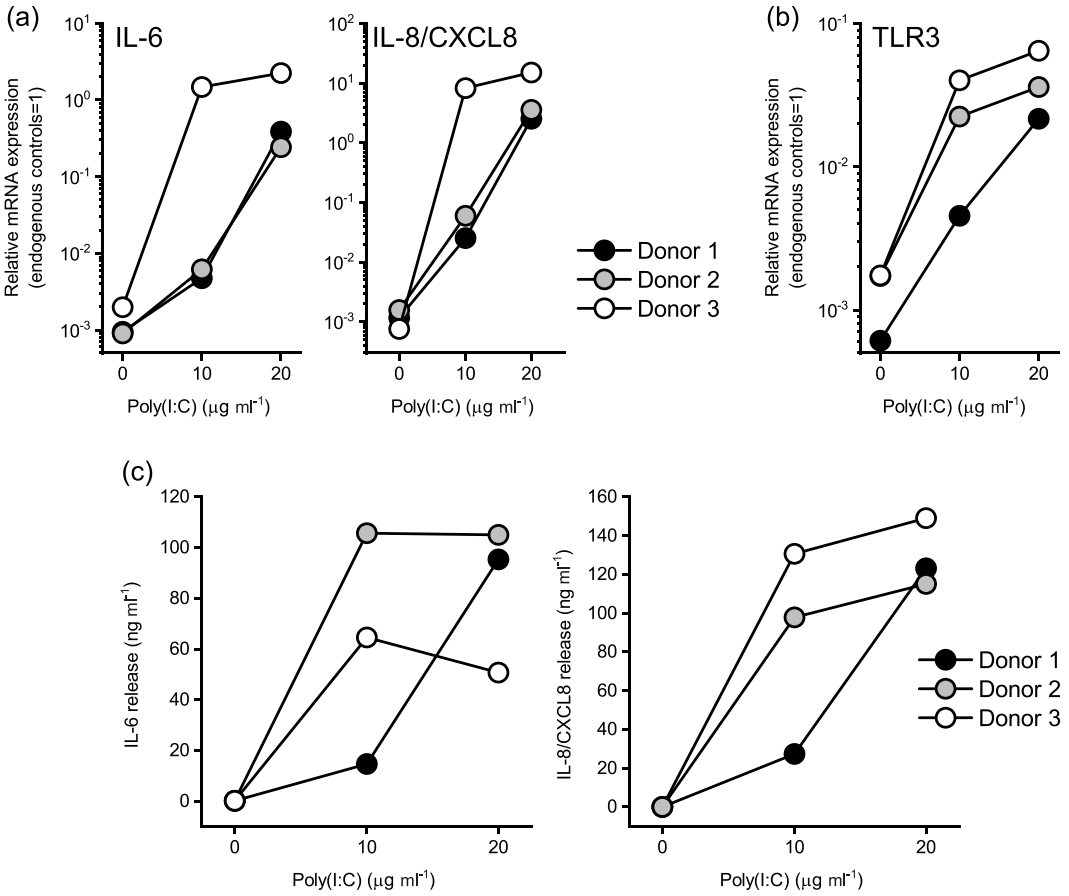


FIGURE 2 Poly(I:C) induces inflammatory responses. (a) Concentration-dependent effect of poly(I:C) on the expression of IL-6, IL-8/CXCL8, and (b) TLR3 transcripts in hDPCs from three different donors. Cells were treated for 24 h with poly(I:C) in the indicated concentrations and gene expression was determined by Q-PCR. PPIA, ACTB, and GAPDH were determined as endogenous controls. (c) Concentration-dependent effect of poly(I:C) on the release of proinflammatory cytokines IL-6 and IL-8/CXCL8. Cells were treated as in (a) and (b) and supernatants were assayed by specific ELISA kits.

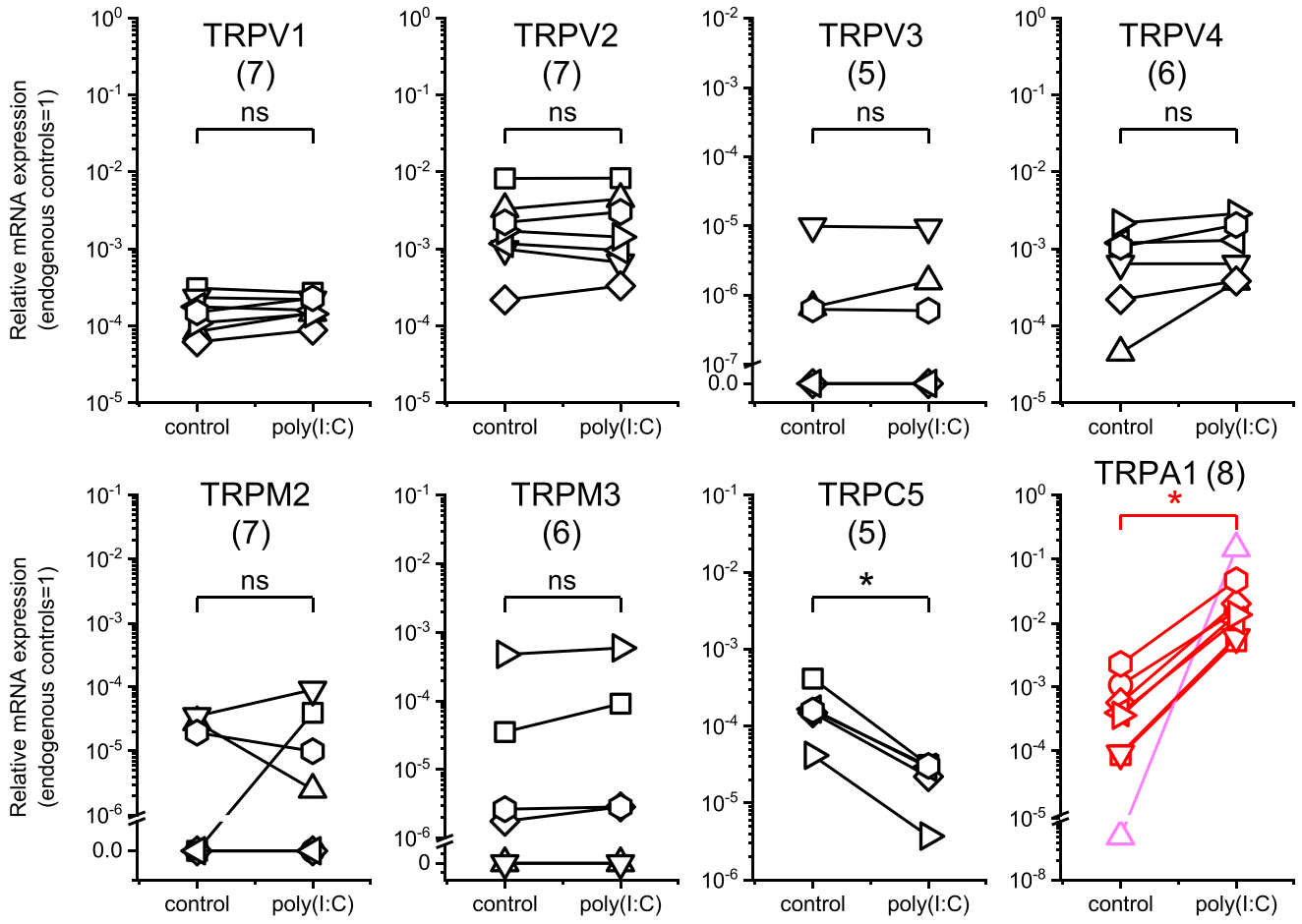


FIGURE 3 Poly(I:C) up-regulates TRPA1 expression in human dental pulp cells. Effect of poly(I:C) (20 μg·ml^{-1}, 24 h) on the expression of thermosensitive TRP ion channel transcripts as measured by Q-PCR from the samples of several donors. Different symbols represent different donors within one panel and the numbers of investigated donors are also marked in parentheses in the corresponding panel. In the TRPA1 panel, the donor marked with light magenta colour was considered an outlier, due to very low expression in the control sample, therefore it was omitted from statistical analysis. *P<0.01, significantly different as indicated; ns, not significant; paired sample t test.

(Figure 2b). We analysed the expression of the above TRP channels in this inflammatory model, treating hDPCs with 20 μg·ml^{-1} poly(I:C) (Figure 3), and we found that it had little effect on the expression of the warm-sensitive TRP channels. Regarding the cold-sensitive channels, the expression of TRPM8 was not induced in inflammatory conditions and, as in control cases, it was not expressed in poly(I:C)-treated samples. Interestingly, TRPC5 expression was markedly down-regulated in each investigated donor (to ≈14% of the control). In contrast, the expression of TRPA1 was markedly up-regulated, with approximately 37-fold higher, on average, in seven donors' samples. In one additional donor, the up-regulation was more than 10,000-fold, although its expression was very low in the control sample of this donor.

We further investigated the functional consequences of the up-regulated TRPA1 gene expression, and we monitored the changes in $[Ca^{2+}]_{IC}$ upon applying TRPA1 channel agonists. We found that AITC and cinnamaldehyde evoked a rapid, concentration-dependent increase in $[Ca^{2+}]_{IC}$ in control hDPCs (Figure 4). These Ca^{2+} signals were markedly increased in the poly(I:C) pretreated cells in good accordance with the elevated TRPA1 expression of TRPA1 channels (Figure 4a–d). Importantly, both AITC- and CA-evoked responses were almost completely inhibited by the TRPA1 antagonists HC030031 and A 967079 supporting the TRPA1-specific action of the applied agonists (Figure 4a–e). Measurements at single cell level indicated that poly(I:C) pretreatment elevated the average amplitude of the AITC-evoked Ca^{2+} responses of the individual cells but did not affect the ratio of the responsive cells, which was found similarly very high in both control and inflammatory conditions (Figure 4f). Investigation of the pharmacological properties of the cinnamaldehyde revealed that both efficacy (maximal response, $\Delta(F_{340}/F_{380})$: 1.94 ± 0.05 vs. 1.06 ± 0.07) and potency (EC_{50}, μM: 19.3 ± 1.6 vs. 62.6 ± 13.9) of the agonist were elevated in the inflammatory group, compared with the control values (Figure 4g).

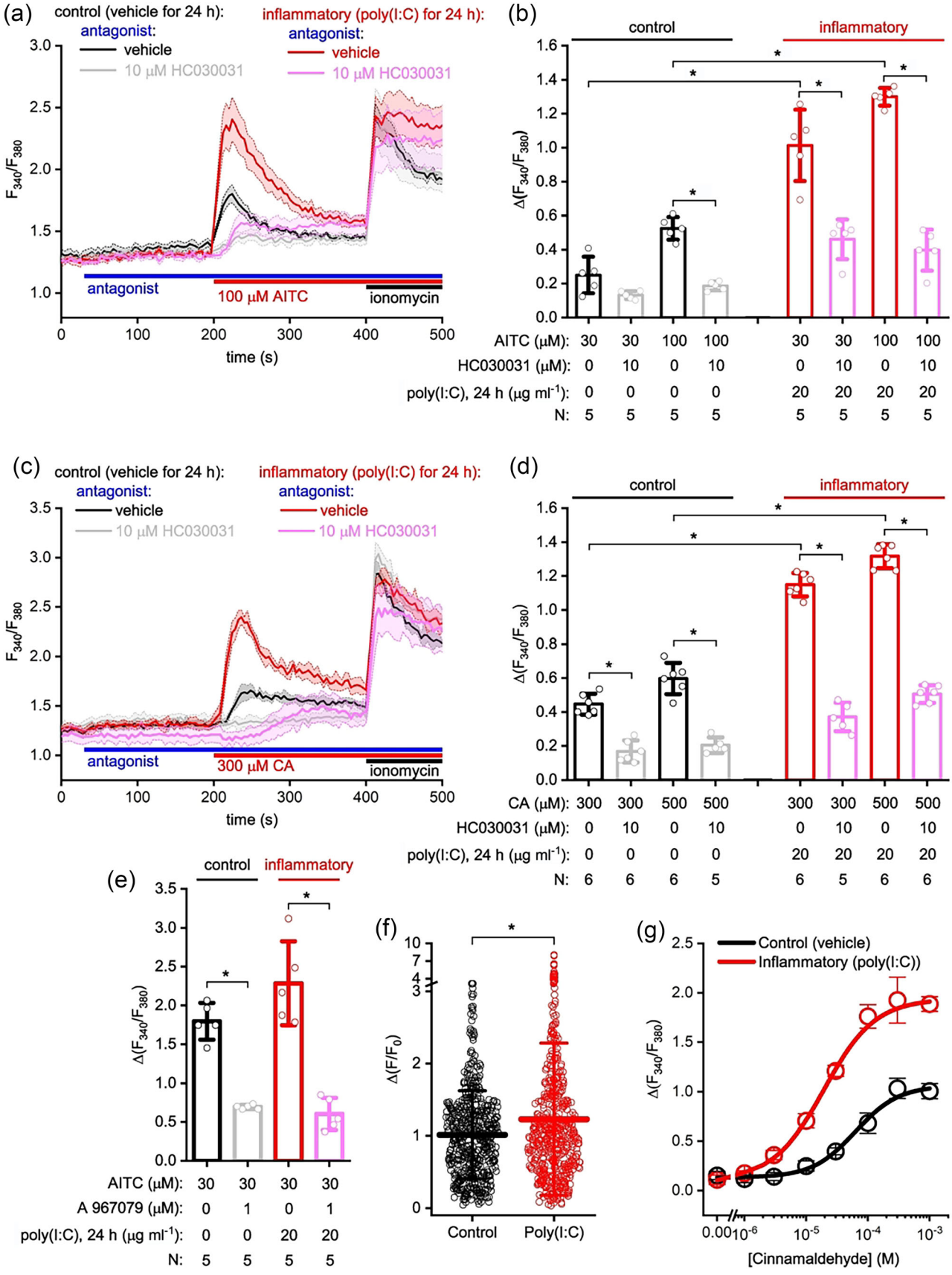


FIGURE 4 Legend on next page.

3.3 | hDPCs are more sensitive to exogenous H_2O_2 in inflammatory conditions due to up-regulated TRPA1

The TRPA1 channel is generally considered to be a cellular sensor for noxious stimuli, and it is activated, among other stimuli, by ROS (Andersson et al., 2008; Talavera et al., 2020). Therefore, we hypothesized that up-regulation of TRPA1 in inflammatory conditions can result in increased sensitivity towards reactive oxygen radicals. To test this hypothesis, we challenged the hDPCs with H_2O_2 and measured its acute effect on $[Ca^{2+}]_{IC}$ (Figure 5). Control cells displayed only moderate Ca^{2+} signals upon H_2O_2 application over 1 mM. In contrast, poly(I:C) pretreated hDPCs were already activated by a lower concentration of H_2O_2 and exhibited much higher responses. Importantly, the H_2O_2-evoked Ca^{2+} signals were abolished in the presence of HC030031, clearly indicating that H_2O_2-evoked elevation in $[Ca^{2+}]_{IC}$ is mediated by TRPA1 channel activity.

3.4 | Poly(I:C) induces oxidative stress and stimulates mitochondrial superoxide production

Mitochondria are the primary places of cellular ROS production, which are neutralized by an antioxidant defence system. A disturbance in the balance between the production of ROS and antioxidant defences results in oxidative stress and increased cellular ROS concentration (Betteridge, 2000). As ROS are important signalling molecules in inflammation and can exacerbate inflammation-related tissue damage (Cooper et al., 2014), we investigated whether poly(I:C) induces oxidative stress and ROS production in our inflammatory model. We found that the expression of the mitochondrial superoxide dismutase 2 (SOD2), the enzyme responsible for the conversion of mitochondrial superoxide radicals into H_2O_2, was markedly up-regulated, but the expression of the cytoplasmic SOD1 and the H_2O_2-neutralizing catalase and glutathione peroxidase 1 (GPX1) was not changed (Figure 6a). These results suggested severe disturbances in the cellular redox homeostasis and indicated mitochondrial oxidative stress. Indeed, we found that poly(I:C) rapidly (in 60 min) induced mitochondrial superoxide production (Figure 6b) in a concentration dependent manner. In contrast to poly(I:C), TRPA1 agonists did not stimulate superoxide production (Figure 6b), suggesting that selective activation of TRPA1 does not induce oxidative stress. Moreover, TRPA1 antagonists had only a partial effect on poly(I:C) induced superoxide production. Although superoxide production evoked by 10 $\mu g \cdot ml^{-1}$ poly(I:C) was partially decreased by HC-030031 and A 967079, it was still significantly elevated even in the presence of the antagonists. Moreover, the effect of 20 $\mu g \cdot ml^{-1}$ poly(I:C) was not influenced by TRPA1 antagonism (Figure 6b). These results indicated that TRPA1 has only a partial role in the mitochondrial generation of ROS in poly(I:C)-induced inflammatory conditions. TRPA1 antagonists did not revert the poly(I:C)-induced inflammation, and they did not inhibit the production of the inflammatory IL-6 and IL-8/CXCL8 (Figure S2).

3.5 | Reduced mitochondrial activity in poly(I:C)-induced inflammation is mediated by ROS production and partially TRPA1

Although TRPA1 only slightly influenced mitochondrial ROS production in inflammatory conditions, we hypothesized that the up-regulated ion channel can serve as a target of the endogenously produced reactive radicals and may contribute to the consequences of oxidative stress in inflammatory conditions. Therefore, we investigated the effect of poly(I:C) induced inflammation on cellular viability and the potential involvement of TRPA1 in the effect. To assess cellular viability, we measured mitochondrial enzyme activity and activity of cytoplasmic esterases, as described in Section 2. We found that poly(I:C) resulted in a marked decrease in mitochondrial functions but hardly affected cytoplasmic enzyme activity (Figure 7). Mitochondrial disturbances were reduced by the ROS scavenger glutathione in a concentration-dependent manner (Figure 7a) indicating that oxidative stress plays a significant role in the negative effect of poly(I:C) on mitochondrial fitness. However, cytoplasmic enzyme activity was not restored by glutathione (Figure 7b), suggesting that nonoxidative pathways also can contribute to some effects of poly(I:C).

As TRPA1 was also highly up-regulated in these conditions, we questioned whether this ROS sensitive ion channel can play a role in

FIGURE 4 TRPA1-mediated responses are enhanced in poly(I:C)-induced inflammatory conditions. (a) Averaged time course of $[Ca^{2+}]_{IC}$ records demonstrating the effect of the TRPA1 channel agonist AITC (100 μM) and antagonist HC-030031 (10 μM) on hDPCs cultured in control (vehicle) or inflammatory (20 $\mu g \cdot ml^{-1}$ poly(I:C) for 24 h) conditions. For the measurements, cells were loaded with fura-2, as described in Section 2. Compounds were applied as indicated in the figure. Traces represent the means ± SD, from 5 records. (b) Statistical analysis on the peak amplitudes of the AITC-evoked Ca^{2+} traces in various conditions, as indicated in the figure. Measurements were carried out as in panel (a). (c) and (d) present $[Ca^{2+}]_{IC}$ measurements as (a) and (b), respectively, but cinnamaldehyde (CA; 300 and 500 μM, as indicated) was applied instead of AITC. *P<0.01, significantly different as indicated; one-way ANOVA and pairwise Tukey HSD post-hoc test. (e) Effect of A 967079 (1 μM), another structurally unrelated TRPA1 channel antagonist on the peak amplitude of Ca^{2+} traces evoked by AITC (30 μM) in control and inflammatory conditions, as indicated in the figure. Statistical analysis was carried out as in panels (b) and (d). (f) Distribution of the peak amplitudes of the intracellular Ca^{2+} traces measured in individual hDPC cells loaded with fluo-4. Cells were cultured in control (n = 554, measured in six experiments) or inflammatory (n = 506, measured in six experiments) conditions and stimulated with 100-μM AITC. Horizontal lines indicate means ± SD of amplitudes. *P<0.01, significantly different as indicated; Student's t test for independent samples. (g) Concentration-dependent effect of the TRPA1 agonist cinnamaldehyde on hDPCs cultured in control and inflammatory (poly (I:C)) conditions. Data shown are means ± SD (from n = 6 experiments) of the amplitude of $[Ca^{2+}]_{IC}$ elevation.

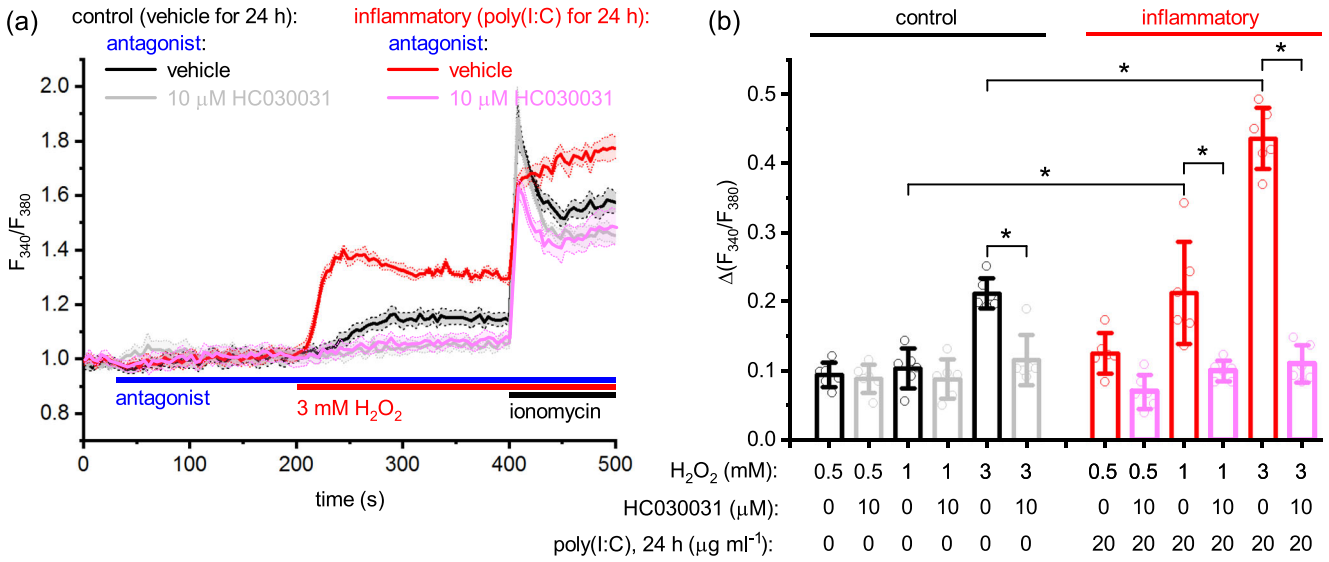


FIGURE 5 TRPA1 channels mediate increased sensitivity towards H_2O_2 in poly(I:C)-induced inflammatory conditions. (a) Averaged time course of $[Ca^{2+}]_{IC}$ records demonstrating the effect of the TRPA1-dependent activation of hDPCs by H_2O_2 in control and inflammatory (pretreated with 20 μg·ml^{-1} poly(I:C) for 24 h) conditions. Compounds were applied as indicated in the figure. Traces represent the means ± SD of 6 records. (b) Statistical analysis on the peak amplitudes of the H_2O_2 evoked Ca^{2+} traces in various conditions showed a concentration-dependent, TRPA1-mediated effect which was significantly amplified in inflammatory conditions induced by poly(I:C). Measurements were carried out as in panel (a). Data shown are the means ± SD from n = 6 records in each group. *P<0.01, significantly different as indicated; one-way ANOVA and pairwise Tukey HSD post-hoc test.

the inflammation-induced damage of mitochondrial viability. We found that poly(I:C) induced mitochondrial damage was partially prevented by the co-application of the TRPA1 antagonists HC-030031 and A 967079 but the cytoplasmic enzyme activity was hardly influenced by these inhibitors (Figure 7c–f). Moreover, the effect of poly(I:C) was markedly reduced following RNAi-based silencing of TRPA1 (Figure 7g,h). We also found a significant interaction between poly(I:C) treatment and A 967079 or siRNA transfection (two-way ANOVA; Figure S3). These results support the conclusion that up-regulated TRPA1 plays role in the inflammation-related damage of the pulpal tissue, although TRPA1-independent mechanisms are also involved.

4 | DISCUSSION

The sensitivity of the dental pulp is primarily related to the innervating trigeminal fibres, but non-neuronal pulpal cells can also significantly contribute to sensory transduction. The sensitivity of the cells is due to various receptor molecules among which TRP channels emerge. Until today, several members of TRP ion channels were reported in the dental pulp and the innervating trigeminal fibres, but their exact role in pulpal inflammation is not fully understood (Hossain et al., 2019; K. Lee et al., 2019).

In our study, we focused on the non-neuronal elements of the human pulp and systematically investigated the expression of thermosensitive members of the TRP family in primary hDPCs cultured in normal and inflammatory conditions induced by the application of poly(I:C), a potent activator of the pattern recognition receptor TLR3. In control conditions, we identified a marked expression of the warm-sensitive channels TRPV1, TRPV2, and TRPV4, the cold-sensitive channel TRPC5, and TRPA1 channels, which are considered to be sensitive to both noxious cold and warm temperature (Moparthi et al., 2016; Vandewauw et al., 2018). TRPM2 and TRPM3 channels were detected only in some of the investigated donors and only at a relatively low level, and TRPV3 was hardly expressed in a few samples. Interestingly, TRPM8 channels, the main cold sensor of the somatosensory fibres (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Paricio-Montesinos et al., 2020; Vriens et al., 2014), was not detected at all in any hDPC samples. Earlier studies investigated simultaneously only a few TRP channels and resulted in some discrepancies between various models. However, in line with our findings, several reports confirmed the expression of TRPV1, TRPV2, TRPV4, TRPC5, and TRPA1 channels in non-neuronal pulp cells in both rodents and human (see Hossain et al., 2019). Similar to our results, TRPM8 channels were not detected in rodent (Son et al., 2009; Yeon et al., 2009) and human odontoblasts (Egbuniwe et al., 2014), although another group reported its functional expression in human odontoblasts (El Karim, Linden, Curtis, About, McGahon, Irwin, & Lundy, 2011) and pulpal fibroblasts (El Karim, Linden, Curtis, About, McGahon, Irwin, Killough, & Lundy, 2011). Immunohistochemical data reported that the TRPM8 channel was expressed in the odontoblast layer but with almost none in the inner pulp of human tooth (Tazawa et al., 2017). This observation might solve the discrepancies, if we assume that TRPM8 expression is restricted to differentiated odontoblasts; then it might not be

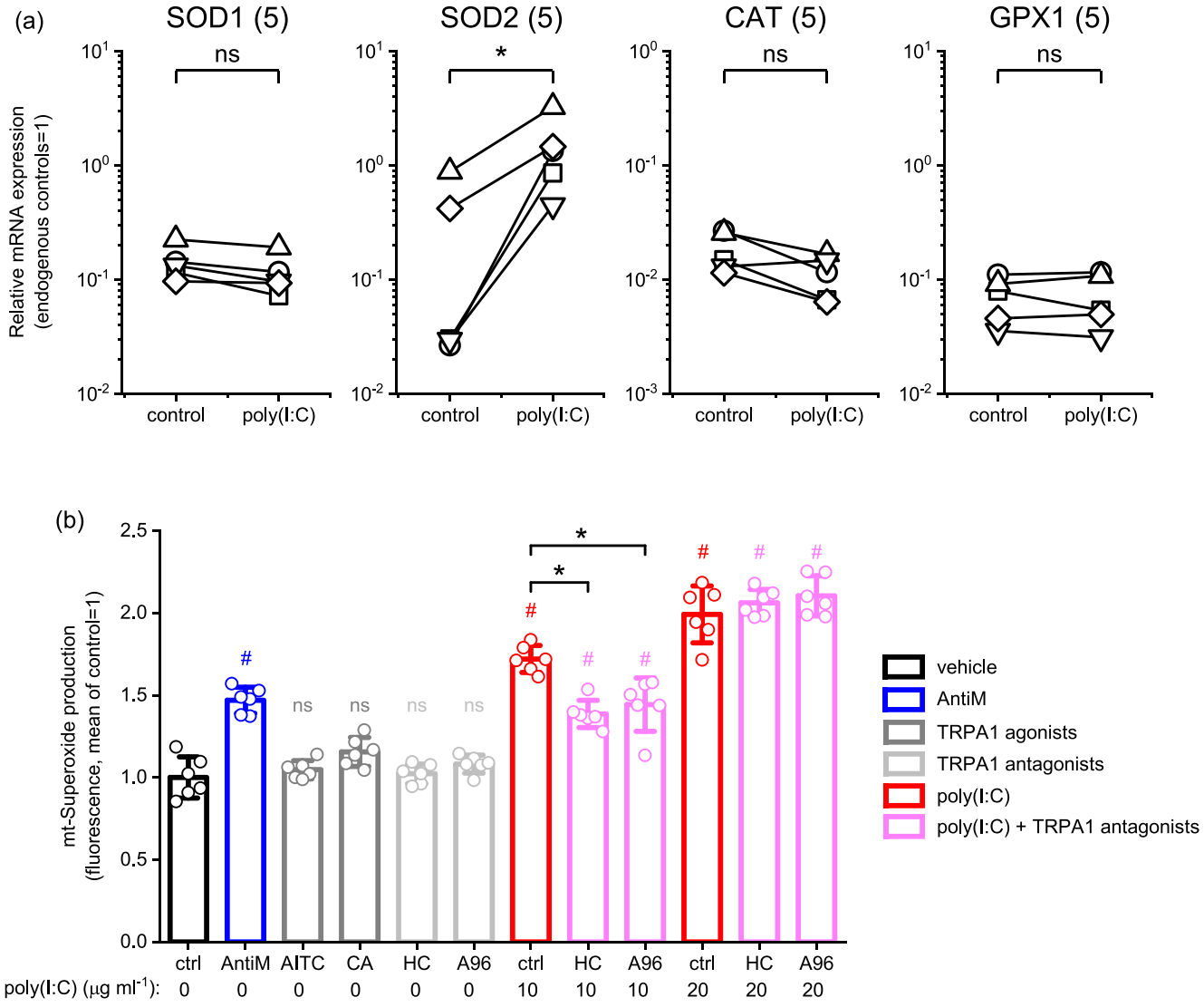
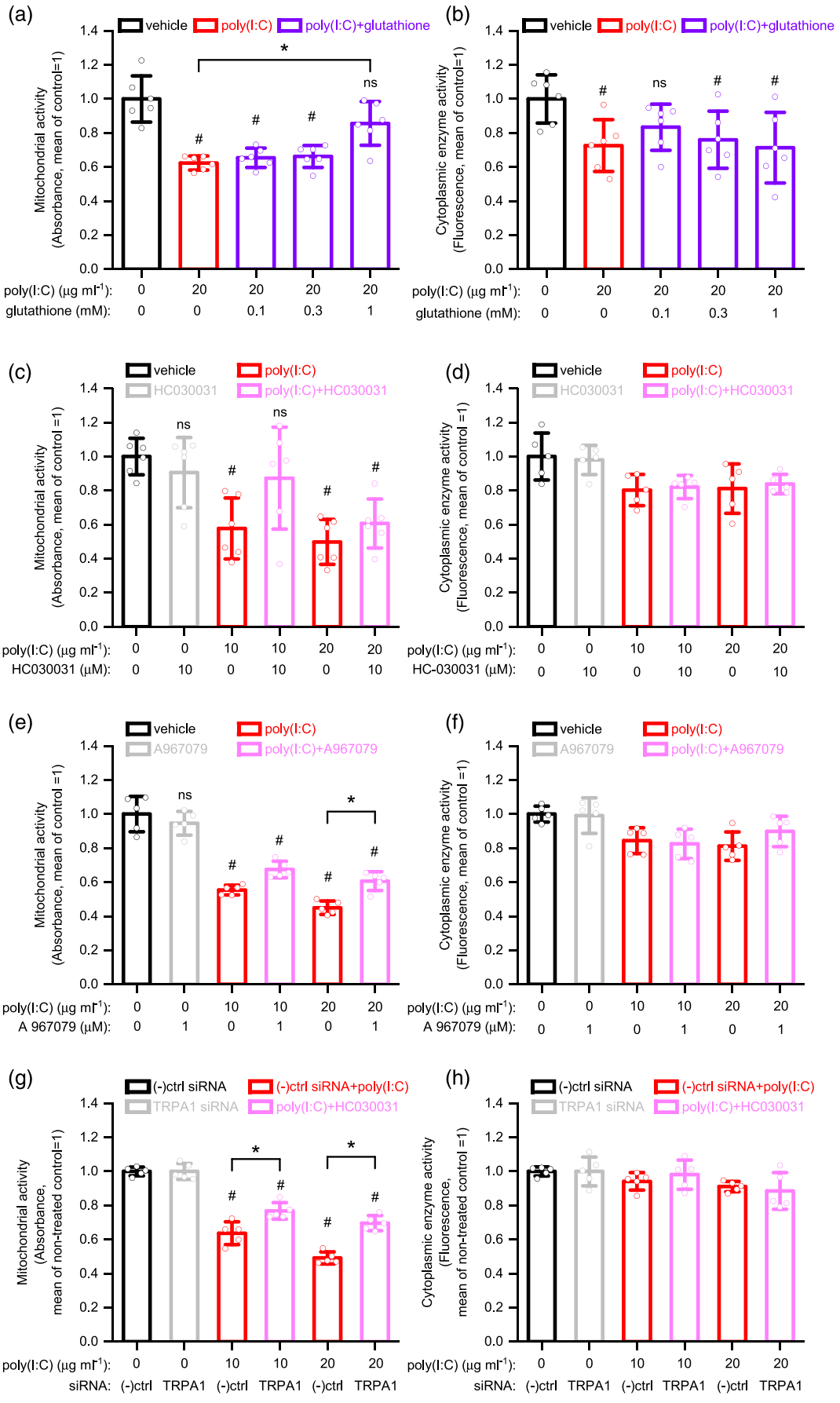


FIGURE 6 Poly(I:C) induces disturbances in redox homeostasis and stimulates mitochondrial superoxide production. (a) Effect of poly(I:C) (20 μg·ml^{-1}, 24 h) on the expression of enzymes involved in the generation and neutralization of reactive oxygen species. Transcripts of cytoplasmic (SOD1) and mitochondrial superoxide dismutase (SOD2), as well as catalase (CAT), and glutathione peroxidase 1 (GPX1) were measured by Q-PCR from the samples of several donors. Different symbols represent different donors within one panel and the numbers of investigated donors are also marked in parentheses in the corresponding panel. *P<0.01, significantly different as indicated; ns, not significant; paired sample t test. (b) Effect of poly(I:C), and TRPA1 channel agonists and antagonists on mitochondrial superoxide production in 60 min. Superoxide production was assayed as described in Section 2. The following treatments were applied, as indicated in the figure: 100-μM AITC (AITC), 300-μM cinnamaldehyde (CA), 10-μM HC-030031 (HC), and 1-μM A 967079 (A96) in the presence or absence of poly(I:C). Antimycin A (AntiM) was used as positive control. Values originally measured in arbitrary unit are normalized to the mean of the vehicle treated control group. Data shown are means ± SD, from n = 6 in each group. #P<0.01, significantly different from the control group, ns, not significant; one-way ANOVA and Dunnett post-hoc test: *P<0.01, significantly different as indicated, one-way ANOVA and Tukey post-hoc test.

detected in proliferating cultures, but species differences and the often questionable quality of the commercially available antibodies against TRP channels (Nilius & Flockerzi, 2014) may complicate the interpretation. Moreover, the TRPM8 channel agonists **icilin** and **menthol** can also activate TRPA1 channels, if applied in higher concentration (Talavera et al., 2020), which makes pharmacological differentiation between TRPM8 and TRPA1 channels a challenging task. In a recent, elegant study, the group of Zimmermann (Bernal et al., 2021) found that the role of TRPM8 channels in cold sensation is rather restricted to the dental primary afferent fibres, and they identified TRPC5 channels as a primary cold sensor in odontoblasts. Moreover, they also demonstrated the contribution of TRPA1 channels to the cold sensitivity of the teeth.

The TRPA1 channel is a multimodal nociceptor sensitive to both thermal and chemical stimuli (Talavera et al., 2020). Several studies have shown it to be generally expressed in the pulp (El Karim



et al., 2015; El Karim, Linden, Curtis, About, McGahon, Irwin, Killough, & Lundy, 2011; Hossain et al., 2019; K. Lee et al., 2019), and we also found a marked expression of TRPA1 in hDPCs. Moreover, in the poly(I:C)-induced inflammatory condition, the expression of TRPA1 transcripts was dramatically (approximately 40-fold) up-regulated in our samples. TRPC5 expression decreased and the expression of other thermosensitive TRPs was not changed significantly.

TRPA1 channels seem to be generally involved in inflammatory signalling in the pulp, as other studies also described its up-regulation, in pulp-derived cells, by **TNFα** (El Karim et al., 2015, 2016; Liu et al., 2020) and bacterial lipopolysaccharide (Tazawa et al., 2020). Although the signalling pathway up-regulating TRPA1 channels is not known in detail, earlier results suggest that activation of NF-κB, a known downstream signalling pathway of TLR3 (Alexopoulouet al., 2001), can contribute to the up-regulation of the channel in our inflammatory model. NF-κB activation by various signals resulted in up-regulation of TRPA1 channels in lung cancer cells (Sakamoto et al., 2023), HaCaT keratinocytes (Luostarinen et al., 2021), allergic contact dermatitis (Kang et al., 2017), and sensory neurons (X. Liu et al., 2023). In fibroblast-like synoviocytes, signalling by inflammatory cytokines, via NF-κB, resulted in a downstream activation of hypoxia-inducible factor-1α (HIF1α) transcription factor which up-regulated TRPA1 expression (Hatano et al., 2012). The clinical relevance of TRPA1 channels in pulpitis is also supported by the findings describing increased TRPA1 immunoreactivity in human carious teeth (El Karim et al., 2015, 2016; Wichaidit et al., 2022). Furthermore, H_2O_2-containing tooth bleaching gel also elevated the expression of TRPA1 channels in dental pulp stem cells (Chen et al., 2021b). Importantly, we found that not only the expression but the specific, functional responses to TRPA1 channel agonists were also highly potentiated in hDPCs cultured in inflammatory condition. In inflammatory conditions, the maximal responses evoked by the TRPA1 agonists were elevated, which is in good accordance with the increased expression of the channel. Moreover, the efficacy of cinnamaldehyde was also elevated as indicated by a decrease in its EC_{50} value, suggesting an additional sensitization of TRPA1 channels. Endogenously produced ROS can contribute to this inflammatory sensitization by reacting with intracellular cysteine residues of the channel (Talavera et al., 2020). However, these reactive residues can serve as targets for additional inflammatory mediators, such as the electrophilic arachidonic acid derivatives (Andersson et al., 2008; Cruz-Orengo et al., 2008). Moreover, **PGE_2** was also shown to sensitize TRPA1 channels via **PKA**- and **PKCε**-mediated pathways (Dall'Acqua et al., 2014). These results indicate multiple connections between inflammatory signalling pathways and TRPA1 channels, which are likely to be present in hDPCs as well.

TRPA1 channels are activated by ROS, and our results clearly demonstrated that inflammatory conditions elevated the sensitivity of hDPCs towards oxidative stress simulated by acute H_2O_2 application. Co-application of a TRPA1 antagonist almost abolished the H_2O_2-evoked Ca^{2+} responses supporting that these oxidative stress-induced Ca^{2+} signals are mediated by TRPA1 channel activity. H_2O_2 is generally applied in tooth bleaching procedures, and it can damage pulpal cells and induce the expression of inflammatory mediators, as well as TRPA1 channels (Chen et al., 2021b; Cintra et al., 2013; Soares et al., 2015). 2-Hydroxyethyl methacrylate, a substance used in dental restorations, was also suggested to activate TRPA1 channels via ROS in an hDPC cell line (Orimoto et al., 2022). Our results have shown that inflamed pulp can be even more sensitive to ROS due to TRPA1 up-regulation.

ROS are released in high concentration and play important roles in inflammation. In pulpitis, ROS are produced by infiltrating immune cells to eliminate pathogenic bacteria but can also damage the surrounding tissue (Cooper et al., 2014). Moreover, dental pulp cells can also produce ROS (Shirawachi et al., 2022). We found that poly(I:C) stimulation also increased mitochondrial superoxide production of hDPCs which was associated with diminished mitochondrial activity and decreased cellular viability within 24 h. Earlier studies suggested that the manipulation of the pulpal ROS signalling may be a promising therapeutic target to prevent inflammation-related tissue damage. ROS scavengers inhibited inflammatory signals and prevented the damage of pulpal cells in various models (Chen et al., 2021a; Jiang et al., 2015; N.-H. Lee et al., 2011). In our study, glutathione also inhibited the inflammation-associated mitochondrial dysfunctions indicating that oxidative stress plays an important role in poly(I:C) induced damages. Moreover, we targeted the ROS-sensitive TRPA1 channels and found that inflammation-associated cellular damage was partly inhibited in hDPCs by antagonizing the channels or silencing TRPA1 expression. These results clearly indicate that inhibition of TRPA1 channels could provide a promising therapeutic approach to reduce the pulpal damage induced by inflammation and oxidative stress.

FIGURE 7 Poly(I:C) decreases mitochondrial activity which involves oxidative stress and TRPA1. (a,b) Effect of poly(I:C) and glutathione on the viability of hDPCs after 24-h treatment. Mitochondrial vitality was assessed by measuring the activity of mitochondrial dehydrogenases of living cells by EZ4U assay (a) and the activity of cytoplasmic esterases was investigated by measuring the conversion of calcein-AM to calcein (b) as described in Section 2. (c) Effect of poly(I:C) and HC-030031 on the mitochondrial activity of hDPCs after 24-h treatment, n = 6 in each group. (d) Effect of poly(I:C) and HC-030031 on the cytoplasmic enzyme activity of hDPCs after 24-h treatment. (e) and (f) are the same as (c) and (d), respectively, but 1-μM A 967079 was applied instead of 10-μM HC-030031. In panel (a)–(f), values are normalized to the mean of the vehicle treated control group (black). (g,h) Effect of poly(I:C) and RNAi-based silencing of TRPA1 on the mitochondrial activity (g) and cytoplasmic enzyme activity (h) of hDPCs. Cells were transfected with either non-targeting negative control RNA ((-) ctrl) or with TRPA1 targeting siRNA. Values are normalized to the mean of the corresponding poly(I:C)-free group (black and grey). In each panel, bars and whiskers represent mean ± SD, n = 5 in each group, unless otherwise mentioned. # $P<0.01$, significantly different; ns, not significantly different, from the control group; one-way ANOVA and Dunnett post-hoc test. *$P<0.01$, significantly different as indicated; one-way ANOVA and Tukey HSD post-hoc test.

Previously, the only possible treatment of pulpitis was root canal treatment with complete extirpation of the pulp. It was the only option for tooth preservation to avoid extraction. Still, it resulted in negative effects on the biomechanical characteristics of the remaining tooth structure, compromising the longevity of the tooth.

In the last decade, vital pulp therapies have become more and more utilised and these were initially considered feasible only with a diagnosis of reversible pulpitis preceding the inflammation of the pulp. However, Ricucci's histological examinations proved that clinical diagnosis might not correlate with the histological condition of the pulp (Ricucci et al., 2014). In many cases, in addition to the classic clinical signs and symptoms of irreversible pulpitis, bacterial invasion and inflammatory infiltration are localized only in the area of the pulp horns, and in such cases, a partial or complete pulpotomy can be a more favourable alternative to root canal treatment (Ricucci et al., 2019). However, the success rate of vital pulp therapies have been reported over a wide range, from 31.8% to 100% in different conditions (Ather et al., 2022; Bjørndal et al., 2010; Ricucci et al., 2019). We believe that the application of potent agents that can significantly reduce pulpal inflammation on the resected pulp wound could improve the outcome of these interventions. Our results suggest that TRPA1 channel antagonists and inhibitors of ROS signalling could be promising candidates to reduce pulpal inflammation. Therefore, our findings may promote the development of more effective treatments for pulpal inflammation, thus extending the tools of regenerative endodontics.

AUTHOR CONTRIBUTIONS

Á. Kunka: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); resources (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **E. Lisztes:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **J. Bohács:** Data curation (supporting); investigation (equal); methodology (supporting); resources (equal); writing—review and editing (equal). **M. Racskó:** Data curation (supporting); formal analysis (equal); investigation (equal); visualization (equal); writing—review and editing (equal). **G. Kovalecz:** Conceptualization (supporting); data curation (supporting); resources (equal); writing—review and editing (supporting). **B. Kelemen:** Data curation (supporting); investigation (supporting); writing—review and editing (equal). **E. D. Tóth:** Data curation (supporting); resources (supporting); writing—review and editing (supporting). **C. Hegedűs:** Data curation (equal); resources (equal); writing—review and editing (equal). **K. Bágyi:** Conceptualization (supporting); data curation (supporting); resources (equal); writing—review and editing (equal). **R. Marincsák:** Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **B. I. Tóth:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); methodology (equal); project administration (equal); supervision (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). All authors gave their final approval and agree to be accountable for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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 and Analysis and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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