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Hydrogen sulfide and inflammatory joint diseases

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Abstract

Background: Rheumatoid arthritis (RA) and osteoarthritis (OA) are widespread rheumatic diseases characterized by persistent inflammation and joint destruction. Hydrogen sulfide (H2S) is an endogenous gas with important physiologic functions in the brain, vasculature and other organs. Recent studies have found H2S to be a mediator in inflammatory joint diseases.

Objective: This review summarizes the recent literature in this area highlighting relevant developments.

Conclusions: Several authors have found that H2S exhibited anti-inflammatory, anti-catabolic and/or anti-oxidant effects in rodent models of acute arthritis and in in vitro models using human synoviocytes and articular chondrocytes from RA and OA tissues. The earliest studies used fast-dissolving salts, such as NaSH, but GYY4137, which produces H2S more physiologically, shortly appeared. More recently still, new H2S-forming compounds that target mitochondria have been synthesized. These compounds open exciting opportunities for investigating the role of H2S in cell bioenergetics, typically altered in arthritides. Positive results have also been obtained when H2S is administered as a sulphurous water bath, an option meriting further study. These findings suggest that exogenous supplementation of H2S may provide a viable therapeutic option for these diseases, particularly in OA.

Keywords: Inflammatory joint diseases; hydrogen sulfide; mitochondria; osteoarthritis; rheumatoid arthritis; sulphurous spring waters

1. INTRODUCTION

Rheumatoid arthritis (RA) and osteoarthritis (OA) represent two of the most common arthritides in the developed world. RA, a chronic inflammatory autoimmune disease affecting diarthrodial joints, is characterized by joint swelling, tenderness and eventual destruction of synovial joints, leading to functional impairment and shortened life expectancy. While the etiology of RA is unknown, one hypothesis is that the disease ensues from an environmental trigger in a genetically predisposed individual [1, 2]. From disease onset, an inflammatory infiltrate' of mononuclear cells exists in the synovial membrane, followed shortly by synovitis. In RA, the two types of cells, macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS), contained in the synovial intimal lining become activated. MLS produce large amounts of tumor necrosis factor-a (TNF), interleukin (IL)-lβ, granuloeyte-macrophage colony stimulating factor (GM-CSF) and chemokines, such as IL-8. These factors further stimulate the FLS to become the primary source of mediators contributing to joint destruction. Important among these mediators are matrix metalloproteinases (MMPs) , particularly collagenases, stromelysins and gelatinases, all of which are responsible for joint structural damage, other molecules that enhance the inflammatory response (IL-6, cyclooxygenase (COX)-2, prostaglandin (PG) E_2 or IL-8) and mediators that increase vascular permeability and facilitate angiogenesis [vascular cell adhesion molecule (VCAM)-l, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)]. The interactions between MLS and FLS involve complex cytokine networks that perpetuate synovitis, TNF-α, IL-1β and IL-6 are key cytokines causing inflammation in RA, with TNF-α having the dominant role because it is a potent paracrine inducer of other inflammatory cytokines [3,4].

In contrast, OA is characterized by the progressive loss of articular cartilage resulting from an imbalance of the catabolic and anabolic processes in chondrocytes. OA characteristically has a significant chronic inflammatory state [5]. Local inflammatory molecules contribute to progression of the disease in the early stages, finally resulting in chronic inflammation. These local inflammatory molecules include IL-lβ, TNF-α, IL-6, nitric oxide (NO) and PGE_2. Particularly in OA, IL-1β is a key pro-inflammatory factor (Reviewed in [6-8]).

While there are two receptors for IL-1β, IL-1RI and IL-1RII, only IL-1RI can transduce the IL-lβ signal [9].

The articular chondrocytes (AC) and FLS of OA patients express high levels of IL-1RI (10, 11] and increased numbers of chondrocytes synthesizing IL-lβ and TNF-α occur in OA cartilage [12]. When IL-lβ binds to IL-1RI, several signalling and transcriptional pathways, such as nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), activation of protein kinase (PK) C, p38, extracellular signal regulated kinases (ERK) 1/2 or c- Jun N-terminal kinases (JNK) are initiated [13, 14]. Therefore, in OA, IL-lβ is responsible for the inhibition of anabolism by the reduction in the production of collagen type II and aggrecan and the induction of catabolism by the upregulation of MMPs and aggrecanases in ACs [15]. IL-lβ also participates in the upregulation of PGE_2 synthesis enzymes, COX-2 and microsomal prostaglandin E synthase-1 (PTGES), which leads to increased PGE_2 production [16, 17].

In both RA and OA, reactive nitrogen species (RNS) and oxygen species (ROS) are also important players involved in inflammation. NO, peroxynitrite, H_2O_2 or O_2 cause deleterious effects in cells, including oxidative damage to proteins, membranes and DNA and activation of the cells apoptotic pathways. NO contributes to the enhancement of the activation and production of MMPs, the inhibition of synthesis of anabolic molecules [18] and the promotion of chondrocyte apoptosis in both RA and OA [19]. During energy metabolism, the mitochondrial respiratory chain is the main source of ROS [20]. Evidence that mitochondrial dysfunction contributes to the pathophysiology of inflammatory joint diseases [21, 22] and to a wide range of other pathologies [23] is accumulating.

Hydrogen sulfide (H_2S), a gas detectable by its characteristic odor of rotten eggs, is very toxic in high concentrations. H_2S exerts its toxicity by the inhibition of cytochrome C oxidase and reduction of ATP production [24]. Early studies were concerned with its environmental toxic effects and with measurement techniques for the detection and prevention of the risks associated with human exposure [25]. We have

since learned that H_2S is an endogenous gasotransmitter capable of penetrating cell membranes without a specific transporter. H_2S is produced in most tissues in considerable levels and participates in many physiologic and pathologic events (reviewed elsewhere [26-29]). Evidence is mounting that H_2S plays an important role in inflammation [30, 31] and that exogenous supplementation of H_2S could be of therapeutic value in several diseases [29]. This review ad- dresses the relevance of H_2S supplementation in the context of acute and chronic inflammation as it relates to RA and OA, including current methods for H_2S exogenous administration, as available in current literature.

2. HYDROGEN SULFIDE SOURCES

2.1. Endogenous Synthesis of Hydrogen Sulfide

Endogenous H_2S is chiefly produced by three enzymes, cystathionine β-synthase (CBS, EC 4.2.1.22), cystathonine γ-lyase (CTH, EC 4.4.1.1) and 3-mercaptopyruvate sulfurtransferase (MPST, EC 2.8.1.2). These enzymes, which utilize cystathionine, homocysteine or L-cysteine to produce hydrogen sulfide, are found in various tissues and their expressíon and distribution are tissue specific [28, 32, 33]. CBS is the predominant enzyme in the brain and heart and CTH is mainly expressed in the liver, kidney and intestine, and by vascular smooth muscle cells [32]. MPST has been reported in the brain, liver, kidney and heart [33]. CBS and CTH are exclusively cytosolic enzymes, while MPST is found both in the cytosol and mitochondria of cells [33].

2.2. Endogenous H_2S and Rheumatic Diseases

Articular tissues also express enzymes associated with H_2S synthesis. A recent publication reports that chondrocyte- like cells (chondrogenically differentiated mesenchymal pro- genitor cells, CH-MPCs) and primary human (h) Aes express both CBS and CTH, and that CTH is induced in both cell types by IL-lβ, TNF-α, IL-6 or lipopolysaccharide (LPS) treatment [34]. Our group bas found these three enzymes at mRNA and protein levels in cartilage, synovial membrane and subchondral bone from OA and normal patients [35]. Other studies have shown that synovial fluid (SF) H_2S concentrations in patients with RA [36, 37] or gout [37] were significantly higher than those in paired H_2S plasma values and those in the SF of age-matched OA patients. In addition, one investigator reported that SF H_2S levels of RA patients correlated with the disease activity score in 28 joints and tender joint count [37].

This same study also reported that plasma H_2S levels in RA and gout did not differ significantly from those of healthy controls, while those of OA patients were significantly higher [37]. In contrast, Whiteman *et al.* [36] and similar studies by our group did not reveal any differences in H_2S concentration in serum between OA patients and healthy controls. On the contrary, we have preliminary evidence that indicates that H_2S biosynthesis in OA joints might be reduced [35].

One hypothesis recently promulgated states that when H_2S is increased in inflammatory diseases, it represents an endogenous compensatory mechanism by cells attempting to overcome inflammation, indicating that H_2S may play an anti-inflammatory role [30, 38-40], although further studies are needed to test this hypothesis.

2.3. Exogenous Administration of H_2S

Many of the early studies investigating the inflammatory role of H_2S in *in vivo* models or in cells *in vitro* used fast-dissolving salts, such as Na_2S, NaSH or Lawesson's reagent (LR), to generate and deliver H_2S. These reagents produce an immediate burst of H_2S that lasts a few seconds. However, this brief concentration is likely not a good model for *in vivo* physiologic H_2S synthesis, which may occur in smaller quantities and much more slowly. In 2008, GYY4137 (morpholin-4-ium-4-methoxyphenyl

(morpholino) phosphino-dithioate), a new type of H_2S-delivering compound, was synthesized [41]. This compound slowly produces H_2S both *in vitro* and *in vivo.* Using an endotoxic shock rat model and also in RAW264. 7 macrophages, GYY 413 7 exhibited anti-inflammatory effects [42]. When GYY4137 was administered to conscious rats 1 or 2 h prior to LPS treatment, the ensuing rise in plasma pro-inflammatory cytokines *(*TNF-α, IL-1β and IL-6) and lung myeloperoxidase (MPO) activity was decreased, while plasma concentration of the anti-inflammatory cytokine IL-1O was increased. In macrophages, GYY 413 7 reduced NFKB activation, the expression of the inducible form of NO synthase (iNOS) and COX-2, as well as the generation of PGE_2 and NO-derived nitrites/nitrates [42].

A more recent series of H_2S-delivering compounds that target mitochondria has been synthesized. These compounds contain a mitochondria-targeting moiety (triphenylphosphonium) that accumulates in the mitochondria [43] coupled to an H_2S-releasing moiety (dithiolethione or thiohydroxybenzamide). The effect of AP39 [(10-oxo-10-(4-(3-thioxo-3H- 1,2-dithiol-5yl)phenoxy)decyl)triphenyl-phosphonium bromide], one of these compounds, was recently tested on bioenergetics, viability and mitochondrial DNA integrity of resting cells and during oxidative stress in bEnd.3 murine microvascular endothelial cells [44]; the results demonstrated anti-oxidant and cytoprotective effects during oxidative stress. These compounds also seem to be more potent than other H_2S-forming compounds; the range of concentrations needed to exert beneficial effects being 30-100 nM, appreciably lower than those reported for NaSH, LR, Na_2S or GYY4137 [44]. These new compounds are novel and useful research tools to investigate the impact of H_2S on cell bioenergetics and are likely to produce important information about the etiology of inflammatory joint diseases, possibly offering new therapeutic approaches.

Finally, we should mention that the pharmacological addition of H_2S-fonning reagents or drugs is not the only way to supply H_2S exogenously. Beneficial effects, in particular, anti-inflammatory and anti-oxidant effects, on patients with chronic lung disease [45, 46], chronic rhinosinusitis [47], allergic disorders [48] and rheumatologic diseases [49] when H_2S is administered in the form of sulphurous mineral water have been reported.

3. H_2S AND ACUTE AND CHRONIC ARTHRITIDES

To date, most studies on the inflammatory effects of H_2S used models of acute inflammation. Some of these studies have found exogenous H_2S treatment to exert anti- inflammatory effects in such classic inflammation models as gastrointestinal inflammation [50] or endotoxic shock [42], although contradictory reports can be found in recent literature. Studies supporting the hypothesis that H_2S may be protective or beneficial that investigate the molecular mechanisms involved in rheumatic inflammatory joint pathologies are relatively recent (for a summary see Table 1).

Table 1. Summary of the literature concerning the effects of H_2S forming compounds on intlammatory joint disease *in vivo* or *in vitro* models.

Reference	Pathology	Animal Model/ Cellular Type	Stimuli	H_2S Compound and Concentration	Main Results
Sieghart *el al.* [51]	OA	FLS	IL-1β	NaSH (up to 1 mM, 20 min)	Reduced spontaneous and IL-1β induced secretion of IL-6, IL-8 and RANTES. Inhibited the formation of hyperplastic lining layer in FLS micromasses.
Burguera *el al.* [52]	OA	hACs	IL-1β or LPS	NaSH and OYY4137 (50-1000 μM, 48 h)	Reduced MMP-3 mRNA and protein. 200 μM OYY4137 reduced LPS-induced mitochondrial ROS. No effect on SOD2 or CAT mRNA.
Burguera *el al.* [53]	OA	hACs	IL-1β (5 ng/ml, 48 h)	NaSH and OYY4137 (50-1000 μ1M, 48h)	Reduced NO and iNOS (mRNA and protein), POE_2 levels, COX·2 and PTOES mRNA; no effect on COX-1. Reduced IL-6 and MMP13 levels and mRNA.
Ha *et al.* [54]	OA	hACs	IL-1β (10 ng/mi, 2 h)	NaSH (0.06-1.5 mM, 30 min pretreatment)	Reduced NO and iNOS (mRNA and protein), POE_2 levels, COX-2 mRNA; MMP13 levels and mRNA, up to 0.6 mM. Best result with 0.3mM NaSH.
Kloesch *el al.* [55]	RA and OA	FLS	-	NaSH (up to 1 mM, 20 min)	NaSH >0.5 mM, 20 min increased IL-6, IL-8, COX-2 in OA and RA-FLS, and increased MMP3 mRNA in RA-FLS. But it also reduced MMP-14 and MMP-2 in RA-FLS.
Kloesch *el al.* [56]	RA	FLS	IL-1β (5 ng/ml, 1 h)	NaSH (0.03-1.0 mM)	NaSH short-term exposure (<0.125 mM, 60 min) reduced constitutive and IL-1β-induced IL-6. NaSH long-term exposure (0.125 mM, 6 h) increased IL-6.
Kloesch *el al.* [57]	-	Chondrocyte cell line C28/I2	IL-1β (5 ng/ml, 1 h)	NaSH (up to 1 mM)	NaSH 0.125 mM, 30 min or 1 mM, 15 min reduced IL-1β-induced IL-6 and IL-8.
Stulhmeier *et al.* [58]	RA	FLS	-	NaSH 0.05-5 mM	NaSH 2mM, 45 min increased HO-1 and HSP70 mRNA and protein levels. But TNF, IL-8, IL-1β and COX-2 gene expression and protein levels were also elevated.
Fox *et al*. [34]	Normal	Chondrogenically differentiated MPCs and hACs	SIN-1 H_2O_2 4-HNE	GVY4137 200 and 500 μM	Reduced SIN·1, H_2O_2 or 4-HNE induced cell death, mitochondrial toxicity (collapse of mitochondrial membrane potential), oxidant stress induced cellular ATP depletion and cytoplasm accumulation of cytochrome C.
Li *et al.* [40]	Normal	hFLS and hCHs	LPS	GYY4137	Reduced NO, POE_2, TNF-α and IL-6, also the catalytic activities of iNOS, COX-2.
Andruski *et al.* [59]	Acute knee inflammation	C57b1/6 mice	Kaolin/carrageenan intraarticular injection	Na_2S 10, 30 or 50 μM	H_2S reduced leukocyte adherence and increased leukocyte velocity, suggesting of an anti-inflammatory effect, but had no influence on pain.

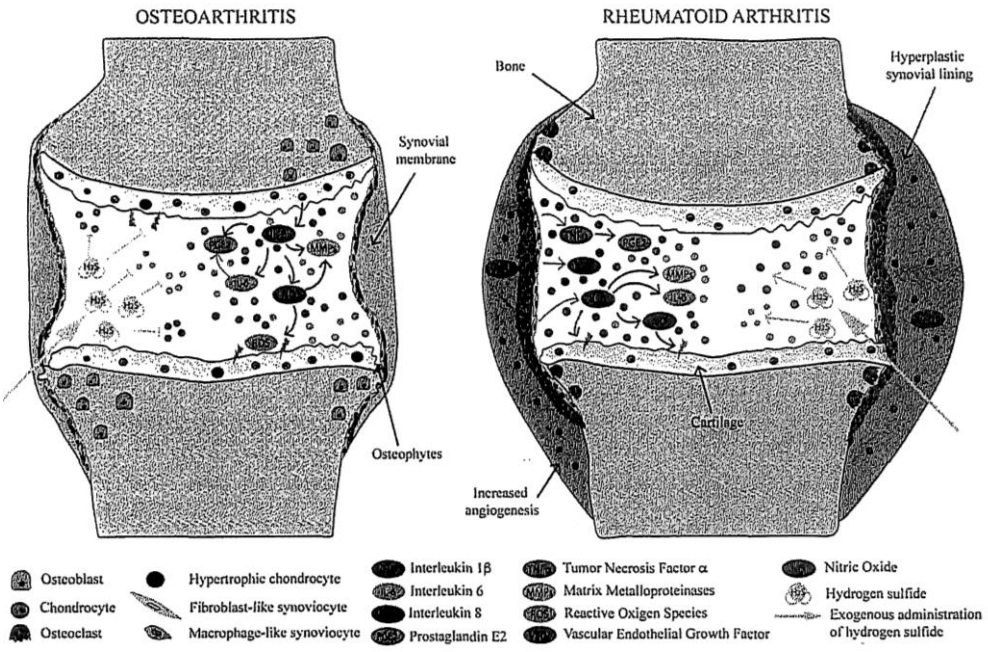

Fig. (1). Summary of the effects of the exogenous administration of hydrogen sulfide (H_2S) in osteoarthritis (OA) and rheumatoid arthritis (RA) using *in vitro* and *in vivo* models. The key pro-inflammatory cytokines are tumor necrosis factor-α (TNF-α) in RA and interleukin-1β (IL-1β) in OA. In RA, macrophage-like synoviocytes produce large amounts of TNF-α and IL-1β, and chemokines, such as IL-8. These activate fibroblast-like synoviocytes, which become the primary source of mediators that contribute to joint destruction. These mediators include matrix metalloproteinases (MMPs), IL-6, cyclooxygenase (COX-2), prostaglandin (PG) E_2 and other mediators that increase vascular permeability and facilitate angiogenesis. In OA, IL-1β is responsible for an increase in the production of lL-6, nitric oxide (NO), PGE_2 and also MMPs. Reactive nitrogen (RNS) and oxygen species (ROS), are also among the critical players involved in inflammation in both RA and OA. Virtually all reports on the administration of exogenous H_2S, in various forms, using *in vitro* and *in vivo* models and human trials related to OA, provide evidence of beneficial effects, including the reduction of some oxidation, inflammation and markers of catabolism mentioned above. On the other hand, most published studies using RA models show exacerbation of inflammation symptoms which, collectively, do not support the idea that H_2S supplementation might be beneficial to RA patients.

3.1. H_2S and In Vitro Models of Chronic inflammation

Several groups, including ours, have used *in vitro* models with FLS or ACs from RA or OA patients to shed light on the role of H_2S in these two rheumatic diseases. To evaluate its effects, these studies have typically used IL-1β or other pro-inflammatory stimuli, including LPS, to produce an inflammatory environment *in vitro* and NaSH or GYY4137 as H_2S-fonning reagents. For a graphic representation of the effects of H_2S on OA and RA cells using *in vitro* models (Fig.1).

3.1.1. Hydrogen Sulfide and OA

Studies by our group [52, 53] and others [54] demonstrated the *in vitro* anti-inflammatory and anti-catabolic effects of NaSH and GYY4137 on OA hACs. Our results showed that co-stimulation of these cells for 48 h with IL-1β (5 ng/ml) and either H_2S generator led to significant reductions in NO, PGE_2, IL-6, MMP3 and MMP13 levels. In these stimulated cells, the mRNA expression of relevant genes involved in the synthesis of these molecules, including iNOS, COX-2 and PTGES, IL-6, MMP3 and MMP13, was also downregulated by the action of H_2S. Protein levels of MMP3, MMP13 and iNOS were also reduced as demonstrated by immunocytochemistry. After pre-treating OA hACs with NaSH for 30 min prior to stimulation with 10 ng/ml of IL-1β for two additional hours, Ha and co-workers [54] found reductions in

the production of NO and PGE_2 and MMP13 proteins by ELISA, as well as reduced mRNA expression of iNOS, COX-2 and MMP13. In our studies, there was also a partial reduction of the nuclear translocation of NFKB p65 induced by IL-1β treatment [53]. In agreement with this observation, Ha *et al.* [54] pretreated cells with 0.3 mM NaSH prior to a 2 h exposure to 10 ng/ml IL-1β and found that the activation of NFKB was inhibited. Table 2 and Fig. (2) provide a summary and a graphical representation, respectively, of the signalling and transcription routes that have been implicated in the effects of H_2S on inflammatory joint cells.

Table 2. Signaling and transcription routes known to be implicated in the effects of H_2S on inflammatory joint cells

Reference	Pathology	Celular Type	Insult	H_2S Compound and Concentration	Signaling and Transcription Routes
Burguera *et al.* [53]	OA	hACs	IL-1β (5 ng/ml, 48 h)	NaSH and GYY4137 (50-1000 μM, 45 min)	Both compounds lead to a partial reduction of NF_KB p66 translocation to the nucleus.
Sieghart *et al.* [51,61]	OA	FLS	IL-1β (10 ng/ml)	NaSH (up to 1 mM, 20 min)	NaSH reduced IL-1β activation (phosphorylation) of several MAPKs (MSK2, MKK6) and GSK-3a/β but p38 was not affected. It also increased phosphorylation of Akt1/2 by 50-60%.
Ha *et al.* [54]	OA	hACs	IL-1β (10 ng/ml, 2 h)	NaSH (0.06-1.5 mM, 30 min prerreatrnent)	Pre-incubation with NaSH (0.3 mM, 30 min) reduced phosphorylation of ERK1/2, $I_KBα$ and NFkB-p65 and inhibited activation of NFkB.
Kloesch *et al.* [56]	RA	FLS	IL-1β (5 ng/ml, 1 h)	NaSH (0.03-1.0 mM)	NaSH short-term exposure (<0.125 mM, 60 min) reduced phosphorylation of ERKI/2, MEK1/2. NaSH long-term exposure (0.125 mM, 6 h) increased phosphorylation of ERK1/2, MEK1/2 and p38.
Kloesch *et al.* [57]	-	Chondrocyte cell line C28/I2	IL-1β	NaSH (up to 1 mM)	NaSH 1 mM, 60 min activated MEK/ERK and was not able to block the degradation of IkBα nor the phosphorylation of NFkB p65.
Stuhlmeier *et al.* [58]	RA	FLS	-	NaSH 0.05-5 mM	NaSH (2 mM) induction of pro-inflammatory genes did not involve NFkB. Rather, it resulted in the phosphorylation of ERK, p38 and JNK. Activation of COX-2 and IL-1β required the activation of p38 and ERK, respectively.
Kloesch *et al.* [55]	RA and OA	FLS	-	NaSH (up to l mM)	NaSH l mM, l h induced the activation of ERK1/2. Inhibitors of MAPKs inhibited H_2S induced ERK1/2 activation.
Fox *et al.* [34]	Normal	CH-MPC and Normal hACs	SIN-1 H_2O_2 4-HNE	GYY4137 200 and 500 μM	GVY4137 induced an increase in the phosphorylation of Akt in hACs. Results suggested that actívatíon of Akt signaling pathway was required for H_2S-mediated cytoprotectíon
Li *et al.* [40]	Normal	hFLS and hCH	LPS	GVY4137	Reduced NFkB activation.

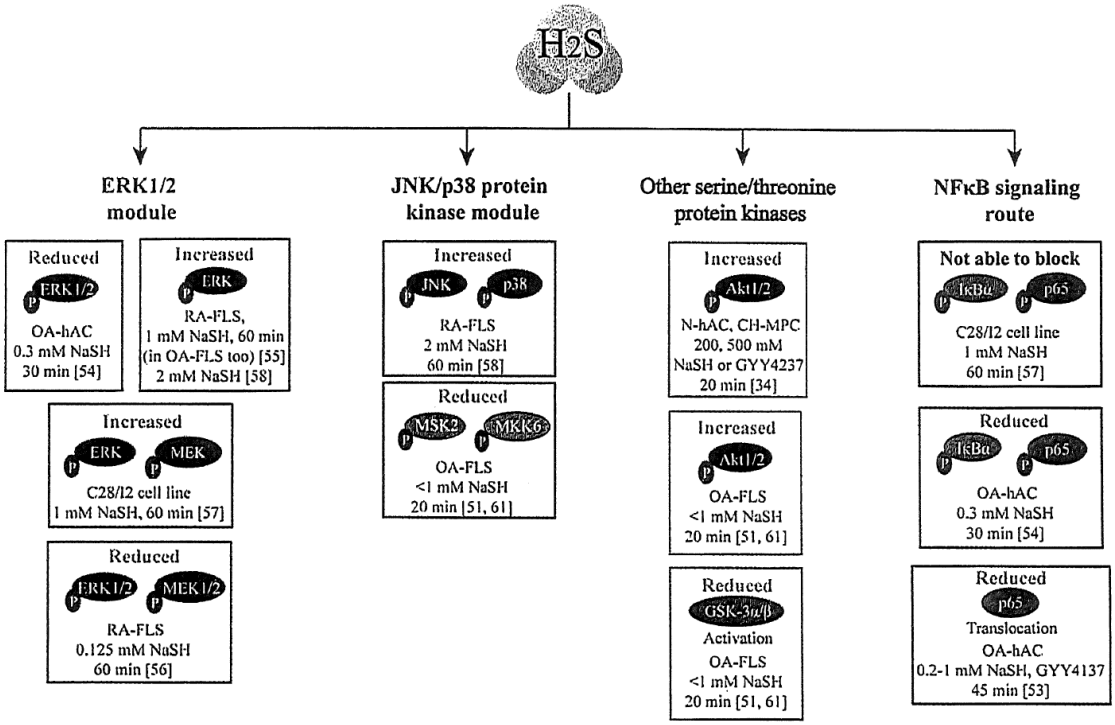

Fig. (2). Schematic representation of the signalling and transcription routes implicated in the effects of the exogenous administration of hydrogen sulfide compounds to articular cells. Hydrogen sulfide has been shown to influence several members of the mitogen activated protein kinase family (incIuding ERK1/2, MEK1/2, JNK or p38), other serine/threonine protein kinases (Akt1/2, GSK-3α/β), as well as to interact with the NFkB signalling route. The reported effects varied depending on the cell type studied, the H_2S forming compound used and its concentration, and the incubation time. Abbreviations: H_2S, hydrogen sulfide; FLS, fibroblast-like synoviocytes; AC, articular chondrocytes; CH-MPC, chondrogenically differentiated mesenchymal progenitor cells; OA, osteoarthritis; RA, rheumatoid arthritis; ERK, extracellular signal regulated kinase; MEK, MAPK/ERK kinase; MSK, mito gen- and stress-activated protein kinase; MKK, mitogen-activated protein kinase kinase; Akt, protein kinase B; GSK-3, glycogen synthase kinase 3; IkB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; JNK, c-Jun N-terminal kinases; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells.

However, a different study found that NaSH treatment was detrimental to OA hFLS [55]. NaSH concentrations above 0.5 mM applied for 20 min treatments stimulated the express ion of IL-6, IL-8 and COX-2 mRNA for as long as 6 h afterward. Nevertheless, further investigations by the same group concluded that pre-treatment with NaSH dose- dependently reduced spontaneous, as well as IL-1β-induced, IL-6, IL-8 and RANTES (regulated on activation, normal T cell expressed and secreted) production in OA hFLS, with 1 mM NaSH proving to be the most effective concentration [51]. These authors also examined signalling routes by spot blot assay and determined the activation of 26 mitogen-activated protein (MAP) kinases and other serine/threonine kinases by IL-1β. Significantly activated were extracellular signal regulated kinases (ERK) 1/2, mitogen- and stress- activated protein kinase (MSK) 2, mitogen-activated protein kinase (MKK) 3/6, heat shock protein (HSP) 27, p38 units α, β, γ, and JNK2, among others, while RAC-beta serine/threonine-protein kinase B (Akt2) phosphorylation was slightly reduced. NaSH treatment significantly reduced the IL-1β-induced phosphorylation of MSK2, MKK.6 and glycogen synthase kinase (GSK) 3α/β. However, p38 MAP kinases, which were highly activated by IL-1β, were not affected. Interestingly, NaSH treatment increased the phosphorylation of Akt1/2 by 50-60% and completely abolished the lining layer hyperplasia of hFLS micromasses stimulated with IL-1β.

3.1.2. Hydrogen Sulfide and RA

There have been a few recent studies reporting of the effects of H_2S on *in vitro* models of RA. Kloesch *et al*. [56] analyzed the effects of treatment of RA hFLS with different concentrations of NaSH for 1 h on IL-6. They found that NaSH concentrations lower than 0.125 mM reduced constitutive IL-6 levels and deactivated p44/42 MAPK (ERK1/2). Pre-incubation with 0.125 mM NaSH also blocked IL-1β- induced.(5 ng/ml for 1 h) IL-6 expression. Conversely, long term exposure of hFLS to H_2S (0.125 mM NaSH for 6h) produced elevated IL-6 expression and activation of p38 MAPK, MEK1/2 and ERK1/2. In another study, Kloesh *et al.* corroborated these results using chondrocyte cell line (C-28/I2) [57]. As found for RA hFLS, these C-28/I2 cells also expressed high constitutive levels of IL-6 and IL-8, .which were decreased following stimulation with NaSH (0.125 mM for 30 min or 1 mM for 15 min). In agreement with their previous findings [56], pretreatment with NaSH (1 mM, for up to 60 min) of C-28/I2 cells transiently activated the MEK/ERK pathway, the effect of which disappeared at 60 min. Additionally, in C-28/I2 cells stimulated with IL-1β, H_2S was unable to block the degradation of IkB (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor) a or the phosphorylation of NFkB p65.

A later study confirmed the adverse effects of NaSH exposure to RA hFLS [55]. The expression of IL-6, IL-8 and COX-2 mRNA was induced in RA hFLS for up to 6 h after 20 min of stimulation with NaSH concentrations above 0.5 mM. MMP3 mRNA was also upregulated in these RA-FLS. In a positive observation, these authors report that MMP14 and MMP2 were downregulated [55]. It is worthy of note that both MMP2 and its molecular regulator MMP14 are enzymes relevant to RA; both are expressed at elevated levels in the synovial membrane and at sites of joint destruction in RA [62-64]. As previously reported [57] in RA and OA hFLS, NaSH treatment at 1 mM for 1 h resulted in phosphorylation of ERK1/2 at 15 min after treatment. Inhibitors of MAPKs inhibited the H_2S-induced expression of IL-6, IL-8 and COX-2 mRNA, as well as ERK1/2 activation in both cell types.

Another study confirming detrimental effects of H_2S on RA hFLS [58] used commercially available RA hFLS treated with 0.05-5 mM NaSH. Short term exposure of the cells to 2 mM NaSH for 45 min induced a stress response characterized by increased mRNA and protein levels of heme-oxygenase (HO)-l and HSP70; although the gene expression and concomitant protein levels of TNF, IL-8, IL-1β and COX-2 were also significantly elevated. However, this NaSH induction of pro-inflammatory genes occurred without the involvement of NFkB transcription factor. Conversely, exposing hFLS to 2 mM NaSH resulted in the phosphorylation of ERK, p38 and JNK. Further experiments in hFLS with MAPK inhibitors demonstrated that the activation by NaSH of the COX-2 gene depends on activation of p38 and that of the IL-1β gene depends on ERK.

Although there are some indications of beneficial results, taken collectively, available evidence from *in vitro* studies does not support any beneficial effects of H_2S for RA (Fig. 1). However, because only NaSH was used to form H_2S in these studies, it is possible that other H_2S sources or concentrations might produce different effects.

3.1.3. Hydrogen Sulfide and Mitochondrial Dysfunction

Mitochondrial dysfunction has emerged as a contributing factor to the pathophysiology of both RA and OA. Compared to normal cells, diverse *in vitro* analyses of mitochondrial respiratory chain activity have shown decreased complexes II and III activities in OA hACs [65]. This mitochondrial dysfunction perhaps potentiates cytokine-induced inflammation in hFLS and hACs [22, 66] and may modulate MMPs expression in hACs [67], Mitochondrial dysfunction may increase ROS production in OA hACs [68]. GYY4137 (200 μM or 500 μM) was employed to investigate the effects of pharmacological H_2S on cellular oxidative stress in hCH- MPC and hACs. In these cells, the mitochondrial toxicity (mitochondrial membrane potential collapse), the decrease in mitochondrial ATP formation and the reduction of the cytoplasmic accumulation of cytochrome C induced by three oxidative stimuli [H_2O_2, 3-morpholinosydnonimine (SIN-1, a peroxynitrite donor) and hydroxynonenal (4-HNE)], as well as in

CTH- or CBS-siRNA treated cells, was significantly inhibited by GYY413. Collectively, these results suggest a cytoprotective role against oxidative injury for H_2S)in human joint cels [34].

Our group has investigated the anti-oxidant properties of GYY4137 and NaSH using OA hACs and found that only 200 μM GYY4137 reduced mitochondrial ROS (measured by 1,2,3-dihydrorhodamine and flow cytrometry), in LPS-, but not IL-1β-, stimulated cells; this level had no effect on the mRNA expression levels of the anti-oxidant enzymes mitochondrial superoxide dismutase (SOD2) and catalase (CAT) [52]. However, in an ischemic/reperfusion model, increased levels of SOD2 and reduced ROS levels have been reported [69]. As previously mentioned, compounds that deliver H_2S directly to the mitochondria, such as AP39, have recently been developed. To the best of our knowledge, these new compounds have not yet been tested on models of joint inflammatory diseases.

3.1.4. Hydrogen Sulfide and Normal Joint Cells

GYY4137 was also employed to investigate the effects of H_2S on cellular death induced by H_2O_2, SIN-l and 4-HNE in hCH-MPC and hACs [34]. When cells were treated with D,L-propargylglycine (PAG, CTH inhibitor), aminooxyacetate (AOAA, CBS inhibitor) or CTH-, CBS-siRNA silencing, GYY4137 preserved cellular viability against all three stimuli, dependent on concentration. A time and concentration-dependent increase in the phosphorylation of Akt, the pro-cell survival protein, was also induced in hACs by GYY4137. Experiments with Akt inhibitors showed that activation of Akt was required for GYY4137 cytoprotection against cell death.

Another *in vitro* study revealed that GYY4137 exerted anti-inflammatory properties on LPS-treated normal hFLS and hACs [40]. When cells were treated with GYY4137 previously (for 1 h prior to LPS addition), and subsequently (at 6 or 18 h after LPS addition) the levels of pro-inflammatory markers PGE_2, TNF-α, IL-6 and NO were reduced. Intracellular levels of COX-2 and iNOS and NFKB activation in both cell types were also reduced when GYY4137 was administered 1 h prior to LPS.

3.2. H_2S and In Vivo Models of Acute Arthritis

In addition to the *in vitro* studies described above, there are a few references using *in vivo* animal models. In 2008, the effects of an intra-articular injection of Na_2S on acutely inflamed joints, induced by kaolin/carrageenan intra-articular injections, in C57B1/6 mice were examined [59]. These effects were evaluated by the following parameters: leukocyte recruitment and trafficking (by intravital microscopy), synovial blood flow (by laser Doppler perfusion imaging), and joint pain (by hindlimb incapacitance and von Frey hair algesiometry). While the local administration of Na_2S caused a dose-dependent reduction in leukocyte adherence and increased leukocyte velocity, suggesting an anti-inflammatory effect, no effect on joint pain was observed. However, the administration of LR to alleviate carrageenan-induced synovitis in rat knees produced a positive effect on pain signs [60]. In this study, Wistar rats were pretreated 60 min prior to induction of synovitis with either a non-selective COX inhibitor (indomethacin), LR, or an inhibitor of endogenous H_2S production (PAG). In these rodents, pre-treatments with indomethacin and LR significantly reduced two common signs of pain, impaired gait and secondary tactile allodynia of the ipsilateral bind paw. In addition, the accompanying inflammatory response, characterized by joint swelling, inflammatory cell infiltration and increased synovial MPO activity, was also reduced. Interestingly, they found that pre- treatment with LR reduced the increased concentration of IL-1β that bad been induced by carrageenan in the rat knee cavity, but not those of TNF-α or IL-6. H_2S pre-treatment, however, bad no effect on carragenan-induced oxidative stress markers, including NO production, iNOS activity and nitration of protein tyrosine residues. Also noted was that the inhibition of endogenous H_2S with PAG enhanced synovial iNOS activity and NO production, but had no effect on other tested markers of inflammation.

In an *in vivo* model of acute joint inflammation produced by complete Freund's adjuvant (CFA) in mice [40], GYY4137 reduced inflammation when injected 6 h after CF A treatment, but did not have a prophylactic effect; it actually increased joint swelling when injected 1 h prior to CFA. Those SF parameters evaluated that were improved with GYY4137 treatment inc1uded SF concentrations of TNF-α, IL-1β, IL-6 and IL-8, as well as reduced MPO activity and N-acetyl-D-glucosaminidase (NAG) concentration. GYY4137 was also anti-inflammatory when injected 18 h after CFA treatment, though to a much lesser extent. Notably, the H_2S concentration in CFA-injected knees was higher than that of the saline-injected control knees.

3.3. H_2S and Human Clinical Studies

Finally, there are several clinical studies reporting positive outcomes when H_2S is administered to patients with rheumatologic diseases by soaking in sulphurous thermal water. A double-blind randomized controlled follow-up trial (RCT) in which hand OA patients received balneotherapy with sulphurous water 5 days a week for 3 weeks, while the control group bathed in tap water, was performed by Kovacs *et al.* [70]. Clinical parameters evaluated included hand pain, morning stiffness in band joints, grip strength of both hands and the Health Assessment Questionnaire Disability Index (HAQ), the Australian/Canadian Hand Osteoarthritis Index (AUSCAN) Hand Osteoarthritis Index, and EuroQol quality of life questionnaire. At treatment termination, the experimental group showed significant improvement in every parameter under the scope of the study. At the end of follow-up at 6 months, the values for pain parameters, and the HAQ and AUSCAN assessments continued to be significantly better in comparison with baseline values. In another prospective, single blinded RCT [71], treatment with intermittent sulphur baths (a 20 min bath 2 times a week for 6 weeks) was applied to patients with bilateral knee OA, while the control group bathed in tap water with the same regime and temperature (35-36°C). Significant improvement in the Lequesne index of OA severity and the Western Ontario and McMaster Universities Arthritis Index (WOMAC) pain and stiffness scores for up to 6 months after treatment, and in self-reported pain (VAS) up to one month after treatment, was seen in the treatment group. The control group showed improvement in the Lequesne index only up to one month after treatment and in pain (VAS) only at the 3-month visit. The SF-36 bodily pain score significantly improved in both groups.

In addition, at least three recent systematic reviews [72- 74], and a Cochrane Collaboration Review [75], have concluded that balneotherapy with sulphurous waters might indeed offer therapeutic value in rheumatic diseases. In fact, the Cochrane Collaboration review found "silver evidence concerning the beneficial effects of mineral baths compared to no treatment." Furthermore, the most recent OARSI guidelines for the nonsurgical management of knee OA in- corporate balneotherapy, including sulphurous waters, for individuals with multiple-joint OA and relevant comorbidities [76]. However, all these reviews of balneotherapy with sulphurous waters also point out that available information remains insufficient and that additional large and well-designed controlled randomized clinical trials are needed.

The mechanisms responsible for the effects of sulphurous waters on rheumatic patients are not fully understood. It has been suggested that the benefits are the result of a combination of several elements. Chemical effects are thought to play an important role, but mechanical and thermal factors also contribute prominently. It is believed that the chemical components present in the water, including H_2S, are absorbed through the skin and then act at a systemic level [77, 78]. For instance, decreased lipid (malondialdehyde) and protein oxidation products (carbonyls and advanced oxidation protein products) along with increased total antioxidant capacity (TAC) were found in the plasma of healthy volunteers that underwent a cycle of hydropinic therapy with sulphurous drinking water, compared with controls that did not [79]. Similar results were obtained in OA patients subjected to sulphur baths [80] or the combination of sulphur based mud baths and hydropinotherapy [49]. In addition, reduced serum TNF-α and cartilage oligomeric matrix protein concentrations were also found and the patients reported significantly lower pain scores [49].

CONCLUSION

The investigation of the involvement of H_2S in inflammatory joint pathologies is a rapidly expanding field. To date, *in vitro* studies using cells from OA patients have found anti- inflammatory, anti-catabolic and anti-oxidant properties for H_2S-producing compounds. The available *in vitro* evidence is still inconclusive in the case of RA.

Several *in vivo* animal models of acute arthritis have shown evidence of anti-inflammatory and pain relieving effects using NaSH, Lawesson's reagent or GYY4137 as H_2S- producing reagents. New mitochondria-targeting H_2S delivering compounds are promising new tools for investigation of the effects of H_2S in the cellular bioenergetics known to be altered in RA and OA. The exogenous supplementation of H_2S using sulphurous mineral water baths might also be an option to deliver H_2S treatment to patients. Overall, although further research is needed to confirm current working hypotheses, studies suggest that exogenous supplementation with H_2S could have therapeutic value in inflammatory joint diseases, particularly OA. Current knowledge suggests that investigations in this field may produce useful information concerning the etiology of inflammatory joint diseases, as well as possibly offering new therapeutic options for treatment of arthritic patients.

LIST OF ABBREVIATIONS

4-HNE	=	Hydroxynonenal
ACs	=	Articular chondrocytes
Akt	=	Protein kinase B
AP39	=	(10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yI)phenoxy)decyl) triphenyl-phosphonium bromide
AUSCAN	=	Australian/Canadian Hand Osteoarthritis Index
CAT	=	Catalase
CBS	=	Cystathionine β-synthase
CFA	=	Complete Freund's adjuvant
CH-MPC	=	Chondrogenically differentiated mesenchymal progenitor cells
COX-2	=	Cyclooxygenase 2
CTH	=	Cystathionine γ-lyase
ERK	=	ExtracelIular signal regulated kinases
FGF	=	Fibroblast growth factor
FLS	=	Fibroblast-like synoviocytes
GM-CSF	=	Granulocyte-macropbage colony stimulating factor
GSK	=	Glycogen synthase kinase
GYY4137	=	Motpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate
h	=	Human origin
H_2S	=	Hydrogen sulfide
HAQ	=	Health Assessment Questionnaire Disability Index
HO	=	Heme-oxygenase
HSP	=	Heat shock protein
IL-1β	=	Interleukin-1β
IL-1R	=	Interleukin-1β receptor
iNOS	=	Inducible NO synthase
IkB	=	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
JNK	=	c-Jun N-terminal kinases
LPS	=	Lipopolysaccharide
LR	=	Lawesson's reagent
MAPK	=	Mitogen-activated protein kinases
MEK	=	MAPK/ERK kinase
MKK	=	Mitogen-activated protein kinase kinase
MLS	=	Macrophage-like synoviocytes

MMPs	=	Matrix metalloproteninases
MPO	=	Myeloperoxidase
MPST	=	3-Mercaptopyruvate sulfurtransferase
MSK	=	Mitogen- and stress-activated protein kinase
NAG	=	N-acetyl-D-glucosaminidase
NFkB	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	=	Nitric oxide
OA	=	Osteoarthritis
PAG	=	D,L-propargylglycine
PGE_2	=	Prostaglandin E-2
PKC	=	Protein kinase C
PTGES	=	Microsomal prostaglandin E synthase-l
RA	=	Rheumatoid arthritis
RANTES	=	Regulated on activation, normal T cell expressed and secreted
RCT	=	Randomized controlled trial
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
SF	=	Synovial fluid
SIN-I	=	3-morpholinosydnonimine
SOD2	=	Mitochondríal superoxide dismutase
TAC	=	Total antioxidant capacity
TNF-α	=	Tumor necrosis factor-α
VAS	=	Visual analogue scale
VCAM	=	Vascular ceIl adhesion molecule
VEGF	=	Vascular endothelial growth factor
WOMAC	=	Western Ontario and McMaster Universities Arthritis Index

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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AUTHOR CONTRIBUTIONS

EF Burguera and R Meijide-Faílde searched the literature and reviewed data for the article. All authors made contributions to the discussion of the content. EF Burguera and FJ Blanco wrote the article. All authors performed re- view/editing of the manuscript before submission.

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