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Polysulfides derived from the hydrogen sulfide and persulfide donor P* inhibit IL-1β-mediated inducible nitric oxide synthase signaling in ATDC5 cells: are CCAAT/enhancer-binding proteins β and δ involved in the anti-inflammatory effects of hydrogen sulfide and polysulfides?

Modesta Trummer [a,b], Erwan Galardon [c], Bernd Mayer [b], Günter Steiner [a,d], Tanja Stamm [a,e], Burkhard Kloesch [a,b,*]

[a] *Ludwig Boltzmann Institute for Arthritis and Rehabilitation, 1090, Vienna, Austria*
[b] *Department of Pharmacology and Toxicology, Institute of Pharmaceutical Sciences, University of Graz, 8010, Graz, Austria*
[c] *UMR 8601, LCBPT, CNRS-Université de Paris, 75270, Paris, France*
[d] *Division of Rheumatology, Department of Internal Medicine III, Medical University of Vienna, 1090, Vienna, Austria*
[e] *Section for Outcomes Research, Center for Medical Statistics, Informatics and Intelligent Systems, Medical University of Vienna, 1090, Vienna, Austria*

A B S T R A C T

Hydrogen sulfide (H_2S) emerged as an essential signaling molecule exerting beneficial effects in various cardiovascular, neurodegenerative, or musculoskeletal diseases with an inflammatory component, such as osteoarthritis. These protective effects were initially attributed to protein S-sulfhydration, a posttranslational modification of reactive cysteine residues. However, recent studies suggest that polysulfides and not H_2S are responsible for S-sulfhydration. To distinguish between H_2S and polysulfide-mediated effects in this study, we used the slow-releasing H_2S and persulfide donor P*, which can be decomposed into polysulfides. The effects of P* on IL-1β-induced inducible nitric oxide synthase (iNOS), a pro-inflammatory mediator in osteoarthritis, were determined by nitrite measurement, qPCR, and Western blotting in the murine chondrocyte-like cell line ATDC5. Decomposed P* significantly reduced IL-1β-induced iNOS signaling via polysulfides, independently of H_2S. In line with this, the fast-releasing H_2S donor NaHS was ineffective. In RAW 264.7 macrophages, similar results were obtained. P*-derived polysulfides further diminished IL-1β-induced CCAAT/enhancer-binding protein (C/E BP) β and δ expression in ATDC5 cells, which might play a critical role in P*-mediated iNOS decline. In conclusion, our data support the view that polysulfides are essential signaling molecules as well as potential mediators of H_2S signaling. Moreover, we propose that C/E BPβ/δmight be a novel target involved in H_2S and polysulfide-mediated anti-inflammatory signaling.

1. Introduction

Osteoarthritis (OA) represents the most common form of arthritis. Pain and loss of function, accompanied by progressive cartilage destruction, form the hallmarks of this degenerative disease. The loss in cartilage results from the imbalance between the synthesis and degra-dation of the extracellular matrix [1,2]. During OA progression, chon-drocytes, but also mononuclear cells, osteoblasts and synovial tissues, actively secrete several inflammatory mediators, with interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) being the most critical [3,4]. Both cytokines induce the expression of inducible nitric oxide synthase (iNOS) [5,6], resulting in the excessive nitric oxide (NO) production found in OA [7–9].

iNOS, in contrast to endothelial (eNOS) and neuronal NOS (nNOS), is only expressed when the cells are induced or stimulated. Upon induc-tion, iNOS generates significant amounts of NO until the enzyme is degraded. iNOS-related NO production is primarily governed by tran-scriptional regulation. The iNOS promoter region contains binding sites for various transcription factors, including signal transducer and acti-vator of transcription 1α (STAT-1α), CCAAT/enhancer-binding proteins (C/EBP), and most importantly, NF-κB. [10,11] In OA, NO contributes to the pathogenesis by activating matrix metalloproteinases, promoting

chondrocyte apoptosis, and inhibiting cartilage matrix synthesis [12].

Despite the high prevalence of OA, the therapeutic options are still limited and lack efficacy [13]. Balneotherapy in sulfur-rich water belongs to the non-pharmacological treatment options for OA included in the guidelines of the OA Research Society [13]. Several studies demonstrated its beneficial effects, and hydrogen sulfide (H_2S) administration exhibited anti-inflammatory, cytoprotective, anti-catabolic, and antioxidant effects in several *in vitro* and *in vivo* models of OA [14–21]. However, the underlying molecular mechanisms are largely unknown.

The numerous effects of H_2S signaling are primarily attributed to its conversion of reactive cysteine residues into their respective hydro-persulfides (-SSH), the so-called S-sulfhydration [22]. S-sulfhydration generally results in increased reactivity of the targeted protein [23]. However, recent studies suggest that per- and polysulfides rather than H_2S are responsible for S-sulfhydration of proteins [24–27]. These sul-fane sulfur species provide a source for H_2S, but at the same time, H_2S is quickly converted to various sulfane sulfur species [28]. In turn, sulfane sulfur species are versatile biologic mediators themselves, highlighting the importance of distinguishing between H_2S and polysulfide-mediated effects.

It remains challenging to differentiate between H_2S and sulfane sulfur species signaling, despite many available H_2S donors. The sulfide salts sodium hydrogen sulfide (NaHS) and sodium sulfide are the most widely used H_2S donors. However, they hardly reflect endogenous H_2S production, as they release H_2S immediately upon dissolution in water. Additionally, sulfane sulfur species are also formed in NaHS solutions [24]. To address these shortcomings, Artaud and Galardon developed P* as a slow-releasing H_2S and persulfide donor [29]. P* forms a meta-stable persulfide in aqueous buffers without H_2S release. H_2S release occurs only in the presence of thiols (e.g., cysteine or gluta-thione). Thus, in the presence of thiols, P* releases H_2S before decom-posing to persulfides and, subsequently, polysulfides. Regarding the biological effects of this compound, we previously reported that P* decreased IL-6 levels in ATDC5 cells which was mainly dependent on H_2S release. Additionally, P* exerted antioxidative effects via Nrf2 and heme oxygenase-1 (HO-1) induction by polysulfide formation [30]. Polysulfides derived from P* decomposition inhibited caspase-3 activity in HeLa cells, thereby suppressing apoptosis [31].

Although H_2S is known to interact with NO, also in inflammatory processes [32], studies differentiating between H_2S and polysulfide-mediated effects are scarce. Therefore, we assessed the anti-inflammatory potential of P* on IL-1β-induced nitrite production and iNOS expression in the murine chondrocyte-like cell line ATDC5. To distinguish between H_2S and polysulfide-mediated effects, P* was decomposed into polysulfides. In addition, we aimed to elucidate the underlying molecular mechanism of P* on iNOS signaling.

2. Materials & methods

2.1. Compounds and biomaterials

P* was synthesized as described [29] and dissolved in H_2O. For the decomposition of P*, the compound was diluted in cell culture medium, and L-cysteine was added in equimolar concentrations. To evoke H_2S evaporation, the obtained solutions were left uncapped overnight at 37 °C. L-cysteine, NaHS, naphthyl ethylenediamine dihydrochloride, phosphoric acid, sulfanilamide, and sodium nitrite were obtained from Sigma-Aldrich (MO, USA). Anti-iNOS antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), all other antibodies were from Cell Signaling Technology Europe (Leiden, Netherlands). The detailed antibody list can be found in Supplementary Table S3. All other substances were purchased from MedChemExpress (THP Medical Products, Vienna, Austria).

2.2. Cell culture

The murine chondrogenic cell line ATDC5 (ECACC 99072806) was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK). We maintained the cells in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 μg/ ml streptomycin (Life Technologies, Thermo Fisher Scientific, Inc. Waltham, Vienna, Austria) in a 37 °C humidified incubator with 5 % CO_2. For each experiment, cells were seeded in 12 well plates (5×10^4 cells/well) and grown for 48 h following different treatments. For iNOS induction, cells were stimulated for 24 h with recombinant murine IL-1β (10 ng/ml) with or without IFN-γ (100 ng/ml), (Peprotech, London, UK).

The murine macrophage cell line RAW 264.7 (ECACC 91062702) was purchased from Cell Lines Service (Eppelheim, Germany). The cells were maintained at 37 °C and 5 % CO_2 in Roswell Park Memorial Institute 1640 medium supplemented with 10 % heat-inactivated FBS, 50 IU/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Thermo Fisher Scientific, Inc. Waltham, Vienna, Austria). To induce iNOS expression, cells were seeded in 12 well plates (6×10^4 cells/well) and grown for 48 h following stimulation for 12 h with LPS (100 ng/mL) and IFN-γ (10 ng/ml).

2.3. NO production

Nitrite levels in cell culture supernatants were measured by the Griess reaction, as nitrite represents a stable NO product. Griess reagent, consisting of 1 % sulfanilamide in 5 % phosphoric acid and 0.1 %naphthyl ethylenediamine dihydrochloride in distilled water, was added to the same amount of cell culture supernatants. Nitrite concentration was calculated by using a standard curve of sodium nitrite.

2.4. Western blot analysis

Following different treatments, ATDC5 cells were directly lysed in 1-fold Laemmli buffer, treated with a short impulse of ultrasound (15 s), and, subsequently, boiled for 5 min at 95 °C. Equal amounts of protein were separated by SDS-Page on 10 % gels and transferred onto PVDF membranes (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria). After-ward, the membranes were blocked for 1 h with 5 % non-fat dried milk in PBST (1 x PBS containing 0.1 % Tween 20) and incubated with pri-mary antibodies overnight at 4 °C. Following 2 h of incubation with horseradish peroxidase (HRP) coupled anti-rabbit secondary antibody, proteins were visualized using WesternBright ECL or WesternBright Sirius HRP substrate (Advansta Inc., Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) and a GeneGnome detection device (Syngene, Cam-bridge, UK). Densitometric analysis was done with Syngene GeneTool software, and relative density was adjusted to tubulin as the loading control.

2.5. Quantitative polymerase chain reaction (qPCR)

Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) was used for total RNA isolation, including DNAse I sample treatment to eliminate genomic DNA. The quality of RNA was determined by measuring OD_{260}/OD_{280} by UV/Vis spectroscopy using a Nanodrop 2000 spectrophotometer (VWR International GmbH). RNA was transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria). Real-time PCR analysis was performed on a StepOnePlusTM Real-Time PCR System (Thermo Fisher Scientific) using TaqMan® Universal PCR Master Mix with pre-designed TaqMan® Gene Expression Assays (Thermo Fisher Scientific) as follows: C/EBPβ (Mm00843434_s1; accession number: NM_009883.4), C/EBPδ (Mm0078671 1_s1; accession number: NM_007679.4), iNOS (Mm00440485_m1; accession number: NM_010927.3) and Cyclophilin

D (Mm07298544_g1; accession number: NM_026352). Cycling conditions were as follows: 2 min 50 °C, 10 min 95 °C, 40 cycles of 15 s 95 °C and for 1 min 60 °C. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method using cyclophilin D as a reference gene, and lack of amplification was verified in no-template controls.

2.6. Statistics

All experiments reported in this study were performed in duplicates independently at least three times. The data are expressed as mean values ± SD and include all performed experiments. ANOVA with Tukey's *post-hoc* test was used to test for statistical significance. The figures depict only the statistical results of the highest concentrations to improve clarity. All *p*-values can be found in Supplementary table S4. *p*-values less than 0.05 (*) were considered statistically significant. Sta-tistical analysis was performed using GraphPad Prism 9.1.1 (GraphPad Software, San Diego, USA).

3. Results

3.1. Induction of iNOS expression in ATDC5 cells

The chondrogenic progenitor cell line ATDC5 represents an established cell culture model for studying chondrocyte differentiation. To induce iNOS expression, ATDC5 cells were incubated for 24 h with IL-1β (10 ng/ml), IFN-γ (100 ng/ml), or a combination of both. Treatment with IL-1β alone induced iNOS expression, and this effect was enhanced under co-treatment with IFN-γ ($p = 0.1711$), (Fig. 1A). IFN-γ had no effect on iNOS expression in the absence of IL-1β. In good concordance with the increased iNOS expression levels, ATDC5 cells released nitrite in response to IL-1β ($p = 0.0046$), and IFN-γ potentiated IL-1β-mediated increase in nitrite formation ($p = 0.0020$), (Fig. 1B). Nevertheless, for all subsequent experiments, we chose to stimulate ATDC5 cells solely with IL-1 β because this reflects more closely the *in vivo* situation in the OA joint where IFN-γ is scarcely produced, in contrast to highly inflamma-tory diseases such as rheumatoid arthritis (RA). Moreover, stimulation with both cytokines may exert stress to the cells which could affect the reliability of the results.

3.2. P* decreases nitrite production as well as iNOS mRNA and protein expression levels

We previously reported that P* exerted antioxidative effects and decreased IL-6 levels in ATDC5 cells [30]. To further elucidate the anti-inflammatory potential of P*, we examined the impact of P* on iNOS signaling. After 1 h of pre-incubation with P*, followed by 24 h stimulation with IL-1β, P* diminished IL-1β-induced nitrite levels strongly and in a concentration-dependent manner by about 75 % (1 mM, $p < 0.0001$), (Fig. 2A). Moreover, P* also decreased iNOS mRNA and protein expression levels by approximately 85 % (1 mM, $p < 0.0001$), (Fig. 2B and C). To exclude toxic side effects, cell viability after 24 h of P* administration was determined (Supplementary Fig. S1).

To confirm these anti-inflammatory findings, we also incubated RAW 264.7 macrophages for 1 h with P*, followed by 12 h of stimulation with LPS/IFN-γ. P* reduced LPS/IFN-γ-induced nitrite production by approximately 75 % (1 mM, $p < 0.0001$), and iNOS expression by about 50 % (1 mM, $p = 0.0001$), (Fig. 2D and E).

To determine whether P* also diminished nitrite levels when the inflamed status had already been initiated, we incubated ATDC5 cells with P* 1 h before (pi), simultaneously (sim), or after (post) cell stimulation. Indeed, P* at 1 mM similarly reduced nitrite levels and iNOS expression, even when the cells were incubated with P* after cell stimulation (Fig. 3A and B).

Furthermore, we compared the anti-inflammatory potential of P* with that of other H_2S donors. ATDC5 cells were incubated for 1 h with the same concentrations (1 mM) of either P*, NaHS, a commonly used fast-releasing H_2S donor, or GYY4137, a well-characterised slow-releasing H_2S donor following IL-1β stimulation for 24 h. As control compound we used penicillamine, the non-sulfane sulfur-containing parental drug of P*. Neither NaHS nor penicillamine significantly reduced IL-1β-induced nitrite levels, while GYY4137 reduced nitrite levels by approximately 30 % ($p = 0.0292$), (Fig. 3C). The compounds showed similar effects on iNOS protein expression, although the effect of GYY4137 did not reach the level of significance ($p = 0.0830$), (Fig. 3D). For better clarification, we also assessed iNOS mRNA levels in response to P* or GYY4137 administration, which were not altered by GYY4137 ($p = 0.8437$), (Fig. 3E).

3.3. P*-mediated iNOS signaling reduction requires polysulfide formation

Accumulating evidence suggests that not only H_2S but also per- & polysulfides, which are formed upon H_2S release, are important biologic mediators [33]. For this reason, we aimed to clarify whether P* de-creases nitrite levels via H_2S release or by the resulting mixture of per- & polysulfides upon H_2S release. As we previously described [30], P* re-leases H_2S in the presence of thiols, subsequently decomposing to pol-ysulfides, thereby providing a tool to differentiate between H_2S- and polysulfide-mediated effects: Thus, overnight decomposed P* (ON P*, prepared as described in 2.1) only consists of polysulfides, while freshly prepared P* (FR P*) releases H_2S upon dissolution before the inevitable formation of polysulfides. ATDC5 cells were incubated for 1 h with FR P* or ON P* prior to 24 h of IL-1β-stimulation. Interestingly, we observed a similar decline in nitrite formation and iNOS protein levels to approxi-mately 20 % by FR P* vs. 30 % by ON P* ($p = 0.8304$; $p = 0.9602$, respectively), (Fig. 4A and B).

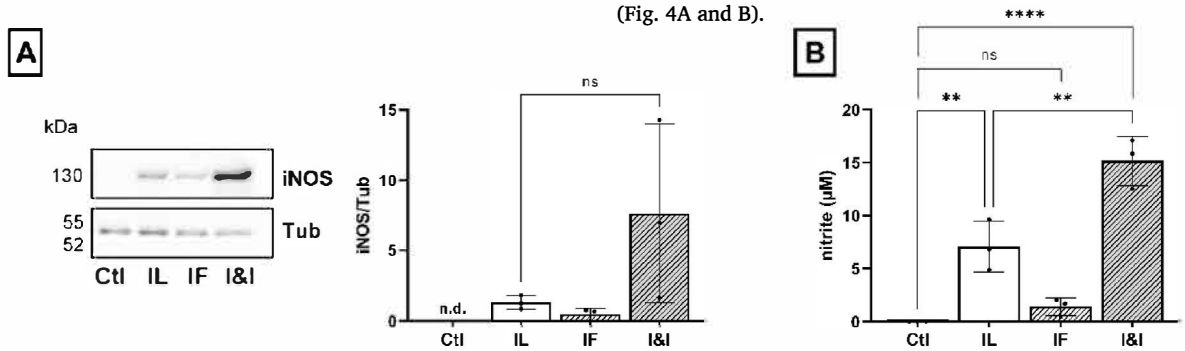

Fig. 1. iNOS induction in response to IL-1β and/or IFN-γ. ATDC5 cells were stimulated for 24 h either with IL-1β (IL, 10 ng/ml) or IFN-γ (IF, 100 ng/ml) or co-stimulated with both (I&I). (**A**) Expression of iNOS was analyzed by Western blotting using α, β-tubulin (Tub) as control. Representative Western blots are shown. Densitometry analysis values were normalized against Tub, n = 3. (**B**) Nitrite production was assessed by the Griess reaction, n = 3.
Ctl = untreated cells, n.d. = non detected, ns = not significant, $^{**}p < 0.01$, $^{****}p < 0.0001$.

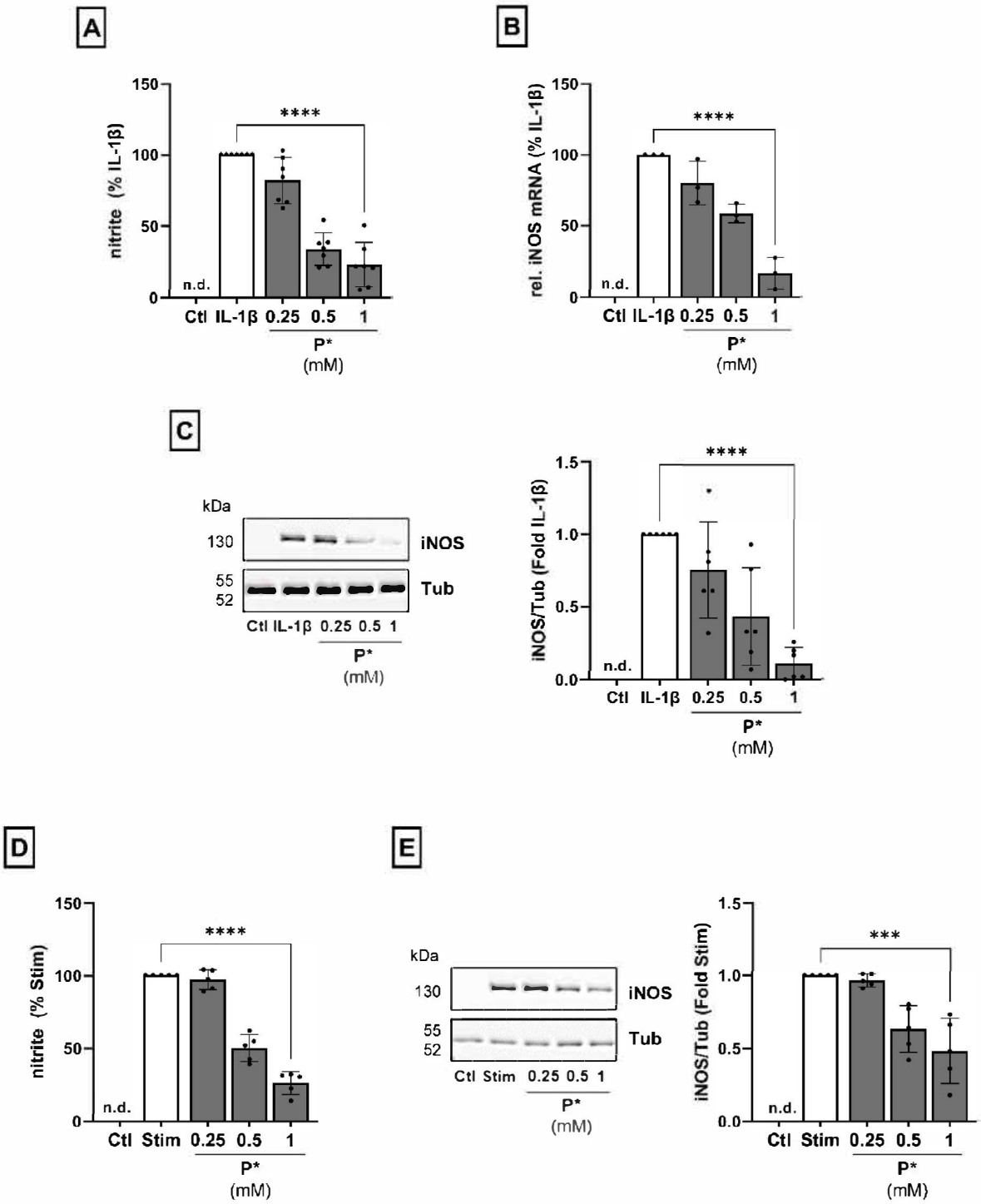

Fig. 2. P* diminishes nitrite production, iNOS mRNA, and iNOS expression levels. (**A-C**) ATDC5 cells were incubated for 1 h with P* followed by 24 h of stimulation with IL-1β (10 ng/ml). (**A**) Nitrite production was assessed using the Griess reagent and normalized to stimulated cells (IL-1β) set to 100 %, n = 6. (**B**) iNOS mRNA levels were determined by qPCR with cyclophilin D as a reference gene and normalized to IL-1β set to 100 %, n = 3. (**C**) Representative Western blots of iNOS and α, β-tubulin (Tub). Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 6. (**D, E**) RAW 264.7 macrophages were incubated with P* for 1 h and stimulated with LPS (100 ng/ml)/IFN-γ (10 ng/ml), (Stim) for 12 h. (D) Nitrite production was assessed using the Griess reagent and normalized to Stim set to 100 %, n = 5. (**E**) Representative Western blots of iNOS and Tub. Densitometry analysis values were normalized against Tub and compared to Stim set to 1, n = 5.
Ctl = untreated cells, n.d. = non detected, $^{**}p < 0.01$, $^{****}p < 0.0001$.

3.4. P decreases iNOS expression independent of HO-1 and p38 MAPK activation*

To clarify the molecular mechanisms of P*-mediated iNOS signaling reduction, we studied the impact of the cytoprotective and antioxidant enzyme HO-1 on NO signaling, as HO-1 exerts anti-inflammatory effects [34,35]. Previously, we demonstrated that P* strongly induced HO-1 expression in ATDC5 cells, which played a role in P*-induced IL-6 in-hibition [30]. ATDC5 cells were incubated for 1 h with or without 10 μM of the HO-1 inhibitor HO-1-IN-1 hydrochloride, then 1 h with P*,

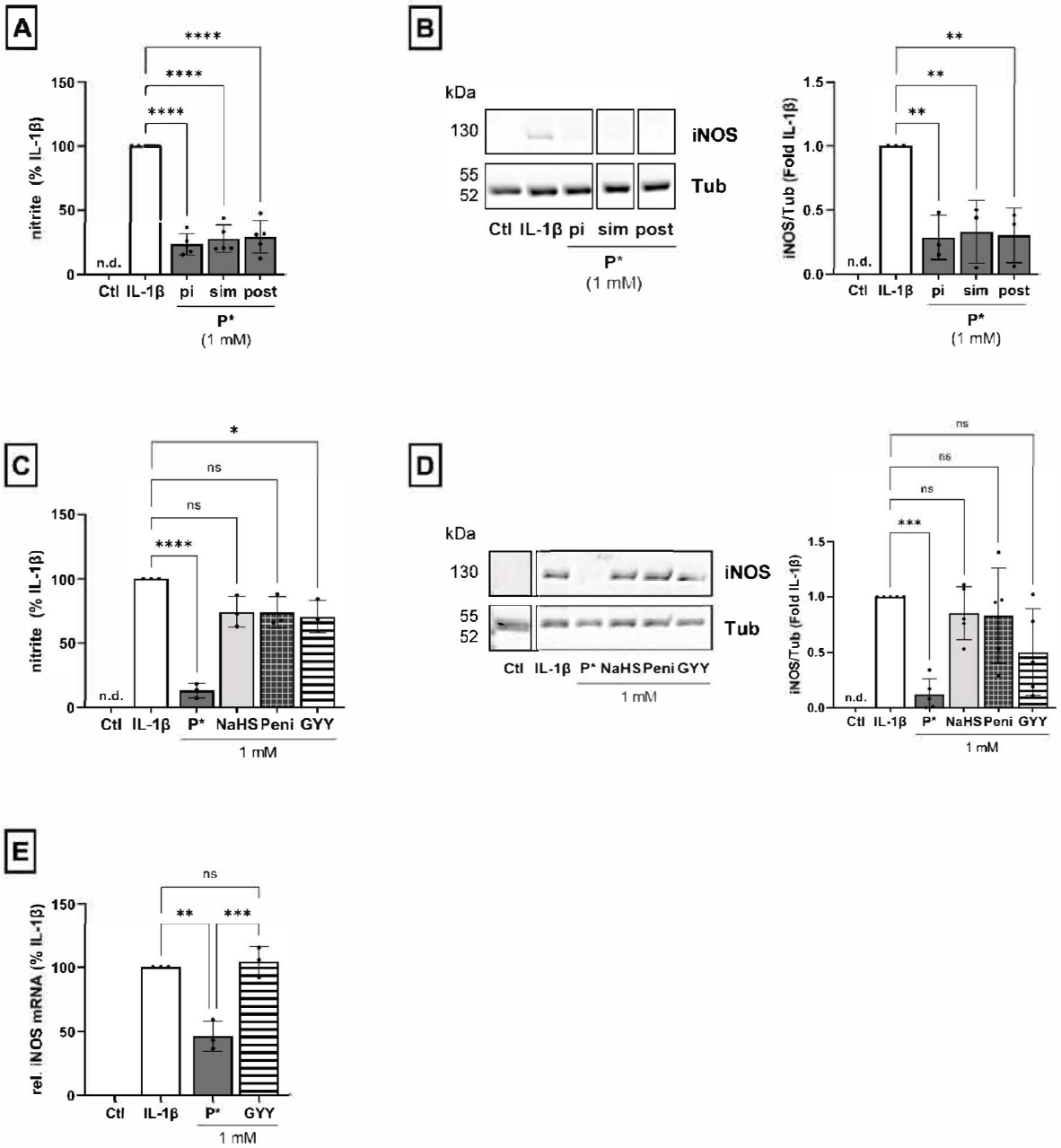

Fig. 3. Comparison of P* to other H_2S donors and different stimulation timepoints. (**A, B**) ATDC5 cells were incubated with 1 mM P* 1 h before (pi), simultaneously (sim), or 1 h after (post) 24 h of stimulation with IL-1β (10 ng/ml). (**A**) Nitrite levels assessed by the Griess reagent were normalized to IL-1β set to 100 %, n = 5. (**B**) Representative Western blots of iNOS and α, β-tubulin (Tub). Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 3. (**C, D, E**) ATDC5 cells were incubated with 1 mM of P*, sodium hydrogen sulfide (NaHS), penicillamine (Peni), or GYY4137 (GYY) for 1 h and afterward stimulated with IL-1β (10 ng/ml) for 24 h. (**C**) Nitrite levels assessed by the Griess reagent were normalized to IL-1β set to 100 %, n = 3. (**D**) Representative Western blots of iNOS and Tub. Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 5. (**E**) iNOS mRNA levels were determined by qPCR with cyclophilin D as a reference gene and normalized to IL-1β set to 100 %, n = 3.
Ctl = untreated cells, n.d. = non detected, ns = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

followed by 24 h of stimulation with IL-1β. With P*, almost identical results on nitrite levels and iNOS expression were obtained, regardless of the presence or absence of HO-1-IN-1 hydrochloride (1 mM: ~85 % *vs.* ~90 % reduction; $p = 0.9889$ and $p = 0.9960$, respectively), (Fig. 5A B) and

Since P* appeared to require p38 MAPK to reduce IL-6 levels in ATDC5 cells [30], we determined whether p38 MAPK might also influ-ence iNOS expression. Surprisingly, pretreatment for 1 h with the p38 MAPK inhibitor SB203580 (10 μM) enhanced nitrite formation by about 35 % ($p = 0.0139$), while iNOS protein levels were not significantly altered ($p = 0.9521$), (Fig. 5C and D). Inhibition of p38 MAPK activity by SB203580 did not alter the effects of P* on nitrite and iNOS expression ($p = 0.1391$ and $p = 0.6121$, respectively), (Fig. 5C and D).

3.5. *P* downregulates the transcription factors C/EBPβ and δ via polysulfides*

Expression of iNOS is primarily controlled by the transcription fac-tors NF-κB, STAT-1α, and C/EBP [10]. In ATDC5 cells, P* had no impact on NF-κB activation [30]. Stimulation with IL-1β for 24 h was not suf-ficient to induce STAT-1α expression (Supplementary Fig. S2), but the expression of C/EBPβ and C/EBPδ was induced. Incubation for 1 h with

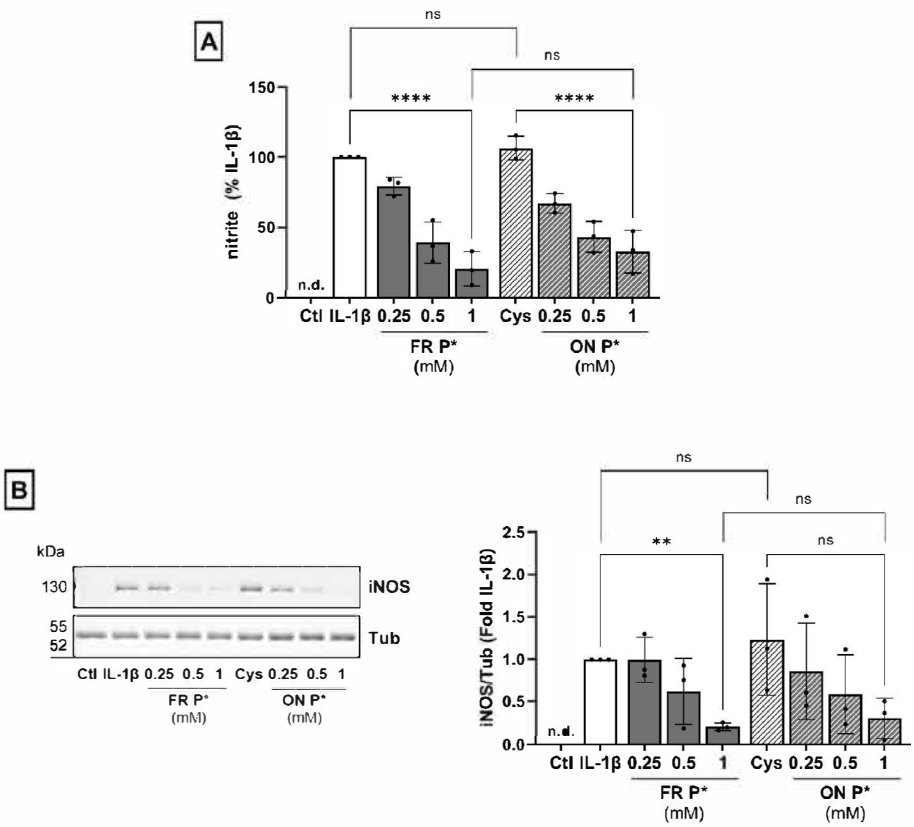

Fig. 4. P* diminishes nitrite formation and iNOS expression levels via polysulfides. ATDC5 cells were treated for 1 h with either freshly prepared (FR P*) or overnight decomposed (ON P*) P* and stimulated for 24 h with IL-1β (10 ng/ml). To decompose P*, respective P* solutions were left uncapped overnight at 37 °C in the presence of L-cysteine (1:1), (Cys). L-cysteine without P* served as a control. (**A**) Nitrite production was assessed using the Griess reagent and normalized to stimulated cells (IL-1β) set to 100 %, n = 3. (**B**) Representative Western blots of iNOS and α, β-tubulin (Tub). Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 3.
Ctl = untreated cells, n.d. = non detected, ns = not significant, $^{**}p < 0.01$, $^{****}p < 0.0001$.

P* following stimulation with IL-1β for 24 h strongly reduced IL-1β-induced C/EBPβ and C/EBPδ expression (1 mM P*: C/EBPβ: ~60 % reduction, $p < 0.0001$; C/EBPδ: ~90 % reduction, $p < 0.0001$), (Fig. 6A and B). In RAW 264.7 macrophages, P* at 1 mM suppressed LPS/IFN-γ-induced C/EBPδ expression by approximately 75 % ($p = 0.0039$), but not C/EBPβ expression ($p = 0.8588$), (Fig. 6C and D).

To gain deeper insights into how P* suppresses C/EBPβ and C/EBPδ expression in ATDC5 cells, mRNA and protein levels were determined after 1 h of incubation with either FR P* or ON P*, followed by 24 h of IL-1β-stimulation. Both FR P* and ON P* reduced IL-1β-induced C/EBPβ-mRNA levels to a similar extent, up to 40 % with FR P* vs. 30 % with ON P* ($p = 0.1582$), (Fig. 6E), as well as C/EBPβ protein levels almost identically (~60 % reduction, $p = 0.9999$), (Fig. 6F). Moreover, C/EBPδ mRNA levels were suppressed by FR P* by ~50 % and, ~45 % by ON P* ($p = 0.9468$), (Fig. 6G). In line with this finding, also C/EBPδ expression was reduced similarly by FR P* (~65 %) or ON P* (~70 %), ($p = 0.9323$), (Fig. 6H).

Since P* suppressed iNOS signaling regardless of the initiation of the inflamed status (see Fig. 3A and B), we further determined C/EBPβ and C/EBPδ expression in ATDC5 cells incubated with P* 1 h before, simultaneously, or 1 h after 24 h of IL-1β-stimulation. As observed with nitrite levels and iNOS expression, P* also significantly diminished C/EBPβ and C/EBPδ expression regardless of the inflamed status initiation (Fig. 7A and B).

Additionally, the potential impact of NaHS, penicillamine, and GYY4137 on C/EBPβ and C/EBPδ expression was assessed. Penicillamine and GYY4137 failed to suppress IL-1β-induced C/EBPβ expression. In contrast, NaHS further augmented IL-1β-mediated C/EBPβ induction by 40 % ($p = 0.0354$), (Fig. 7C). Regarding C/EBPδ expression, NaHS and penicillamine administration displayed no effect, while GYY4137 reduced C/EBPδ by 70 % ($p = 0.0003$), (Fig. 7D).

4. Discussion

Aberrantly high iNOS expression associated with excessive NO formation is one of the characteristics of the development and progression of OA. H_2S, in turn, is an established modulator of inflammation. In this study, the slow-releasing H_2S and persulfide donor P* greatly diminished cytokine-induced nitrite formation and iNOS expression in the chondrocyte-like cell line ATDC5 and RAW 264.7 macrophages. Strongly correlating with our findings, the fast-releasing H_2S donor NaHS was reported to inhibit IL-1β-mediated NO formation as well as iNOS mRNA and protein expression in chondrocytes derived from OA patients [17,36,37]. Furthermore, several slow-releasing H_2S donors, including GYY4137, displayed akin inhibitory effects on NO in different *in vitro* and *in vivo* models of OA [14,36,38,39]. Remarkably, P* proved to be substantially more effective than NaHS and GYY4137 in this study.

Despite the accumulating evidence of the importance of polysulfides, we are not aware of any study investigating the impact of polysulfides on NO production and signaling in chondrocytes. Herein, we demonstrate that P* diminished IL-1β-induced iNOS signaling via polysulfides in ATDC5 cells. Previous studies examining the effect of garlic-derived polysulfides or N-acetylcysteine (NAC)-polysulfides in RAW 264.7 macrophages also observed a decline in NO signaling in response to polysulfide administration [40–42]. These data indicate that poly-sulfides, not H_2S, are responsible for the reported inhibition of iNOS signaling by H_2S donors.

Since it is becoming increasingly evident that polysulfides, rather than H_2S, signal via S-sulfhydration, thereby mainly reducing the target protein's activity [43], a direct S-sulfhydration of iNOS appears conceivable. However, it seems unlikely that P* inhibits iNOS signaling primarily via inhibition of iNOS activity by S-sulfhydration as P* diminished iNOS mRNA levels similarly to nitrite levels. Moreover, iNOS-derived NO formation is mainly regulated by post-/transcriptional modifications, while S-sulfhydration represents a posttranslational modification. Nonetheless, a recent proteomic study identified 994

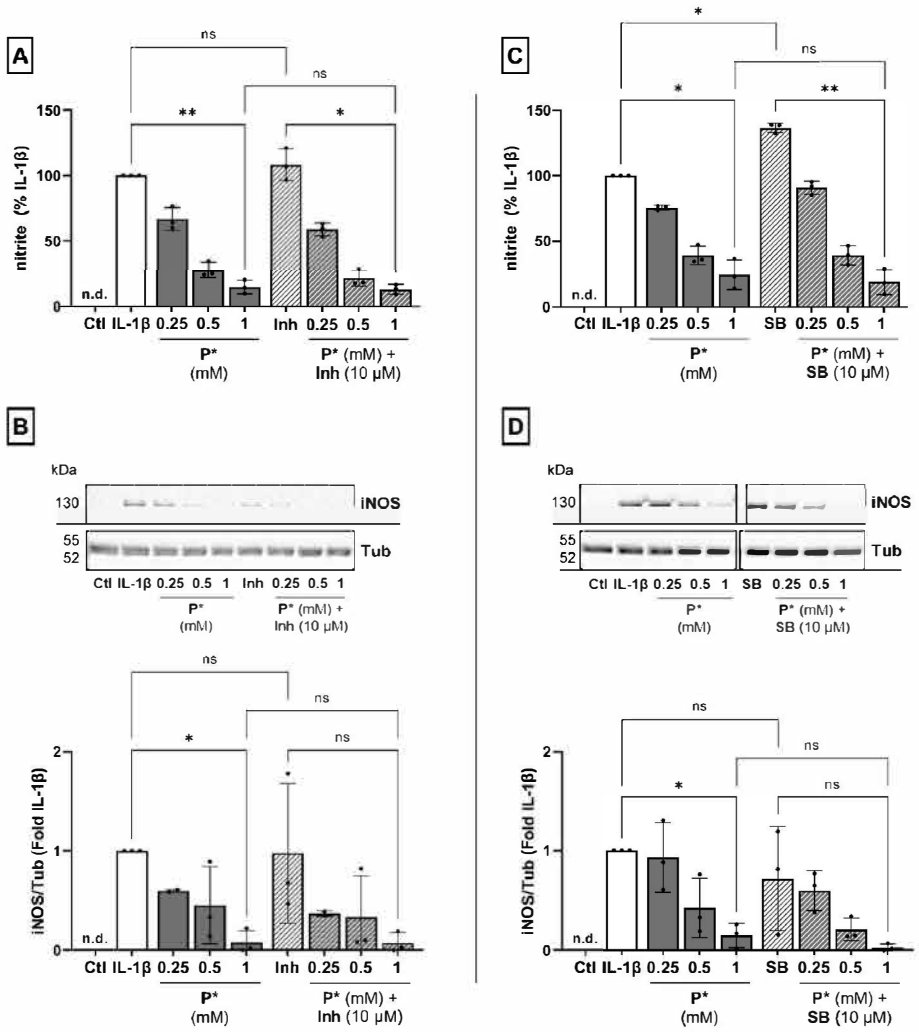

Fig. 5. P* decreases iNOS signaling independent of P*-mediated HO-1 and p38 MAPK activation. ATDC5 cells were incubated for 1 h either with 10 μM HO-1 inhibitor HO-1-IN-1 hydrochloride (Inh), (**A, B**) or 10 μM p38 inhibitor SB203580 (SB), (**C, D**) and subsequently with P* for 1 h, followed by 24 h of IL-1β (10 ng/ml). (**A, C**) Nitrite production was assessed using the Griess reagent and normalized to stimulated cells (IL-1β) set to 100 %, n = 3. (**B, D**) Representative Western blots of iNOS and α, β-tubulin (Tub). Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 3.
Ctl = untreated cells, n.d. = non detected, ns = not significant, $*p < 0.05$, $**p < 0.01$.

S-sulfhydrated proteins, demonstrating the vast amount of S-sulfhydration targets [44]. To date, only eNOS is reported to be an S-sulfhydration target [45], although NaHS inhibited the activity of all three recombi-nant NOS isoforms [46,47]. Further investigations on S-sulfhydration target proteins might eventually reveal an upstream target involved in iNOS signaling.

Nonetheless, P*-derived polysulfides may inhibit iNOS signaling further upstream by directly suppressing inflammatory cytokine production. Glutathione polysulfides were reported to directly inhibit IL-1β transcription in retinal pigment epithelium cells [48], and NAC-polysulfides suppressed LPS-induced IFN-β-and TNF-α-production in RAW 264.7 macrophages [42]. However, other underlying molecular mechanisms likely also contribute to P*-mediated effects since P* attenuated IL-1β-induced upregulation of iNOS signaling regardless of whether P* had been added before or after cell stimulation.

Besides, considering that H_2S and polysulfides interact with NO or nitrite, forming polysulfides and bioactive S/N-hybrid species [49,50], these S/N-hybrid species are presumably formed in response to P* administration which may also affect iNOS signaling. At present, the precise identity of the polysulfide that is responsible for P*-mediated iNOS decline remains unknown.

A vital transcription factor involved in iNOS transcription is C/EBP [10]. In agreement with other studies performed in different cell lines, we found that IL-1β significantly induced the expression of C/EBPβ/δ in ATDC5 cells [51,52]. However, to our knowledge, no studies exist on a possible interaction between C/EBP and/or H_2S/polysulfides in the context of inflammation. Remarkably, P* strongly diminished IL-1β-mediated C/EBPβ/δ mRNA and protein expression levels in ATDC5 cells depending on polysulfides. Moreover, P* suppressed not only iNOS expression when the inflamed status had already been initiated but also C/EBPβ/δ expression. In RAW 246.7 macrophages, C/EBPδ, but not C/EBPβ expression was decreased in response to P*-administration. Given the importance of C/EBP in iNOS transcription, it seems plausible that P* diminishes IL-1β-induced iNOS expression at least partly via C/EBPβ/δ.

Interestingly, GYY4137 also suppressed C/EBPδ expression significantly in ATDC5 cells, but not C/EBPβ expression. In contrast to the slow-releasing H_2S donors, NaHS increased C/EBPβ expression and did not alter C/EBPδ expression. In agreement with our results, garlic-derived polysulfides were reported to decrease C/EBPβ mRNA or expression levels in several models of adipogenesis [53–55], while NaHS did not S-sulfhydrate C/EBPβ or δ [56]. In murine hepatocytes, NaHS also induced C/EBPβ expression [57]. Taken together, these findings indicate that the transcription factors C/EBPβ and δ are involved in H_2S and polysulfide signaling. However, the exact molecular mechanisms are so far unknown.

Multiple C/EBPβ/δ regulation mechanisms have been reported, including gene transcription and translation, as well as phosphorylation and methylation, protein-protein interactions, protein degradation, and autoregulation [58,59]. From these regulation possibilities affecting C/EBPδ, the transcription factors specificity protein 1 (SP1), STAT-3, and Runt-related transcription factor 2 (RUNX2), among others, have

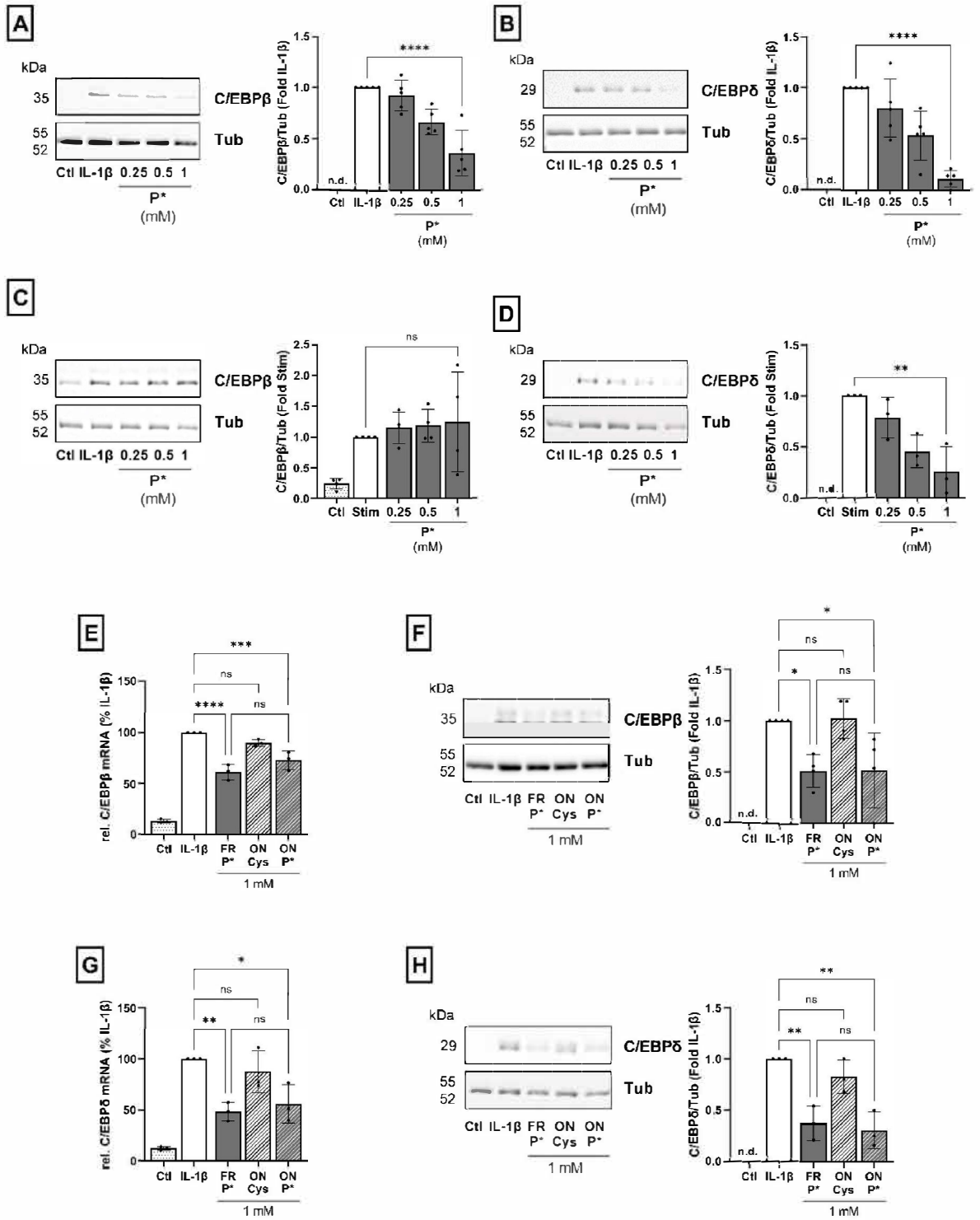

Fig. 6. P* suppresses C/EBPβ and C/EBPδ expression requiring polysulfides. (**A, B**) ATDC5 cells were incubated for 1 h with P*, followed by 24 h of IL-1β (10 ng/ml). (**C, D**) RAW 264.7 cells were incubated for 1 h with P*, followed by 12 h of LPS (100 ng/ml)/IFN-γ (10 ng/ml), (Stim). Representative Western blots of C/EBPβ (**A, C**), C/EBPδ (**B, D**), and respective α, β-tubulin (Tub) are shown. Densitometry analysis values were normalized against Tub and then compared to IL-1β or Stim, respectively, set to 1, n = 3–5. (**E – H**) ATDC5 cells were treated for 1 h with either freshly prepared (FR P*) or overnight decomposed (ON P*) P* and stimulated for 24 h with IL-1β (10 ng/ml). To decompose P*, respective P* solutions were left uncapped overnight at 37 °C in the presence of L-cysteine (1:1), (Cys). L-cysteine without P* served as a control. (**E, G**) C/EBPβ (**E**) and C/EBPδ (**G**) mRNA levels were determined by qPCR with cyclophilin D as a reference gene and normalized to IL-1β set to 100 %, n = 3. (**F, H**) Representative Western blots of C/EBPβ (**F**) and C/EBPδ (**H**) and Tub. Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 3–4.
Ctl = untreated cells, n.d. = non detected, ns = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

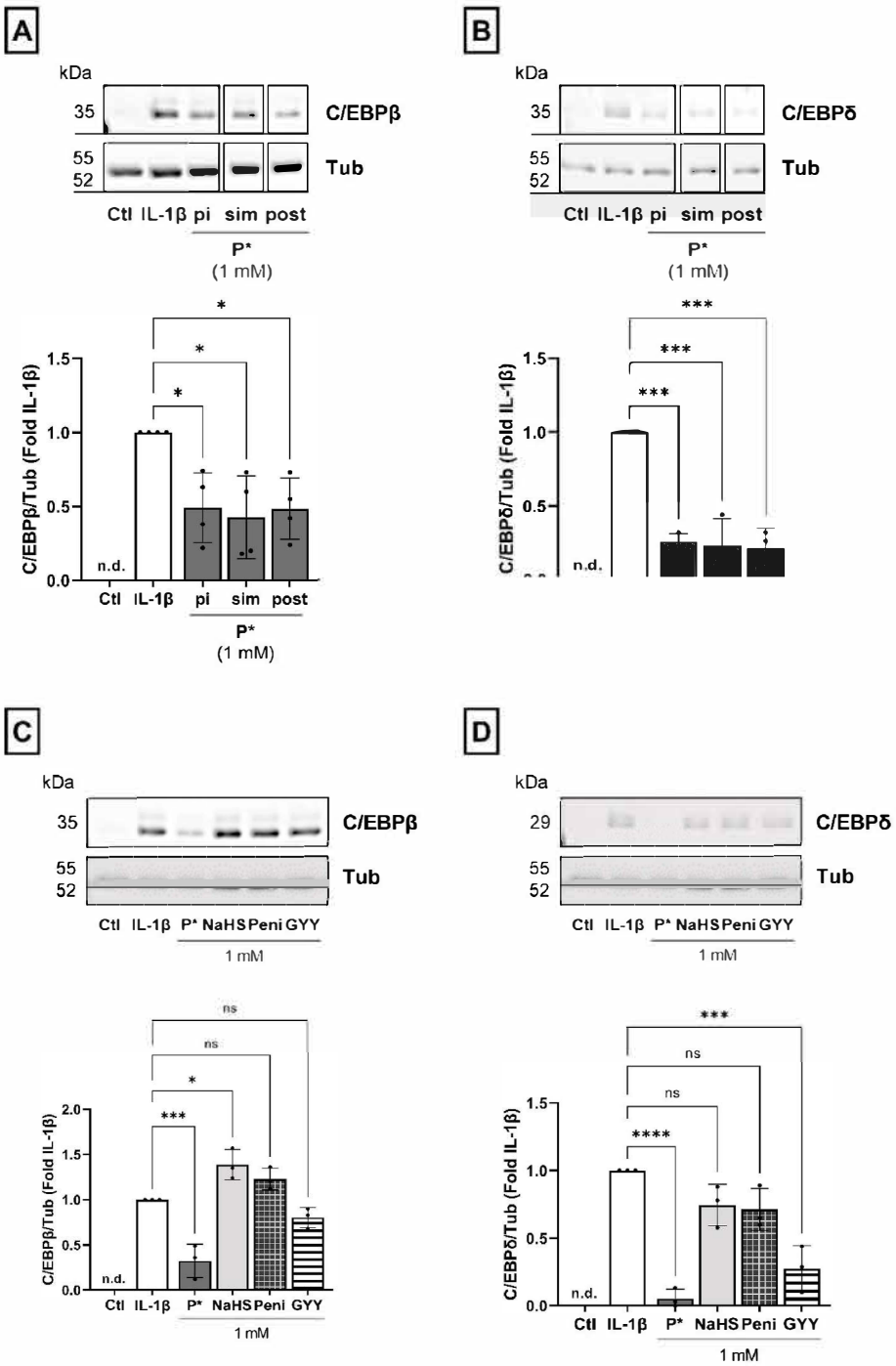

Fig. 7. Impact of different incubation timepoints and other H_2S donors on C/EBPβ and C/EBPδ expression in ATDC5 cells. (**A, B**) ATDC5 cells were incubated with 1 mM P* 1 h before (pi), simultaneously (sim), or 1 h after (post) 24 h of stimulation with IL-1β (10 ng/ml). (**C, D**) ATDC5 cells were incubated with 1 mM of P*, sodium hydrogen sulfide (NaHS), penicillamine (Peni), or GYY4137 (GYY) for 1 h and afterward stimulated with IL-1β (10 ng/ml) for 24 h. Representative Western blots of C/EBPβ (**A, C**), C/EBPδ (**B, D**), and respective α, β-tubulin (Tub) are shown. Densitometry analysis values were normalized against Tub and then compared to IL-1β set to 1, n = 3–4.
Ctl = untreated cells, n.d. = non detected, ns = not significant, $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

been described to be altered by H_2S/polysulfide administration [60–65]. Since these transcription factors play a role in inflammatory signaling and OA development, they offer potential mechanistic targets underly-ing P*-signaling. Notwithstanding, P* may diminish C/EBPβ expression in a calcium-dependent manner. C/EBPβ was found to be activated via an intracellular Ca^{2+}-increase modulated by the activation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) [66]. H_2S, in turn, is known to regulate Ca^{2+}-channels [67], and S-sulfhydration mediated by the H_2S donor S-propyl-L-cysteine reduced the activity of CaMKII [68]. Elucidating the impact of these potential pathways in P*-mediated C/EBPβ/δ-suppression will be the subject of our future research.

Besides, the inhibition of C/EBP signaling offers a promising therapeutic target also in OA. The expression and DNA-binding activity of C/EBPβ and C/EBPδ were found elevated in synovial tissues of patients with OA and RA [69,70]. C/EBPβ promoted hypertrophic differentiation of chondrocytes via cyclin-dependent kinase inhibitor 1C, and both C/EBPβ/δ activated the transcription of osteocalcin gene, a marker of mature osteoblasts [71,72]. Additionally, C/EBPβ was described to be a crucial regulator of chemokine gene expression in response to IL-1β in chondrocytes [73]. Nonetheless, further studies are needed to confirm the possible role of C/EBPβ/δ in the anti-inflammatory effects of P* and the crosstalk between C/EBP and H_2S/polysulfides in OA.

Although ATDC5 cells have been reported to be both a reliable and

effective *in vitro* model for studying NO signaling in chondrocytes [74], it remains essential to validate our findings in other *in vitro* chondrocyte models, particularly in primary chondrocytes obtained from patients with OA or RA. However, we do not expect that comparable potent anti-inflammatory results will be obtained *in vivo* due to the relatively high required concentrations of P* that make pharmacological appli-cation difficult.

Taken together, P* offers a valuable tool for studying not only H_2S, but also polysulfide signaling *in vitro*. Thus our results obtained with decomposed P* support the view that polysulfides act as essential signaling molecules and may be considerably more effective than H_2S. Moreover, we propose that C/EBPβ/δ are novel targets involved in H_2S and polysulfide-mediated anti-inflammatory signaling. Further investigations on this possible interaction might help to better understand the exact mechanisms by which H_2S and polysulfides exert their phys-iological functions. Moreover, C/EBPβ and C/EBPδ regulate numerous target genes, involved in cellular differentiation, proliferation, apoptosis, energy metabolism, and inflammation, and H_2S and poly-sulfides affect the cardiovascular, nervous, and gastrointestinal systems. Thus, the design of novel polysulfide donors based on P* with enhanced pharmacodynamic properties might pave the way for new effective drugs against OA and other diseases involving dysregulation of C/EBP and H_2S signaling pathways.

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Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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