

Effect of hydrogen sulfide sources on inflammation and catabolic markers on interleukin 1 β -stimulated human articular chondrocytes

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Summary

Objective. Hydrogen sulfide (H₂S), the third gasotransmitter together with NO and CO, is emerging as a regulator of inflammation. To test if it might offer therapeutic value in the treatment of osteoarthritis (OA) we evaluated the effects of two exogenous sources of H₂S, NaSH and GYY4137, on inflammation and catabolic markers that characterize OA.

Method. Human chondrocytes (CHs) were isolated from OA tissue. Cells were stimulated with a pro-inflammatory cytokine (interleukin-1 β , IL1 β , 5 ng/ml) and the ability of the two H₂S sources to ameliorate its effects on the cells was tested. Nitric oxide (NO) production was quantified through the Griess reaction. Protein levels of inducible NO synthase (NOS2) and matrix metalloproteinase 13 (MMP13) were visualized through immunocytochemistry (ICC). Relative mRNA expression was quantified with qRT-PCR. Prostaglandin-2 (PGE-2), interleukin 6 (IL6) and MMP13 levels were measured with specific EIAs. NF κ B nuclear translocation was visualized with immunofluorescence.

Results. Both H₂S sources led to significant reductions in NO, PGE-2, IL6 and MMP13 released by the cells and at the protein level. This was achieved by downregulation of relevant genes involved in the synthesis routes of these molecules, namely NOS2, cyclooxygenase-2 (COX2), prostaglandin E synthase (PTGES), IL6 and MMP13. NF κ B nuclear translocation was also reduced.

Conclusion. NaSH and GYY4137 show anti-inflammatory and anti-catabolic properties when added to IL1 β activated osteoarthritic CHs. Supplementation with exogenous H₂S sources can regulate the expression of relevant genes in OA pathogenesis and progression, counteracting IL1 β pro-inflammatory signals that lead to cartilage destruction in part by reducing NF κ B activation.

Keywords. Human articular chondrocytes; Osteoarthritis; Hydrogen sulfide; Inflammation; Metalloproteinase

Introduction

Osteoarthritis (OA) is a degenerative joint pathology that results in severe pain and loss of function. With a high prevalence among the elderly population, it is one of the three most common causes of incapacitation in people younger than 60^{1, 2 and 3}. OA is characterized by a progressive loss of articular hyaline cartilage in the joints and although its complete etiology is not known, it is generally accepted that OA is caused by a deregulation of the catabolic and anabolic processes that regulate cartilage matrix synthesis. Chondrocytes (CHs), the only cell type present in cartilage, are responsible for producing and maintaining the extracellular matrix (ECM) which, normally, has a very low turnover rate. Loss of this equilibrium is not the result of a single mechanism but of a complex interplay between abnormal mechanical stresses, increased amounts of proinflammatory cytokines and/or other insults.

OA has not traditionally been considered an inflammatory disease⁴, but many studies confirm the implication of several pro-inflammatory mediators, such as interleukin-1 β (IL1 β) and tumor necrosis factor- α (TNF- α), in the progression of OA⁵. For instance, elevated IL1 β levels are found in the synovial fluid of OA patients and IL1 β gene expression is upregulated in OA tissue. Additionally, IL1 β activates the synthesis and release of matrix metalloproteinases (MMPs) leading to matrix breakdown⁶.

Nitric oxide (NO) has also been implicated in the pathophysiology of OA. NO is produced from l-arginine by NO synthases. Three isoforms are known, two are constitutively expressed (Neuronal NO, nNOS and endothelial NO, eNOS) and the expression of the third isoform, NOS2, is induced in cellular stress situations and by IL1 β and TNF- α . At normal physiological concentrations NO influences vascular tone, cell adhesion and vascular permeability⁷, but, it can also exert potential toxic effects. Like IL1 β , NO has been shown to inhibit the synthesis of cartilage matrix components. Excess production of NO is also linked to oxidative damage⁸.

In addition to the effects above, OA is also characterized by an increase in PGEs production. PGEs are produced from arachidonic acid in a cascade that involves cyclooxygenases 1 and 2 (COX1 and COX2) which produce the intermediate form PGE H-2. PGE E synthase (PTGES) converts PG (H)-2 to PGE E-2 (PGE-2) downstream of COX1 and COX2⁹. OA cartilage produces high quantities of PGE-2 and PTGES is also upregulated, together with COX2, in IL1 β and TNF- α stimulated CHs^{5 and 9}.

All these factors, probably synergistically¹⁰, contribute to the increase in catabolic processes that lead to cartilage destruction in OA.

Hydrogen sulfide (H₂S) is emerging as a potential regulator of inflammation and it might offer therapeutic value in the treatment of OA. H₂S has been traditionally classified as a toxic gas¹¹ but in the past 10–15 years, scientific opinion has changed as more reports on its biological activity were published and it is now considered as a biologically relevant molecule^{12 and 13}. H₂S is synthesized endogenously in mammals from l-cysteine by at least two pyridoxal-5'-phosphate dependent enzymes, cystathionine β -synthase (CBS) and cystathionine γ -lyase (CTH) and to a lower extent by mercaptopyruvate sulfurtransferase (MPST). Together with NO and CO, H₂S has been identified as a gasotransmitter^{14 and 15} and as such it can permeate cellular membranes without a specific transporter. Low (micromolar) levels of H₂S have been found to be cytoprotective in several disease models, whereas several inflammation animal models have found increased endogenous levels of H₂S (reviewed in^{12 and 13}). With respect to inflammatory rheumatic diseases, Whiteman *et al.*¹⁶ were able to detect H₂S in the synovial fluid from patients with rheumatoid arthritis (RA) and OA as well as in plasma from normal controls (37.6 μ M). Levels from RA patients (62.41 μ M) were significantly increased over those of the OA group (25.1 μ M). This group has also recently shown that human articular CHs and mesenchymal progenitor cells synthesize CBS and CTH, and that their expression was induced by various cytokines, including IL1 β ¹⁷. Therefore, even though its role has not been clearly elucidated yet, evidence suggests that H₂S may have an important function in inflammation and it might be of interest as a therapeutic agent in diseases that have an inflammatory component, such as OA.

In this study, two exogenous sources of H₂S, NaSH (a fast dissolving salt) and GYY4137 (as a slow releasing agent), were added to cultures of IL1 β -stimulated human articular CHs isolated from osteoarthritic tissue. Concentrations of the H₂S sources ranged from 0.05 to 1 mM and their effects on several markers of inflammation and cartilage degradation were investigated. Also, since activation of nuclear factor- κ B (NF κ B) is directly responsible for the upregulation of several proinflammatory cytokines and enzymes, including NOS2, COX2 and IL6^{18 and 19}, this signaling route was investigated as a possible mechanism of action for H₂S.

Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Penicillin/Streptomycin (P/S) were from Gibco (Gibco, Madrid, Spain). Culture flasks and other disposable plastic were purchased from BD (BD Bioscience, Madrid, Spain). Sodium hydrosulfide (NaSH), sodium nitrite, human recombinant interleukin-1 β (IL1 β), trypsin and collagenase were purchased from Sigma–Aldrich (Sigma–Aldrich Química S.A., Madrid Spain). GYY4137 (Morpholin-4-ium 4-methoxyphenyl(morpholino phosphinodithioate)) was purchased from Santa Cruz Biotechnology, Heidelberg, Germany. AlamarBlue[®] was purchased from Invitrogen S.A., Barcelona, Spain. The Griess reagent was from Enzo Life Science, Madrid, Spain. The PGE-2 EIA was purchased from GE Healthcare, Barcelona, Spain and the ELISAs for matrix metalloproteinase-13 (MMP13), and interleukin-6 (IL6) were purchased from R&D systems (Madrid, Spain). Antibodies for NOS2, MMP13 and NF κ B p65 were from Abcam (Abcam plc, Cambridge, UK), Thermo Scientific (Cienytech, A Coruña, Spain) and Santa Cruz Biotechnology, respectively. Reagents for immunocytochemistry (ICC) were from Dako (Dako, Barcelona, Spain) and reagents for molecular biology were from Invitrogen (Invitrogen S.A. Barcelona, Spain), unless otherwise stated.

Tissue selection and cell culture

Osteoarthritic cartilage donors were patients of the Orthopedic Service of the University Hospital A Coruña (CHUAC) that had knee or hip replacement surgery (seven men and six women, age 77.5 \pm 10.0 years old). The local ethics committee approved the study and written informed consent was obtained from all the patients. CHs were isolated by sequential enzymatic digestion from human articular cartilage

as described previously²⁰. Briefly, cartilage sections were washed with saline and minced into small pieces. This was then incubated with trypsin at 37 °C for 10 min, followed by 12–16 h incubation with type IV clostridial collagenase (2 mg/ml) in DMEM with 5% FBS and 1% P/S. Isolated cells were centrifuged and re-suspended in expansion medium (DMEM containing 10% FBS and 1% P/S) and allowed to grow until pre-confluent at 37 °C in a humidified incubator with 5% CO₂. The cells used for the experiments were routinely checked for production of collagen type II and aggrecan to ensure they maintained a chondrocytic phenotype.

Cell stimulation and treatment experiments

Only cells from passage two were used. For all experiments cells were incubated in expansion medium during 24 h for adherence, then the medium was changed to depleted medium (DMEM containing 0.5% FBS and 1% P/S) with the different concentrations, 0 (Basal), 100, 200, 500 or 1,000 μM of one of the sulfide sources (NaSH or GYY4137) and with or without IL-1β (5 ng/ml), for an additional incubation time of either 24 h (cell viability) or 48 h (rest of the experiments).

Cell viability assay

The effect of the sulfide-releasing compounds on CH viability was determined with AlamarBlue[®] cell viability reagent following manufacturer instructions. Cells were seeded ($5 \cdot 10^4$ /well, 96 well plates) and treated as described above. AlamarBlue[®] was added to the cells in a volume equivalent to 10% of the total volume. Reduction of AlamarBlue[®] was measured with a spectrophotometer (Nanoquant Infinite M200, Tecan Ibérica Instrumentación S.L Barcelona, Spain) at 570 nm and with 600 nm as a reference, after 4 h of incubation.

NO production assay

The Griess reaction was used to determine the effects of NaSH and GYY4137 on NO production in IL1β-stimulated CHs²¹. CHs were plated ($5 \cdot 10^4$ /well, 96 well plates) and treated as described above. Culture supernatants (50 μl) were collected for nitrite measurements and mixed with 50 μl of Griess reagent. NO formation was detected by nitrite accumulation using sodium nitrite as standard. Optical density was measured at 570 nm in a Nanoquant Infinite M200 spectrophotometer (Tecan).

NOS2 and MMP13 ICC

The effect of NaSH and GYY4137 on the accumulation of NOS2 and MMP13 proteins in IL1β-stimulated CHs was measured through ICC. Cells were seeded ($3 \cdot 10^4$ /well, 8 well chamber slides), treated as described above and then fixed in acetone at 4 °C for 10 min. The endogenous peroxidase activity was blocked with Dako Real[™] peroxidase blocking solution during 10 min at room temperature. Cells were washed with phosphate buffer solution (PBS) and incubated with either rabbit-antihuman polyclonal NOS2 antibody or mouse-antihuman MMP13 monoclonal antibody. The rabbit/mouse peroxidase/DAB DAKO REAL[™] EnVision[™] detection kit was used to determine antigen–antibody interactions. Slides were dehydrated in graded alcohol, cleared in xylene and mounted in DePeX (Guri[®], VWR International Eurolab S.L., Barcelona, Spain). A negative control was included by omitting the primary antibody in one of the wells. Slides were then visualized in an Olympus Dx61 optical microscope (Olympus España S.A.U., Barcelona, Spain).

NFκB immunofluorescence

For the NFκB immunofluorescence assay cells were seeded ($3 \cdot 10^4$ /well, 8 well chamber slides) and treated as described above, but for only 45 min, to be able to detect NFκB translocation to the cells nuclei and then fixed in paraformaldehyde at 4 °C for 10 min. Cells were then treated with 3% Triton-X for 5 min, washed subsequently with water and PBS. Cell were incubated with the antibody for 3 h in the dark, counterstained with DAPI for 30 min and mounted with Glycergel mounting medium.

PGE-2, IL6 and MMP13 ELISA assays

The effect of the addition of the H₂S sources on PGE-2, MMP13 and IL6 production in IL1 β -stimulated CHs was quantified on cell supernatants using specific enzyme immunoassays, following their manufacturer instructions. Cells were seeded ($5 \cdot 10^4$ /well, 96 well plates) and treated as described above. The calibration curves for PGE-2 EIA, the MMP13 ELISA and the IL6 ELISA spanned from 50 to 6,400 ng/ml, from 33 to 4,200 pg/ml and from 9.4 to 1,200 pg/ml, respectively. Samples were diluted accordingly.

qRT-PCR analyses

The effect of NaSH and GYY4137 on the expression of the genes included in Table I in IL1 β -stimulated CHs was quantified using qRT-PCR. Cells were seeded ($3 \cdot 10^5$ /well, 12 well plates) and treated as described above. Total RNA was isolated using Trizol[®] Reagent, following the manufacturer protocol. RNA concentration was quantified at 260 nm using a NanoDrop[™] spectrophotometer (Thermo Scientific, Madrid, Spain). The A260/A280 ratio was calculated to verify quality and purity. Integrity of RNA was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies Spain S.L., Madrid, Spain). Total RNA was treated with DNase enzyme (Fermentas, Fisher Scientific, Madrid, Spain) to degrade residual genomic DNA. Complementary DNA was synthesized from 0.5 μ g of total RNA using SuperScript[®] VILO[™] Master Mix in a total volume of 10 μ l in a Thermocycler (Gene Amp PCR System 9700, Applied Biosystems, Madrid, Spain). qRT-PCR experiments were performed on a LightCycler1 480 Instrument (Roche, Mannheim, Germany) using Taqman probes (Universal Probe Library set, Roche). Primers and probe assays were designed using Roche Assay Design Center available at www.universalprobelibrary.com. Designed probe assays are included in Table I. PCR reactions consisted of a pre-incubation (95 °C, 10 min) (up to) 45 cycles of amplification including incubation (95 °C 10 s), extension (60 °C, 30 s) and cooling (72 °C, 1 s) and final cooling ramp (40 °C, 20 s). HPRT1 (Hypoxanthine-guanine phosphoribosyltransferase) and TBP (TATA box binding protein) genes were used as the reference genes. These genes were previously selected from a reference gene panel using GeNorm software. Relative levels of expression were calculated using qBase + software (www.BioGazelle.com). Data were normalized against the mean value obtained in unstimulated cells (Basal), which was normalized to 1, and were expressed as relative expression levels.

Table I. Primers and probes used for the qRT-PCR experiments

Gene	RefSeq (mRNA)	Primers sequences	Position	Probe #	Amplicon length (bp)
HPRT1	NM_000194.2	5'-tgatagatccattcctatgactgtaga-3'	434-460	22	127
		5'-caagacattcttccagttaaagtg-3'	535-560		
TBP	NM_003194.4	5'-gccatagtgatcttgcagt-3'	104-124	67	139
		5'-cgctggaaactgctcacta-3'	223-242		
INOS	NM_000625.4	5'-gctccaagctgaaattga-3'	3,562-3,580	68	73
		5'-gatagcgcttctggctcttg-3'	3,615-3,634		
PTGES	NM_004878.4	5'-ctgggatgacagcctgaat-3'	1,237-1,256	83	69
		5'-gactcacatgggagcctttt-3'	1,286-1,305		
COX2	NM_000963.2	5'-cttcacgcatcagttttcaag-3'	707-728	23	96
		5'-tcaccgtaaatatgatttaagtccac-3'	777-802		
IL-6	NM_000600.3	5'-gctgagtacaaaagtcctgatcca-3'	548-571	40	130
		5'-ctgcagccactggttctgt-3'	659-677		
MMP13	NM_002427.2	5'-ccagtcctccgaggagaaca-3'	909-928	73	85
		5'-aaaaacagctccgcatcaac-3'	974-993		

Statistical analyses

All qRT-PCR experiments were performed with cells from $n \geq 3$ biological replicates and duplicate technical repeats. Relative expression values were calculated with qBase + software and are expressed as the geometric mean and 95% lower and upper limit confidence intervals. Statistical analysis (one-way ANOVA followed by a *post-hoc* test) of these results was done with qBase + software too. For the rest of

the experiments, results are expressed as mean and 95% lower and upper limit confidence intervals of at least three biological replicates and duplicate technical repeats. Statistical analyses were performed with R software (version 2.15.2) ²². Data were assayed with one-way ANOVA and a Tukey multiple comparisons test. In all cases, a *P* value lower than 0.05 was considered significant.

Results

Cell viability

Reduction of AlamarBlue[®] values were normalized with respect to the basal condition which was given an arbitrary value of 100%. None of the concentrations of either NaSH or GYY4137 used reduced the viability of the OA CHs (Table II).

Table II. CH viability, as measured with AlamarBlue reagent. Results are mean and 95% confidence intervals (Lower limit, ll, upper limit ul) of *n* = 3 independent experiments performed in duplicate. A Kruskal Wallis rank sum test was performed; for NaSH, *P* = 0.80 and for GYY4137, *P* = 0.78

% Viability Mean (Ll 95% CI, ul 95% CI), <i>n</i> = 3	Basal	IL1 β	H ₂ S compound concentration (μ M)				
			50	100	200	500	1,000
NaSH	100.0 (46.3, 153.7)	106.5 (34.5, 181.4)	107.2 (54.0, 156.7)	103.4 (43.8, 161.2)	104.4 (43.6, 163.1)	100.0 (42.7, 157.7)	98.2 (41.3, 156.1)
GYY4137	100.0 (53.7, 146.3)	106.5 (45.7, 170.8)	98.9 (50.4, 150.5)	94.9 (51.0, 143.2)	93.7 (48.1, 143.5)	98.6 (54.1, 143.8)	99.4 (50.2, 149.4)

Sulfide compounds reduce NO production by downregulating NOS2 gene expression

Basal production of NO (measured as nitrite) ranged from around 0.06 μ M–12 μ M (3.92 (1.64, 6.20), *n* = 7, performed in duplicate). Stimulation with IL1 β (5 ng/ml) increased in production of NO to values that ranged between 23 μ M and 133 μ M (79.85 (63.70, 96.00), *n* = 7, performed in duplicate) [Fig. 1(A) and (B)]. Addition of the different concentrations of H₂S compounds did not induce the formation of NO by itself (results not shown). Both NaSH and GYY4137 induced a dose-dependent reduction on NO production in IL1 β stimulated cells, with values for 500 μ M and 1,000 μ M NaSH and GYY4237 being significantly lower [Fig. 1(A) and (B)].

NOS2 mRNA expression was quantified with qRT-PCR. Stimulation with IL1 β induced NOS2 mRNA expression and the addition of H₂S-releasing agents dose-dependently attenuated this increase, however, none of the values were significantly different from the IL1 β value. For both NaSH and GYY4137, 1,000 μ M was the most effective concentration in reducing NOS2 mRNA expression [Fig. 1(C) and (D)]. Results obtained through the Griess reaction and those of qRT-PCR were corroborated also by ICC [Fig. 1(E)–(M)]. In addition, to ensure that these results were due to the H₂S released from NaSH and GYY4137, we performed these same experiments with decomposed NaSH and GYY4137 (stock solutions were left uncapped in a chemical hood for 10 days). In this case, all effects were lost, no reduction was observed in NO production or NOS2 relative expression from the IL1 β -stimulated values (results not shown).

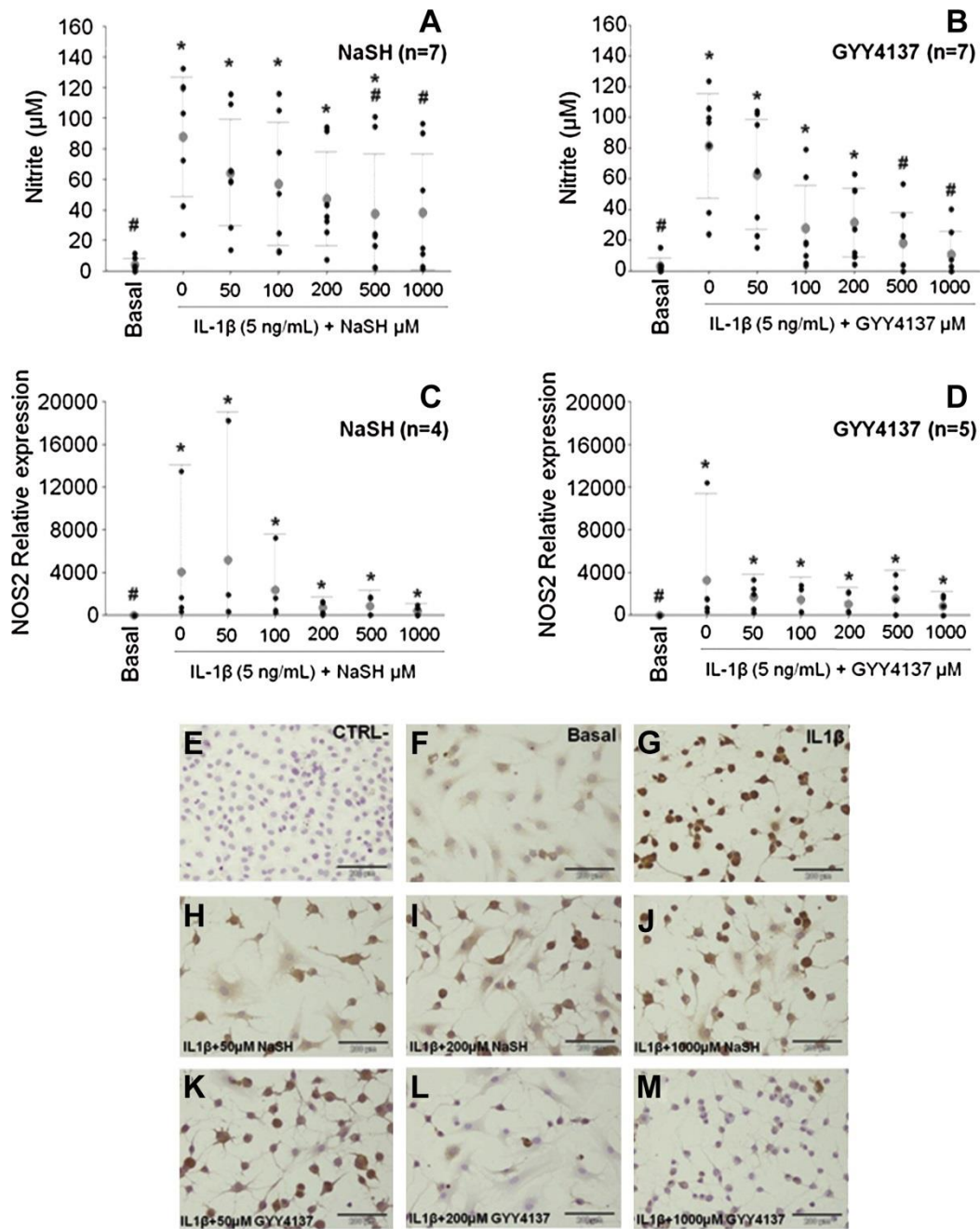


Fig. 1. Addition of NaSH (A, C) and GYY4137 (B, D) to IL1 β -stimulated CHs reduced nitrite accumulation, as measured through the Griess reaction (A and B), NOS2 gene expression, by qRT-PCR (C and D) and NOS2 protein accumulation, by ICC (E–M). Values are mean and 95% CI (Lower limit, upper limit, n number in parenthesis, performed in duplicate). In A and B, Kruskal–Wallis rank sum test was performed followed by multiple comparisons. In C and D, one way ANOVA was performed followed by a Tukey *post-hoc* multiple comparisons test. * $P < 0.0001$ (cf. Basal group); # $P < 0.0001$ (cf. IL1 β stimulated group), except for C where $P = 0.0001$. ICC images include IL1 β -stimulated CHs (G) treated with different concentrations of NaSH (H–J) and GYY4137 (K–M). A negative ICC control (without primary antibody (E)), as well as unstimulated cells (F), were included for comparison.

H₂S compounds reduce PGE-2 levels by downregulating COX2 and PTGES enzymes

PGE-2 basal level was 0.25 (0.13, 0.36) ng/ml ($n = 3$) in the cells. Stimulation with IL1 β led to an increase up to 64.7 (41.78, 87.66) ng/ml PGE-2. Different concentrations of NaSH and GYY4137 were added to the IL1 β -stimulated cells to investigate their effect on PGE-2 production [Fig. 2(A) and (B)]. All concentrations tested, except for 1,000 μ M NaSH and 50 μ M GYY4137, resulted in a significant decrease from the IL1 β -stimulated cells. When looking at the mRNA expression of the main enzymes involved in PGE-2 synthesis, we found a notable dose-dependent reduction in COX2 mRNA expression [

Fig. 2(C) and (D)] from the IL1 β -induced increase. In particular, the IL1 β -induced value was 97.5 (19.5, 487.9) fold vs Basal and 1,000 μ M NaSH and GYY4137 reduced this value to 12.0 (1.6, 89.13) and 7.6 (0.4, 142.1), respectively, $n = 4$. Interleukin-1 β also induced an elevation of PTGES mRNA expression (68.5 (19.8, 236.7) vs Basal) and 1,000 μ M NaSH and GYY4137 considerably reduced this value to 16.3 (3.8, 69.2) and 31.7 (8.3, 120.7), respectively, $n = 4$ [Fig. 2(E) and (F)].

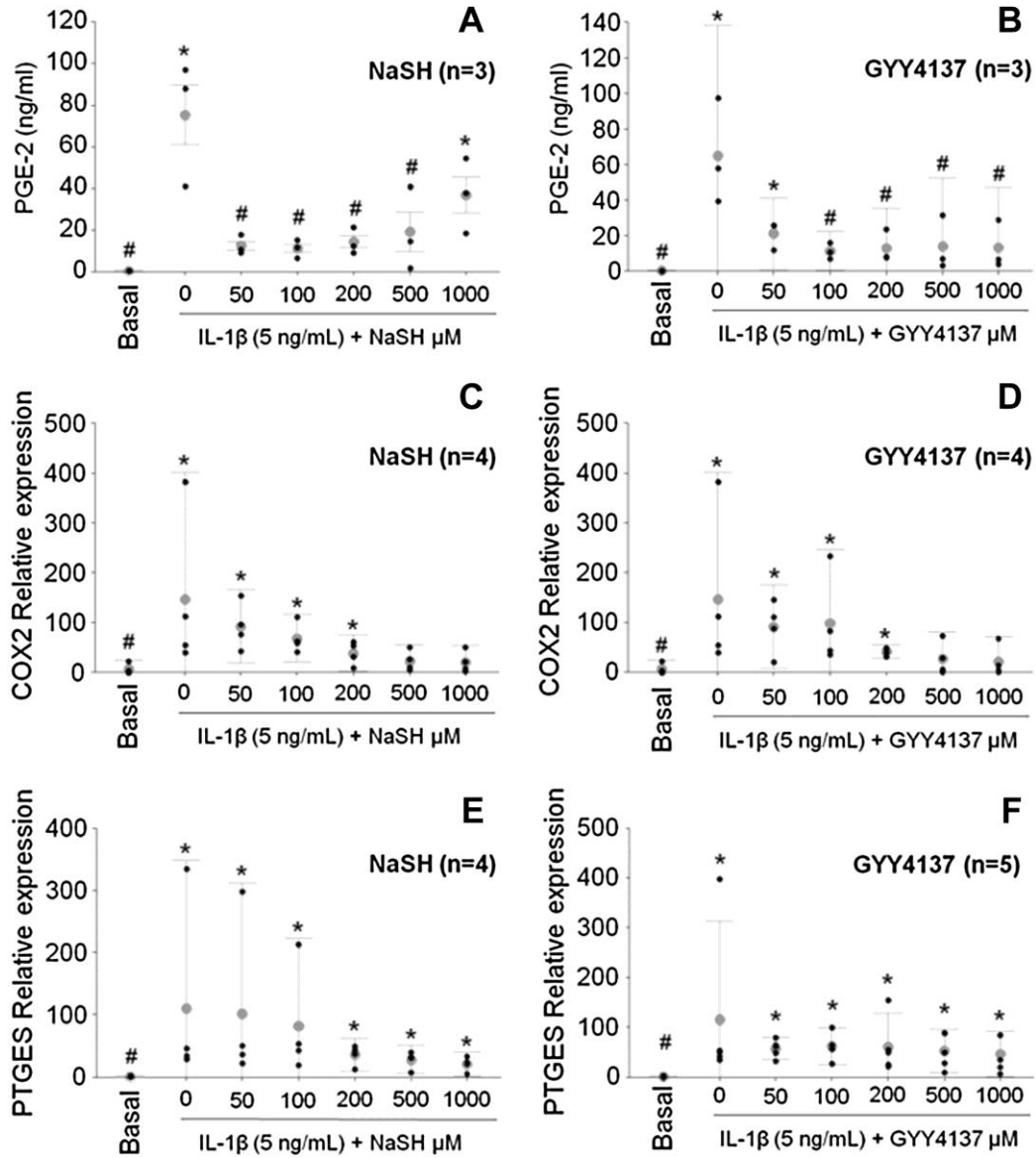


Fig. 2. PGE E-2 production in IL1 β -stimulated CHs declined after treatment with different concentrations of NaSH (A) and GYY4137 (B). Concurrently, COX2 (C, D) and PTGES (E-F) mRNA expression in the cells was also reduced due to the addition of NaSH (C, E) and GYY4137 (D, F). PGE-2 values, measured with a specific EIA are mean and 95% CI (Lower limit, upper limit, $n = 3$). Relative mRNA expression values were calculated, with respect to the Basal condition with qBase + software, n indicated in parenthesis and performed in duplicate. One way ANOVA and *post-hoc* multiple comparisons tests were performed to identify significant differences. In A-C, * $P < 0.001$ (cf. Basal group) and # $P < 0.001$ (cf. IL1 β stimulated group); in D, * $P = 0.003$ (cf. Basal group) and # $P < 0.001$ (cf. IL1 β stimulated group) and in E-F, * $P < 0.0001$ (cf. Basal group) and # $P < 0.0001$ (cf. IL1 β stimulated group).

H₂S compounds also reduce IL6 levels and mRNA expression

IL6 levels were increased due to stimulation with IL1 β (184.3 (146.6, 222.1) ng/ml vs 106 (36, 176) pg/ml in the unstimulated cells). Measured levels decreased in cells co-stimulated with IL1 β and NaSH or GYY4137. The best results were obtained for concentrations 1,000 μ M NaSH (86.2 (65.2, 151.5) ng/ml) and 1,000 μ M GYY4137 (89.1 (57.13, 121.1) ng/ml) [Fig. 3(A) and (B)]. Similar results were obtained for the mRNA expression levels; IL6 expression was also induced by IL1 β (7,743 (3,250, 18,447)) and co-stimulation with the H₂S sources reduced it in a dose-dependent manner. In this case, both 1,000 μ M NaSH and GYY4137 were the best concentrations and their values were significant reductions from the IL1 β value (616.0 (33.2, 11,430) and 543.3 (34.6, 8,540) vs Basal, respectively) [Fig. 3(C) and (D)].

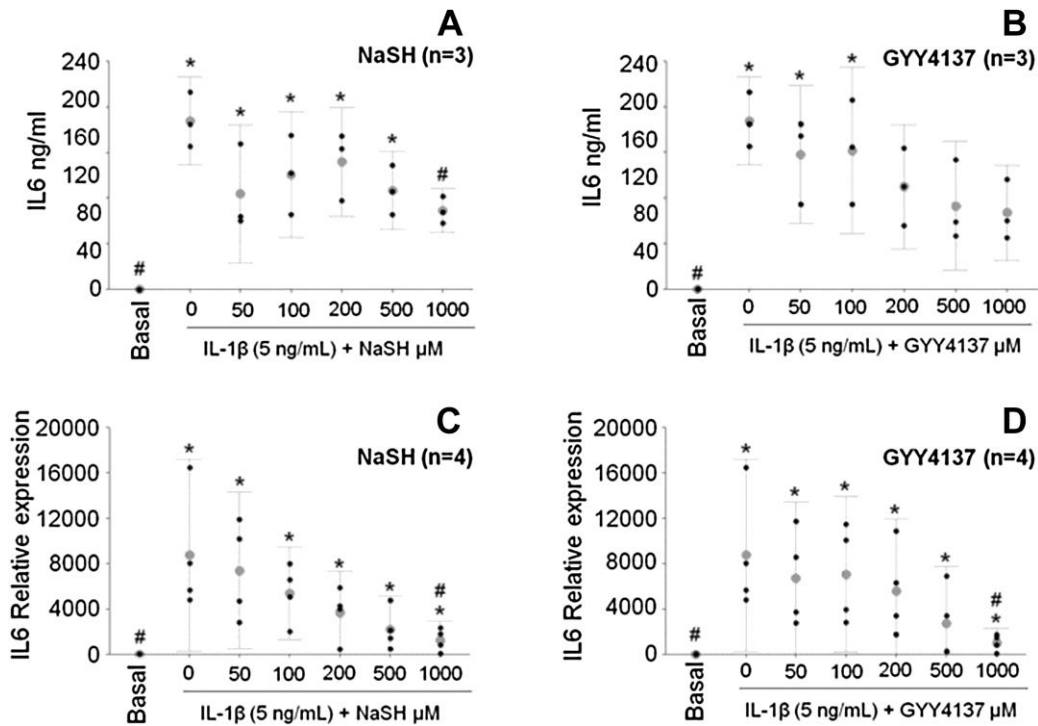


Fig. 3. Interleukin-6 levels (measured with a specific ELISA, A-B) and mRNA expression (by qRT-PCR, C, D) were both reduced in IL1 β -stimulated CHs after treatment with different concentrations of NaSH (A, C) and GYY4137 (B, D). For the ELISA experiments, values are mean and 95% CI (Lower limit, upper limit, $n = 3$, with duplicate measurement). For the qRT-PCR, values were calculated with qBase + software, $n = 4$ with duplicate technical repeats. One way ANOVA was performed followed by a *post-hoc* multiple comparisons tests, except in B where a Kruskal–Wallis rank sum test followed by multiple comparisons was performed. For A, C and D * $P < 0.0001$ (cf. Basal group) and # $P < 0.0001$ (cf. IL1 β stimulated group); for B * $P < 0.001$ (cf. Basal group) and # $P < 0.001$ (cf. IL1 β stimulated group).

H₂S compounds show anti-catabolic properties

We also looked at the effects of H₂S compounds on MMP-13 production. Interleukin-1 β increased MMP13 levels to 44.1 (26.7, 61.6) ng/ml (Basal level was 556 (438, 674) pg/ml, $n = 3$, performed in duplicate) that were subsequently reduced to 33.5 (22.1, 44.9) ng/ml for 1,000 μ M NaSH and 17.3 (9.9, 24.6) ng/ml for 1,000 μ M for GYY3147 [Fig. 4]. Also due to the IL1 β addition, MMP13 mRNA expression increased up to 48.4 (17.7, 132.2) (fold vs Basal) [Fig. 4(C) and (D)]. For both NaSH and GYY4137, 1,000 μ M was the most efficacious concentration in reducing MMP13 expression (down to 12.2 (3.6, 42.0)) for NaSH, and 8.2 (1.68, 40.1) for GYY4137 [Fig. 4(C) and (D)]. Also at the protein level in ICC, MMP13 levels were clearly decreased by co-stimulation with both H₂S-sources [Fig. 4(E)–(M)].

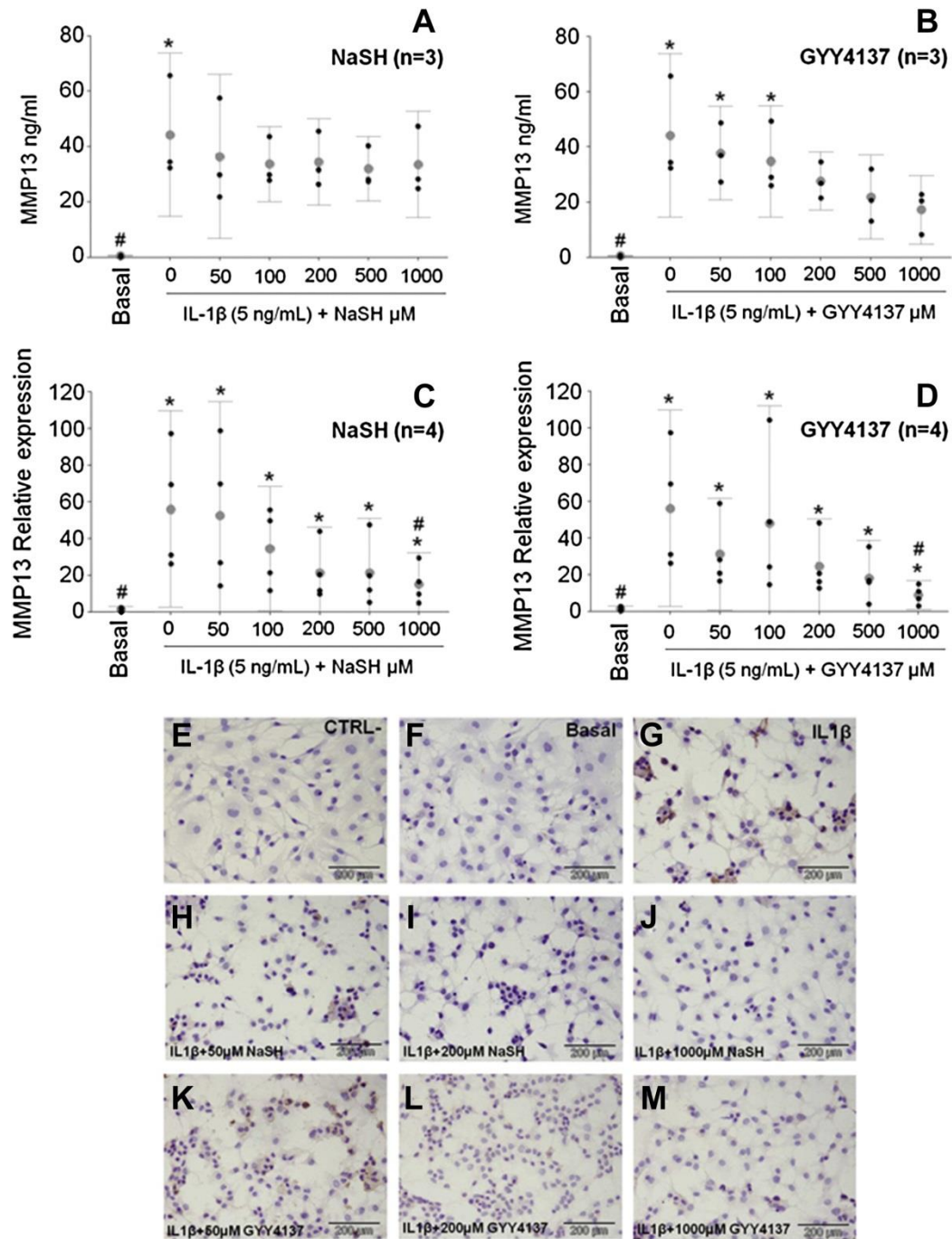


Fig. 4. Addition of NaSH (A, C) and GYY4137 (B, D) to IL1 β stimulated CHs resulted in reduced MMP13 production, measured with a specific ELISA (A and B), reduced MMP13 gene expression by qRT-PCR (C and D), and in less MMP13 protein accumulation, by ICC (E–M). Values are mean and 95% CI (Lower limit, upper limit, $n = 3$, with duplicates). One way ANOVA and a *post-hoc* multiple comparisons test were performed to identify significant differences, except in A where a Kruskal–Wallis rank sum test followed by multiple comparisons was done. ICC images include IL1 β -stimulated CHs (G) treated with different concentrations of NaSH (H–J) and GYY4137 (K–M). A negative ICC control (without primary antibody (E)), as well as unstimulated cells (F), was included for comparison. For B–D $*P < 0.0001$ (cf. Basal group) and $^{\#}P < 0.0001$ (cf. IL1 β stimulated group), and for A $*P < 0.00467$ (cf. Basal group) and $^{\#}P < 0.00467$ (cf. IL1 β stimulated group).

H₂S compounds reduce NFκB p65 translocation to the nucleus

In an attempt to elucidate the possible mechanism by which H₂S might be exerting the observed effects, an immunofluorescence assay was performed to visualize NFκB p65 nuclear translocation in the cells. Results are included in Fig. 5. In the basal condition, p65 was located mostly in the cytoplasm of the cells [Fig. 5(A)]. Stimulation with IL1β resulted in the translocation of p65 to the nuclei of the cells, as can be clearly seen in Fig. 5(B). Co-stimulation with the different concentrations of H₂S compounds resulted in a reduction in the fluorescence intensity in the nuclei [Fig. 5(C)–(G), for GYY4137 and Fig. 5(H)–(L) for NaSH], although p65 could still be seen in the nuclei and the basal condition was not recovered.

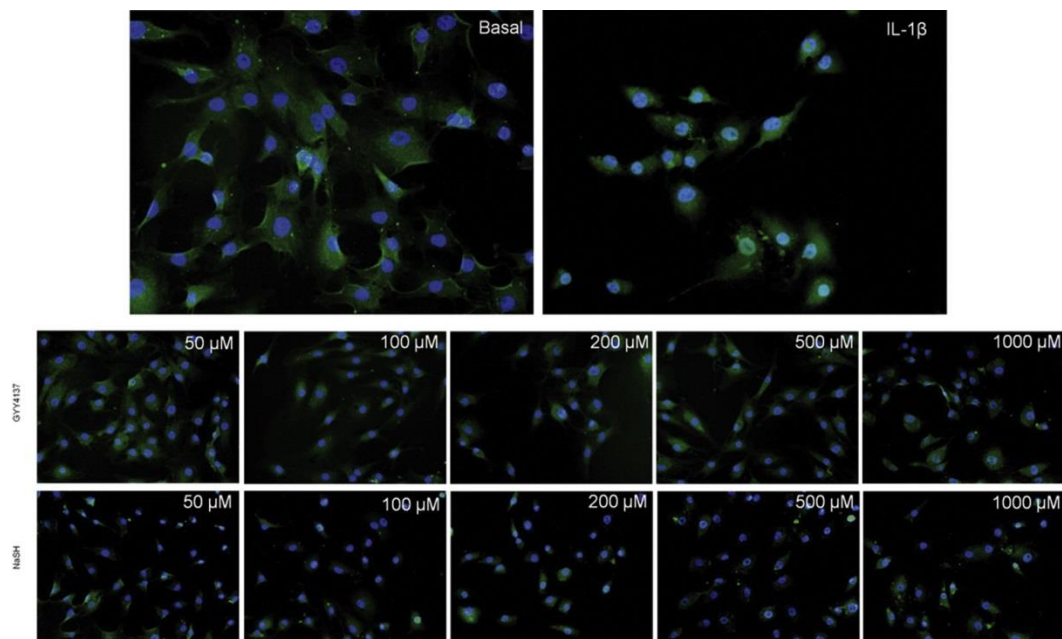


Fig. 5. Immunofluorescence images of NFκB p65 protein in IL1β stimulated CHs (45 min). In the basal condition (no stimulation) p65 can be seen mostly in the cells cytoplasm. IL1β stimulation lead to p65 translocation to the nuclei and the different concentrations of GYY4137 (C–G) and NaSH (H–L) resulted in a slight reduction of the nuclear fluorescence intensity, although p65 can still be seen in the nuclei and the basal situation was not fully recovered.

Discussion

This paper evaluates the ability of two exogenous H₂S sources as anti-inflammatory and anti-catabolic agents in human articular CHs. Interleukin-1β was used as prototype pro-inflammatory cytokine to reproduce the “OA-like effect” in the cells. As expected^{5 and 6}, the stimulation of OA CHs with IL1β induced the production of markers of inflammation and catabolic activity such as NO, PGE-2, IL6 and MMP13.

NO is a multifunctional molecule that mediates various biological processes and is a well-known mediator in OA. It has been reported that OA CHs die by NO-induced apoptosis²¹, that OA CHs produce NO spontaneously²³ and that human articular CHs express NOS2 when stimulated in culture²⁴. Later studies reported that the most direct effect of NO in CHs seemed to be the suppression of energy metabolism²⁵, but it has also been suggested that NO can inhibit matrix synthesis by CHs²⁶. Our results show a dose-dependent reduction of NO levels and the inducible synthesis enzyme, NOS2, with both NaSH and GYY4137. Our GYY4137 results are in line with those recently published for lipopolysaccharide (LPS)-activated hCHs¹⁸ treated with this compound, but those of NaSH contradict a recent publication²⁷ in which its use led to an increase in NO and NOS2 in rat vascular smooth muscle cells. Also, another study described that NaSH had a biphasic effect and at high concentrations (>200 μM) increased the synthesis of NO in a macrophage cell line²⁸. Some authors reported that H₂S can react directly with ROS and reactive nitrogen species (RNS)^{12, 29 and 30} which might account for part of the reduction they observed in NO, but in our case the NO decline is accompanied by a downregulation of the

NOS2 gene and a reduction in the NOS2 protein. Therefore the reduction in NO cannot only be the result of a redox reaction and our results in the present *in vitro* model suggest that the H₂S compounds can directly regulate the NOS2 gene.

The role of H₂S in inflammatory signaling has not been elucidated yet. Many reports attribute H₂S both pro- and anti-inflammatory properties depending on the pathology studied, the experimental model used or the source of H₂S^{13 and 31}.

The effects of NaSH and GYY4137 on the release of pro- and anti-inflammatory mediators in LPS-treated murine RAW264.7 macrophages were recently investigated²⁸. GYY4137 inhibited LPS-induced release of pro-inflammatory mediators IL1 β , IL6, TNF- α , and PGE-2 by increasing the anti-inflammatory chemokine IL-10. In contrast, NaSH had a biphasic effect and at high concentrations (>200 μ M) increased the synthesis of IL1 β , IL6, TNF- α , and PGE-2. On the other hand, reductions in PGE-2 and COX2 levels were also found in LPS-activated normal human fibroblast-like synoviocytes (hFLS) and hCHs¹⁸.

Our results show a dose-dependent reduction in COX2 for both H₂S sources. COX2 is considered the “inducible” COX isoform, activated by many factors, and current anti-inflammatory therapy for OA makes use of COX2 inhibitors. These unfortunately, have other disadvantages, including an accompanying inhibition of physiologic COX1³². In the present results, COX2 inhibition was achieved at the highest H₂S-compound concentration used, without significantly affecting COX1 (results not shown). In addition, our results also show a reduction of PTGES gene expression due to the H₂S co-stimulation. PTGES was demonstrated as a key enzyme in the regulation of PGE-2 production in cytokine stimulated CHs from patients with OA⁹, suggesting that the terminal PG synthase downstream of COX is important for deciding which PG is synthesized, and that the induction of PTGES might be necessary for substantial PGE-2 production by CHs in response to pro-inflammatory stimuli. Therefore, the H₂S-sources can regulate the two most important enzymes implicated in PGE-2 production in activated hCHs.

Furthermore, in our model, IL6 production was stimulated by IL1 β and the co-stimulation with H₂S-releasing compounds both downregulated IL6 gene mRNA expression and reduced IL6 levels produced by the cells. Two different studies have recently showed that NaSH could transiently block IL6 expression, first in RA fibroblast-like synoviocytes³³ and later in a CH cell line C-28/I2³⁴. IL6 can show both pro-inflammatory and anti-inflammatory effects (since, by itself, it can induce the production of tissue inhibitor of metalloproteinase, TIMP, but not MMPs) but IL1-induced IL6 is required for the inhibition of proteoglycan synthesis by IL1 in human articular cartilage³⁵; also IL6 is synergistically responsible for IL1-induced MMPs increases⁶ and IL6 or its receptor are therapeutic targets for OA³⁶. Therefore, the H₂S-sources as used here can further reduce inflammation by regulating IL6 gene expression, resulting in lower free IL6 cytokine released by the cells.

Collagenase 3 (MMP13) is the major type II collagen-degrading collagenase. It is regulated by stress-, inflammation- and differentiation-induced signals. Work by Goldring and colleagues recently reviewed¹⁰ has singled out MMP13 as the key gene upon which many of the signaling pathways (i.e., NF κ B, JNK or p38 MAPK) and transcription factors (such as NF κ B, HIF2 α , Ihh, or Runx2) involved at the different stages of OA converge. Most of these regulatory factors directly or indirectly, impact on MMP13 transcription and activity. In our model, 1,000 μ M of the two H₂S-compounds used, reduced MMP13 below the IL1 β -induced levels at the mRNA and at the protein level also. There are, to the best of our knowledge, no other reports available on the effects of NaSH or GYY4137 on MMP13 activity on human osteoarthritic CHs. However, it has been reported that NaSH (20 min, 1 mM) upregulated the expression of the MMP3 gene in RA-FLS, while MMP3 was not detected at the mRNA level in the OA-FLS³⁷. On the other hand, DAS, an oil-soluble organosulfur compound extracted from garlic, reduced MMP13 synthesis in IL1 β -activated rabbit CHs³⁸.

Additionally, the H₂S compounds used in the present model seem to reduce the translocation of NF κ B to the CHs nuclei, although not to prevent it completely. Activation of NF κ B is directly responsible for the upregulation of several proinflammatory cytokines and enzymes, including NOS2, COX2 and IL6^{18 and 19}, so it is a plausible mechanism of action for NaSH and GYY4137. Nevertheless further studies are needed to confirm H₂S implication in the NF κ B signaling pathway and to investigate what other signaling routes might be implicated.

Collectively, our results show that supplementation with exogenous H₂S taps on important markers in OA and indicate that OA cartilage might benefit from the exogenous supplementation of H₂S. However, the current results obtained *in vitro* might not translate to an *in vivo* situation, and this should be tested directly. Nevertheless, current data should grant further study into the implication of H₂S, both exogenously added and/or endogenously controlled, in OA as a potential therapeutic target.

Author contributions

All authors were involved in drafting or critically reading the manuscript for important intellectual content, and all authors approved the final version. Conception and design: EF Burguera, R Meijide-Failde, FJ Blanco. Data acquisition: Á Vela-Anero, EF Burguera. Analysis and interpretation of data: Á Vela-Anero, J Magalhaes, FJ Blanco. Obtaining of funding: R Meijide-Failde, FJ Blanco.

Competing interest statement

FJB has received Grants (Clinical Trials, conferences, advisor and publications) from: Abbvie, Amgen, Bioiberica, Bristol Mayer, Celgene, Celltrion, Cellerix, Grunenthal, Gebro Pharma, Lilly, MSD, Merck Serono, Pfizer, Pierre-Fabra, Roche, Sanofi, Servier, UCB.

EFB, AVA, JM and RMF declare they do not have any conflict of interest.

No competing financial interests exist.

Funding sources

This work was supported in part through funding from the Fondo Investigación Sanitaria, Madrid, Spain (CIBER-CB06/01/0040; PI12/00329; RETIC-RIER-RD12/0009/0018; and Proteo-Red/ISCIII); Ministerio Ciencia e Innovación PLE2009-0144, FEDER (European Community) and Xunta de Galicia: grant 10 PXIB 310153 PR and Red Gallega REDICENT. The funders did not contribute to data collection, analysis or interpretation of the data, manuscript preparation or submission.

Acknowledgments

Authors want to express their gratitude to the patients that made possible this study and to the Rheumatology and Orthopedic Services of the University Hospital A Coruña (CHUAC) for their help in obtaining the cartilage samples. EFB was supported by Axencia Galega de Innovación (IPP program) and Ciber-BBN/ISCIII (grant no. CB06/01/0040).

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