Involvement of hydrogen sulphide and sulphur-containing compounds in the pathogenesis and therapy of rheumatic disorders

Background

Sulphur bath therapy has long been in use for the therapy of patients suffering from rheumatic disorders and is still considered helpful for the treatment of diseases such as rheumatoid arthritis (RA) or osteoarthritis (OA). However, scientific investigations dealing with the beneficial as well as adverse effects of this kind of treatment are rare and have sometimes led to controversial results (1). Moreover, the underlying molecular mechanisms are poorly understood (2, 3). In vitro, H_2S exerts a host of effects on various biological targets, resulting in responses that range from cytotoxicity (4, 5) to cytoprotective effects (6). Several studies have demonstrated cytoprotective effects of H_2S at micromolar concentrations, which may be related to its ability to neutralize a variety of reactive species including oxyradicals (7), peroxynitrite (8), hypochlorous acid (9) and homocysteine (10). Exposure to higher concentrations (millimolar) of H_2S tends to be cytotoxic due to free radical and oxidant generation, calcium mobilization (11), glutathione depletion (5), as well as the induction of mitochondrial cell death pathways (4, 12).

Our own data obtained in the past three years suggest that H_2S may have anti- as well as pro-inflammatory properties depending on concentration and cell type (13-15). At high concentrations (0.5 – 1.0 mM NaHS), H_2S upregulated pro-inflammatory genes (e.g. IL-6, IL-8 and COX-2) in fibroblast-like synoviocytes (FLS) derived from RA and OA patients (15). In contrast, H_2S shows pronounced anti-inflammatory properties in monocytes and macrophages (manuscript in preparation).

Effects of hydrogen sulphide on the inflammmatory status of fibroblast-like synoviocytes of rheumatoid arthritis and osteoarthritis patients

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To explore the effects of H_2S, two FLS lines (one from a patient with RA and one from a patient with OA) were incubated for 20 min with 1.0 mM of the H_2S-donor NaHS. After changing the culture medium, incubation was continued for 12 h. At different time points, cell culture supernatants were collected and IL-6 release was quantified by ELISA. IL-6 expression was significantly increased by H2S treatment. Furthermore, quantitative real-time PCR (qRT-PCR) revealed that in RA-FLS also IL-8, COX-2 and MMP-3 were also upregulated by NaHS treatment (Fig. 1, left panel), whereas MMP-2 and MMP-14 were negatively regulated (Fig. 1, right panel).

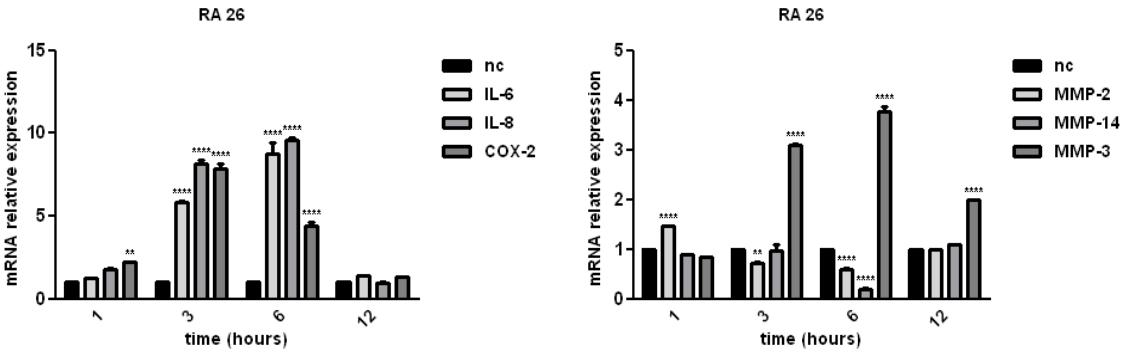

Fig. 1: H2S upregulates mRNA levels of IL-6, IL-8, COX-2 (left panel) and MMP-3 (right panel) in RA-FLS. 1, 3, 6 and 12 h after initial H2S exposure, total RNA was isolated and mRNA levels were quantified by qRT-PCR. Significant changes are indicated by asteriks: **p<0.01, ****p<0.0001.

To clarify the underlying mechanism leading to the induction of expression of pro-inflammatory genes by H2S, phosphorylation of extracellular signal-regulated kinase (ERK1/2) was analyzed by Western blotting in RA and OA-FLS; already 15 min after initial H2S exposure ERK1/2 was activated in both cell lines.

Inhibitors of p38 and ERK1/2 MAPK, SB203580 and U0126, respectively, and of NF-κB (BAY-117082) completely blocked H2S-induced IL-6 expression.

Taken together, these results demonstrate that high concentrations of H_2S can stimulate gene expression of IL-6 and other pro-inflammatory genes such as IL-8 and COX-2 as well as MMP-3 in FLS from RA and OA patients via activation of p38 and ERK1/2 MAPK and of NF-κB pathway.

Effects of hydrogen sulfide on the inflammmatory status of LPS-stimulated monocytes

During synovial inflammation, monocytes/macrophages play a pivotal role by synthesis and release of cytokines and chemokines important in induction and amplification of the inflammatory response. Hence, we wanted to investigate whether H2S would prevent or rather stimulate the activation of monocytes by lipopolysaccarides (LPS) and whether NF-κB and MAPKs were involved in this process.

The human pro- monocyte cell line U937 was differentiated for 24 h with PMA and then treated for 30 min with increasing concentrations of NaHS (0.125 – 1.0 mM) before being stimulated for 6 h with LPS (100 ng/ml). A significant reduction in IL-6 expression was obtained at 0.25 mM NaHS (Fig. 2). The highest NaHS concentration used in the experiments reduced IL-6 production at about 30 – 40 % (Fig. 1, left panel). Similar results were obtained when TNF-α release was monitored (data not shown). Notably, an almost complete suppression (~ 80 %) of IL-6 release was observed when the cells were treated 3 h after initial LPS treatment with the same dose of NaHS again (Fig. 2, right panel).

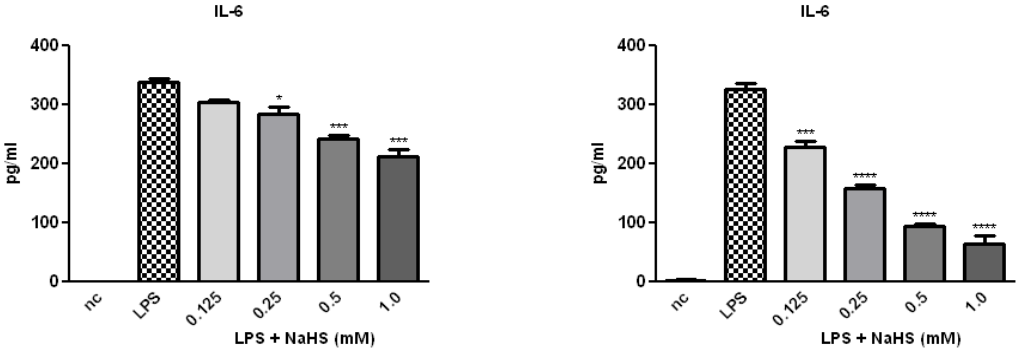

Fig. 2: H_2S blocks IL-6 expression in U937 cells. Cells remained untreated or were incubated for 6 h with LPS in the absence or presence of increasing concentrations of NaHS. IL-6 release was quantified by ELISA. Significant changes are indicated by asterisk: *p<0.05, ***p<0.001.

Remarkably, in contrast to the inhibitory effects of H2S on TNF-α and IL-6 expression, H2S stimulated the expression of IL-1β (Fig. 3). This stimulation was only evident when LPS plus NaHS were present in the culture medium while NaHS alone was not able to induce IL-1β expression (data not shown).

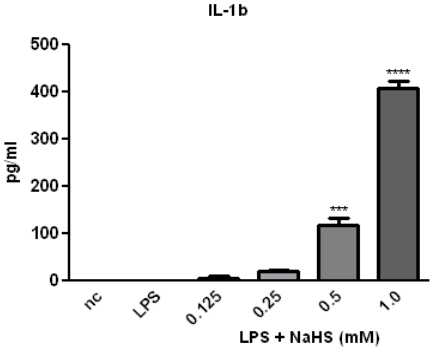

Fig. 3: H_2S induces the release of IL-1β in U937 cells. Cells remained untreated or were incubated for 6 h with LPS in the absence or presence of increasing concentrations of NaHS. IL-1β release was quantified by ELISA. Significant changes are indicated by asterisk: ***p<0.001, ****p<0.0001.

ersial effects in LPS-stimulated monocytes. On the one hand, H_2S ; by inhibition of TNF-α and IL-6 expression, on the other hand, it induced phosphorylation of ERK1/2 (data not shown) and stimulated the expression or the release (possibly via caspase-1 activation) of IL-1β. Further studies are necessary to clarify the involvement of

H_2S in p38 and ERK1/2 MAPK activation/deactivation as well as the impact of H_2S on transcriptional and post-translational processes of pro-inflammatory gene expression in activated monocytes.

Effects of hydrogen sulfide and the sulphur-containing anti-oxidants dimethyl sulphoxide and dimethyl sulphone on IL-6 and IL-8 expression in a human chondrocyte cell line (C-28/I2)

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Next, we were interested to study the effects of H_2S on chondrocytes which are centrally involved in cartilage synthesis and metabolism. In addition, we studied the effects of two sulfur containing anti-oxidants dimethyl sulphoxide (DMSO) and dimethyl sulphone (DMS). DMSO is a powerful water miscible solvent that dissolves most water insoluble drugs. DMSO possesses anti-inflammatory properties, as well as the ability to act as a free radical scavenger (16, 17). Furthermore, it is capable of inducing or inhibiting cell proliferation, apoptosis and/or differentiation. Thus, its properties have been exploited in the treatment of dermatological, rheumatic, and renal manifestations of amyloidosis. However, limited data are available on the underlying molecular mechanism. DMS is found in small amounts in many foods, including unpasteurized milk, grains, meat, eggs, and fish. It is also present in popular dietary supplements. Reported effects claimed to be associated with DMS include relief of pain, reduction of inflammation, arthritis, allergies and asthma (18, 19).

We studied the effects of NaHS, DMSO and DMS on IL-6 and IL-8 expression in C-28/I2 cells, a human chondrocyte cell line, originally derived from a young patient with OA (20). In the case of H_2S treatment, cells were left untreated or were incubated for 15 min with different concentrations of NaHS (0.125 and 1.0 mM). After changing the culture medium, incubation was continued for 12 h. In contrast to H_2S treatment, cells were incubated in the presence of DMSO (0.5 and 1.0 %) or DMS (10 and 100 mM) over a total period of 12 h. At different time points, cell culture supernatants were collected and IL-6 and IL-8 levels were quantified by ELISAs. Reduced levels of IL-6 and IL-8 were detected from 1 to 6 h after initial H_2S exposure (14). DMSO (1.0 %) and DMS (100 mM) were much more efficient in blocking IL-6 expression than H_2S: after 12 h of incubation almost 70 % inhibition was obtained with both substances.

Taken together, these studies show that NaHS and the sulphur containing anti-oxidants DMSO and DMS are potent inhibitors of constitutive as well as IL-1β-induced cytokine expression in a human chondrocyte cell line (21). Identification of the components of the signal transduction pathways that are sensitive to anti-oxidants may eventually open a new territory to more selective treatment of inflammatory disorders.

Effects of the polyphenols curcumin and resveratrol on cytokine expression in fibroblast-like synoviocytes

Curcumin (diferuloylmethane) is a yellow pigment found in the rhizome of turmeric (*Curcuma longa L. Zingiberaceae*) which has a wide range of pharmacological and biological activities (22). The anti-oxidant, anti-carcinogenic, anti-inflammatory and apoptotic effects of this compound have been assessed in various *in vitro* and *in vivo* systems. Although several studies have reported that curcumin may modulate numerous aspects of cell function relevant to inflammatory arthritis, the underlying molecular mechanisms are incompletely understood (23).

Resveratrol (*trans*-3,49,5-trihydroxystilbene) is a natural phytoalexin found in large quantities in grapes and other food products. Resveratrol was found to have a potent anti-carcinogenic activity in several animal models of cancer (24). The anti-carcinogenic properties of resveratrol are closely associated with its antioxidant activity and ability to inhibit cyclo-oxygenase, hydroperoxidase, protein kinase C, Bcl-2 phosphorylation, Akt, NF-κB, MMP -9, and programmed cell death (25, 26).

Since little is known about the effects of curcumin and resveratrol on FLS derived from RA patients, we investigated possible anti-inflammatory and apoptotic properties of these substances in FLS obtained from two RA patients. Particular attention was paid to the influence of curcumin and resveratrol on IL-6 and VEGF-A expression in IL-1β and PMA-stimulated FLS, as well as to its modulation of NF-κB and MAPK such as p38 and ERK1/2.

FLS were stimulated with IL-1β (10 ng/ml) or treated with IL-1β plus increasing concentrations of curcumin (12.5 – 50 µM) or resveratrol (12.5 – 100 µM). As shown in Fig. 4, even low concentrations of curcumin (12.5 µM) significantly blocked IL-6 release in FLS. Higher concentrations (25 – 50 µM) almost completely suppressed IL-6 expression.

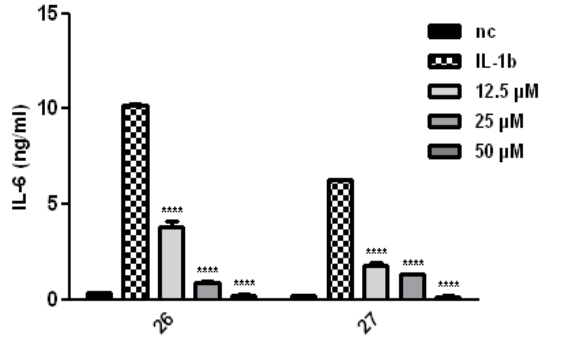

Fig. 4: Curcumin blocks IL-1β -induced IL-6 expression in FLS. Cells remained untreated or were stimulated with IL-1β or with IL-1β plus increasing concentrations of curcumin. IL-6 release was quantified by ELISA (nc, negative control; 26, RA-26; 27, RA-27). Significant changes are indicated by asteriks: ***p<0.001, ****p<0.0001.

Resveratrol had much lower inhibitory capacity on IL-1β -induced IL-6 expression compared to curcumin, and in fact, a concentration of 100 µM resveratrol was required to obtain the same degree of suppression of IL-6 production as observed with 12.5 µM curcumin (Fig. 5).

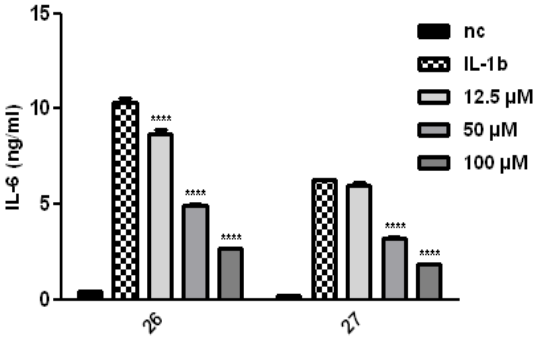

Fig. 5: Resveratrol blocks IL-1β -induced IL-6 expression in FLS. Cells remained untreated or were stimulated with IL-1β or with IL-1β plus increasing concentrations of resveratrol. IL-6 release was quantified by ELISA (nc, negative control; 26, RA-26; 27, RA-27). Significant changes are indicated by asteriks: ****p<0.0001.

Phorbol esters such as PMA stimulate protein kinase C and activate the Ras/Raf/ERK1/2 signalling pathway. Therefore the capacity of curcumin and resveratrol to block PMA-induced IL-6 expression was studied. The results obtained were similar to those obtained with IL-1β stimulated cells (data not shown).

To investigate the influence of curcumin and resveratrol on MAPK activation, FLS were incubated for 30 min with increasing concentrations of curcumin (12.5 – 50 µM) or resveratrol (12.5 – 100 µM) before being stimulated for 20 min with IL-1β. As shown in Fig. 6, both curcumin and resveratrol induced dephosphorylation of ERK1/2 in a concentration-dependent manner; however, p38 MAPK activation was unaffected by either substance.

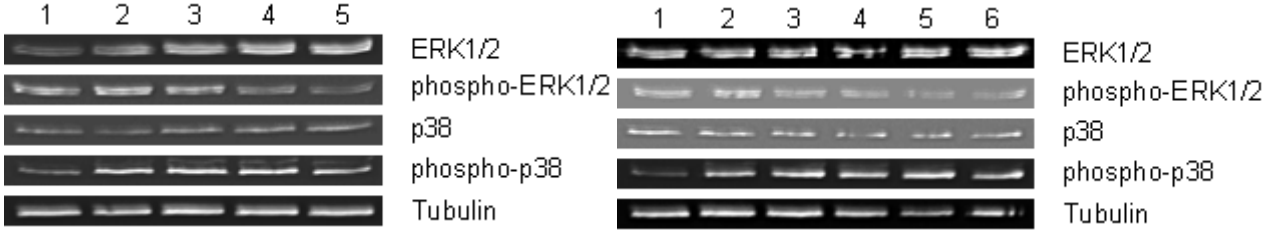

Fig. 6: Curcumin and resveratrol induce ERK1/2 deactivation in FLS. Cells remained untreated or were incubated for 30 min with increasing concentrations of curcumin (left panel) or resveratrol (right panel) before being stimulated for 20 min with IL-1β (1, negative control; 2, IL-1β; 3, 12.5 µM curcumin/resveratrol; 4, 25 µM curcumin/resveratrol; 5, 50 µM curcumin/resveratrol; 6, 100 µM resveratrol).

Taken together, curcumin and resveratrol effectively suppressed IL-1β and PMA-induced IL-6 and VEGF-A expression in FLS. Higher concentrations of curcumin (50 μM) induced activation of caspases which finally led to induction of apoptotis whereas resveratrol did not (data not shown). Although further studies are needed, these results provide important new insights into the biological effects of curcumin and resveratrol, and suggest both substances as natural remedies for the treatment of chronic inflammatory diseases like RA.

Summary

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovial membrane, hyperplasia of the synovial lining and overactivation of osteoclasts, resulting in the irreversible destruction of articular cartilage and bone. Recently, several studies have highlighted the importance of H_2S in inflammation. H_2S suppresses leukocyte adherence to the vascular endothelium and migration of leukocytes into the subendothelial space, as well as reducing plasma exudation. H_2S has been shown to reduce expression of many pro-inflammatory cytokines, chemokines, and enzymes, most likely related to its ability to suppress activation of nuclear transcription factor-κB (NF-κB). H_2S is also a potent anti-oxidant and can induce apoptosis in neutrophils.

Fibroblast-like synoviocytes (FLS), located in the intimal lining layer of the synovial membrane, have been shown to promote secondary synovitis by the release of a series of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) as well as matrix-metalloproteinases (MMPs).

It was our aim to investigate possible anti-inflammatory effects of exogeneous H_2S (NaHS) on FLS derived from patients with RA and OA. We showed that low concentrations of H_2S (μM) may have anti-inflammatory properties whereas high concentrations of H_2S (mM) can stimulate expression of IL-6 and other pro-inflammatory genes such as IL-8 and COX-2 as well as MMP-3. Therefore, we conclude that H_2S is a gaseous transmitter molecule with bivalent properties. Evaluation of H_2S-releasing drugs in an *in vivo* setting, i.e the collagen-induced arthritis model, will provide insight as to whether or not the exploitation of H2S as a therapeutic agent will live up to the promise.

Beside H_2S, naturally occuring anti-oxidants such as DMS, and the polyphenols curcumin and resveratrol get an increasing importance in the treatment of chronic inflammatory diseases. The anti-oxidant, anti-carcinogenic, anti-inflammatory and apoptotic effects of these compounds have been assessed in various *in vitro* and *in vivo* systems. Since little is known about the effects of curcumin and resveratrol on FLS derived from RA patients, we investigated possible anti-inflammatory and apoptotic properties of curcumin and resveratrol in FLS obtained from two RA patients.

Our data demonstrate that curcumin and resveratrol effectively suppressed IL-1β and PMA-induced IL-6 and VEGF-A expression in FLS derived from RA patients. Furthermore, curcumin and resveratrol induced activation of different caspases and induced apoptotic events in FLS.

Although further studies are needed, these results provide important new insights into the biological effects of curcumin and resveratrol, and suggest both substances as natural remedies for the treatment of chronic inflammatory diseases like RA.

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