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Hydrogen sulfide stimulates activation of hepatic stellate cells through increased cellular bio-energetics

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ABSTRACT

Hepatic fibrosis is caused by chronic inflammation and characterized as the excessive accumulation of extracellular matrix (ECM) by activated hepatic stellate cells (HSCs). Gasotransmitters like NO and CO are known to modulate inflammation and fibrosis, however, little is known about the role of the gasotransmitter hydrogen sulfide (H_2S) in liver fibrogenesis and stellate cell activation. Endogenous H_2S is produced by the enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH) and 3-mercaptopyruvate sulfur transferase (MPST) [1]. The aim of this study was to elucidate the role of endogenously produced and/or exogenously administered H_2S on rat hepatic stellate cell activation and fibrogenesis. Primary rat HSCs were culture-activated for 7 days and treated with different H_2S releasing donors (slow releasing donor GYY4137, fast releasing donor NaHS) or inhibitors of the H_2S producing enzymes CTH and CBS (DL-PAG, AOAA). The main message of our study is that mRNA and protein expression level of H_2S synthesizing enzymes are low in HSCs compared to hepatocytes and Kupffer cells. However, H_2S promotes hepatic stellate cell activation. This conclusion is based on the fact that production of H_2S and mRNA and protein expression of its producing enzyme *CTH* are increased during hepatic stellate cell activation. Furthermore, exogenous H_2S increased HSC proliferation while inhibitors of endogenous H_2S production reduce proliferation and fibrotic makers of HSCs. The effect of H_2S on stellate cell activation correlated with increased cellular bioenergetics. Our results indicate that the H_2S generation in hepatic stellate cells is a target for anti-fibrotic intervention and that systemic interventions with H_2S should take into account cell-specific effects of H_2S.

1. Introduction

Chronic inflammation occurs in many liver diseases, e.g. non-alcoholic steatohepatitis (NASH), viral infection or chronic alcohol consumption. Liver fibrosis can be viewed as an uncontrolled wound healing response. Hepatic stellate cells (HSCs) play an important role in the onset and perpetuation of liver fibrosis. Under normal conditions, HSCs are quiescent and are the principal vitamin A storing cells in the liver [1]. In conditions of chronic inflammatory liver injury, quiescent hepatic stellate cells (qHSCs) transform into proliferative myofibroblast-like cells called activated HSCs (aHSCs). During activation, HSCs lose their vitamin A content and start to produce large amounts of extracellular matrix (ECM) [2]. When the inflammatory response is not suppressed, the excessive accumulation of ECM can lead to hepatic fibrosis, cirrhosis and eventually hepatocellular carcinoma. At present, there is no effective treatment for hepatic fibrosis, leaving liver transplantation as the only viable treatment option. Therefore, it is important to understand the mechanisms that lead to hepatic stellate cell activation and hepatic fibrosis [3,4]. Gasotransmitters like nitric oxide (NO) and carbon monoxide (CO) have been shown to play an important role in chronic liver inflammation and liver fibrosis [5,6]. Recently, interest has been focused on another gasotransmitter, hydrogen sulfide (H_2S) [7–9].

In the last two decades, H_2S has been identified as a gasotransmitter that is generated in many mammalian cells and is involved in various physiological and pathophysiological processes as a signaling molecule similar to NO and CO [10]. H_2S has also been implicated to modulate inflammation and fibrosis, although its role in liver fibrosis and hepatic stellate cell activation is still not completely elucidated.

H_2S is produced intracellularly from cysteine and methionine by the

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Abbreviations

Akt	protein kinase B
AOAA	amino-oxyacetic acid
ATP	adenosine triphosphate
BDL	bile duct ligation
BrdU	5-bromo-2′-deoxyuridine
CBS	cystathionine β-synthase
cDNA	complementary deoxyribonucleic acid
CO	carbon monoxide
CTH	cystathionine γ-lyase
Col1α1	collagen type 1 alpha 1
DL-PAG	dl-Propargylglycine
ECAR	Extra-cellular acidification rate
ECM	extracellular matrix
FCCP	trifluoromethoxy carbonylcyanide phenylhydrazone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H2O2	hydrogen peroxide
HBSS	Hanks' balanced salt solution
H2S	hydrogen sulfide
HSCs	hepatic stellate cells
MPST	3-mercaptopyruvate sulfur transferase
NaHS	sodium hydrosulfide
NASH	nonalcoholic steatohepatitis
NO	nitric oxide
mRNA	messenger ribonucleic acid
OCR	Oxygen consumption rate
p38 MAP-Kinase	P38 mitogen-activated protein kinases
PDGF-BB	platelet-derived growth factor BB
PLP	Pyridoxal phosphate
TGFβ1	transforming growth factor beta 1

enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CTH) and 3-mercaptopyruvate sulfur transferase (MPST) [11,12]. It has been shown to regulate hepatic fibrosis via its anti-oxidative and anti-inflammatory properties and by inducing cell-cycle arrest, apoptosis, vasodilation and reduction of portal hypertension [8,9,13–16]. However, most of these experiments were performed in *vivo* conditions and did not focus directly on the process of fibrogenesis and HSCs activation. Furthermore, conflicting results have been reported depending on the concentration or type of H_2S donor used. Based on the H_2S release rate, H_2S releasing donors can be categorized as fast (NaHS; Na_2S) or slow (GYY4137; ADT-OH) releasing donors, often yielding contrasting results [17–19]. For instance, some studies reported pro-inflammatory and anti-apoptotic properties of H_2S and in some studies H_2S was shown to increase mitochondrial bioenergetics and promote cell proliferation [20–23]. Therefore, there are still major gaps in our understanding of the actual effects of H_2S on HSCs and liver fibrosis.

The aim of the current study was to elucidate the effects of H_2S on HSCs by investigating how endogenously produced and/or exogenously administered H_2S affects primary rat HSCs and its proliferation. Furthermore, we tried to elucidate the dynamics of endogenous production of H_2S and H_2S synthesizing enzymes during HSCs activation.

2. Materials and methods

2.1. Hepatic stellate cell isolation and culture

Specified pathogen-free male Wistar rats were purchased from Charles River (Wilmington, MA, USA) and housed in a 12hr light-dark cycle under standard animal housing conditions with free access to chow and water. HSCs were isolated from rats weighing 350–450 g, anesthetized by isoflurane and a mixture of Ketamine and Medetomidine. The liver was perfused via the portal vein with a buffer containing Pronase-E (Merck, Amsterdam, the Netherlands) and Collagenase-P (Roche, Almere, the Netherlands). The HSC population was isolated by density centrifugation using 13% Nycodenz (AxisShield POC, Oslo, Norway) solution. Isolated HSCs were cultured in Iscove's Modified Dulbecco's Medium supplemented with Glutamax (Thermo Fisher Scientific, Waltham, MA, USA), 20% heat inactivated fetal calf serum (Thermo Fisher Scientific), 1% MEM Non Essential Amino Acids (Thermo Fisher Scientific), 1% Sodium Pyruvate (Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics: 50 µg/mL gentamycin (Thermo Fisher Scientific), 100 U/mL Penicillin (Lonza, Vervier, Belgium), 10 µg/mL streptomycin (Lonza) and 250 ng/mL Fungizone (Lonza) in an incubator containing 5% CO_2 at a 37 °C [24]. Quiescent HSCs (day 1) spontaneously activate when cultured on tissue culture plastic and reached complete activation (increased proliferation, loss of retinoids and increased synthesis of extracellular matrix components) after 7 days of culture. Day 3 cultured HSCs are considered intermediately activated.

2.2. Experimental design

Culture-activated HSCs (aHSCs) were treated with H_2S donors or inhibitors for 72 h. All treatments with H_2S donors and inhibitors were performed in fresh medium containing 20% FCS and other supplements. H_2S releasing donors GYY4137 (kind gift from Prof. Matt Whiteman, University of Exeter, United Kingdom) and NaHS (Sigma-Aldrich, Zwijndrecht, the Netherlands) were diluted in distilled water and prepared freshly. NaHS was added every 8 h to the cells because of its rapid evaporation. The CBS inhibitor O-(carboxymethyl) hydroxylamine, AOAA (Sigma-Aldrich) was prepared as a 200 mmol/L stock solution and diluted in distilled water at neutral pH. CTH inhibitor DL-propargylglycine, DL-PAG (Sigma-Aldrich) was freshly prepared.

2.3. Measurement of H_2S concentration

The accumulation of H_2S in the culture medium was measured as described previously [25,26]. After 72 h incubation, medium samples were collected in 250 µL of 1% (wt/vol) zinc acetate and distilled water was added up to 500 µL. Next, 133 µl of 20 mmol/L N-dimethyl-*p*-

Table 1
Primer sequences.

Gene	Sense 5′-3′	Antisense 5′-3′	Probe 5′-3′
18s	CGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	CGCGCAAATTACCCACTCCCGA
Col1a1	TGGTGAACGTGGTGTACAAGGT	CAGTATCACCCTTGGCACCAT	TCCTGCTGGTCCCCGAGGAAACA
Acta2	GCCAGTCGCCATCAGGAAC	CACACCAGAGCTGTGCTGTCTT	CTTCACACATAGCTGGAGCAGCTTCTCGA
Cth	TACTTCAGGAGGGTGGCATC	AGCACCCAGAGCCAAAG	no probe, qPCR with Sybr green
Cbs	GCGGTGGTGGATAGGTGGTT	CTTCACAGCCACGGCCATAG	no probe, qPCR with Sybr green
Mpst	TGGAACAGGCGTTGGATCTC	GGCATCGAACCTGGACACAT	no probe, qPCR with Sybr green
36b4	GCTTCATTGTGGGAGCAGACA	CATGGTGTTCTTGCCCATCAG	TCCAAGCAGATGCAGCAGATCCGC
Tgfβ1	GGG CTA CCA TGC CAA CTT CTG	GAG GGC AAG GAC CTT GCT GTA	CCT GCC CCT ACA TTT GGA GCC TGG A

phenylenediamine sulfate in 7.2 mmol/L hydrogen chloride and 133 µl 30 mmol/L ferric chloride in 1.2 mmol/L hydrogen chloride were added. After incubation for 10 min at room temperature, protein was removed by adding 250 µL trichloroacetic acid and centrifugation at 14000*g* for 5 min. Spectrophotometry was performed at 670 nm light absorbance (BioTek Epoch2 microplate reader) in 96 well-plates. All samples were assayed in duplicated. Concentrations were calculated against a calibration curve of NaHS (5–400 µmol/L) in culture medium.

2.4. Quantitative real-time polymerase chain reaction

Hepatic stellate cell RNA was isolated using Tri-reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNA concentrations were measured by Nano-Drop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) and 1.5 µg of RNA was used for reverse transcription (Sigma-Aldrich). cDNA was diluted in RNAse-free water and used for real-time polymerase chain reaction on the QuantStudio™ 3 system (Thermo Fisher Scientific). All samples were analyzed in duplicate using 18S and 36b4 as housekeeping genes. The mRNA levels of *Cth*, *Cbs*, *Mpst* (Invitrogen) were quantified using SYBR Green (Applied Biosystems), other genes were quantified by TaqMan probes and primers. Relative gene expression was calculated via the $2^{-?Ct}$ method. The primers and probes are shown in Table 1.

2.5. Cell toxicity determination by Sytox Green

Cell necrosis was measured by Sytox Green nucleic acid staining (Invitrogen, the Netherlands) at a dilution of 1:40.000 in culture medium or HBSS for 15 min at a 37 °C. Necrotic cells have ruptured plasma membranes, allowing entrance of non-permeable Sytox green into the cells. Sytox green then binds to nucleic acids. Fluorescent nuclei were visualized at an excitation wavelength of 450–490 nm by a Leica microscope. Hydrogen peroxide 1 mmol/L was used as a positive control.

2.6. Cell proliferation measurement

Proliferation of aHSCs was measured by Real-Time xCELLigence system (RTCA DP; ACEA Biosciences, Inc., CA, USA) and by colorimetric BrdU cell proliferation ELISA kit (Roche Diagnostic Almere, the Netherlands). Cells were seeded in a 16-well E-plate and treated as indicated. Cell index was determined by measuring the change of impedance on the xCELLigence system.

For BrdU incorporation assay, aHSCs were seeded in a 96-well plate and treated as indicated. BrdU incorporation was determined according to manufacturer's instructions and quantified by light emission chemiluminiscence using the Synergy-4 machine (BioTek).

2.7. Western blot analysis

Cells were seeded in 6-well plates and treated as described. Protein lysates were collected by scraping in cell lysis buffer (HEPES 25 mmol/L, KAc 150 mmol/L, EDTA pH 8.0 2 mmol/L, NP-40 0.1%, NaF 10 mmol/L, PMSF 50 mmol/L, aprotinin 1 µg/µL, pepstatin 1 µg/µL, leupeptin 1 µg/µL, DTT 1 mmol/L). Total amount of protein in lysates was measured by Bio-Rad protein assay (Bio-Rad; Hercules, CA, USA). For Western blotting, 20–30 µg protein was loaded on SDS-PAGE gels. Proteins were transferred to nitrocellulose transfer membranes using *Trans*-Blot Turbo Blotting System for tank blotting. Proteins were detected using the following primary antibodies: monoclonal mouse anti-GAPDH 1:5000 (CB1001, Calbiochem), polyclonal goat anti-COL1α1 (1310-01, Southern Biotech), monoclonal mouse anti-ACTA2 1:5000 (A5228, Sigma Aldrich), polyconal rabbit anti-CTH 1:1000 (12217-1-AP, Proteintech), monoclonal mouse anti-CBS 1:1000 (sc-271886, Santa Cruz), monoclonal mouse anti-MPST 1:1000 (sc-374326, Santa Cruz). Protein band intensities were determined and detected using the Chemidoc MR (Bio-Rad) system.

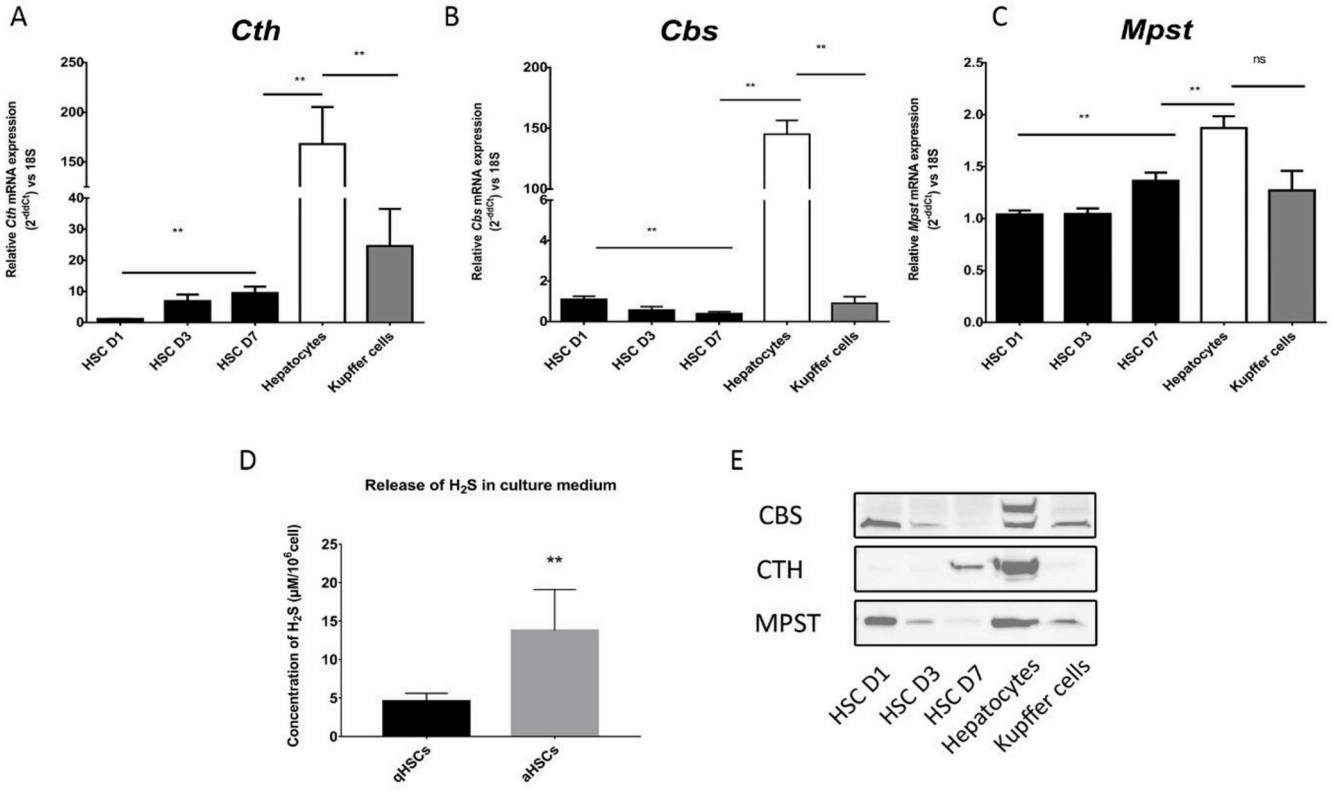

Fig. 1. Expression of H_2S producing enzymes and H_2S production in hepatic stellate cells.

2.8. Cellular bioenergetics analysis

Mitochondrial activity and production of ATP was assessed by XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara SA, USA). aHSCs were seeded in Seahorse XF24 cell culture plates and treated as indicated for 48hrs. Oxygen Consumption Rate (OCR) and Extra-Cellular Acidification Rate (ECAR) were assessed after the addition of glucose (5 mmol/L), oligomycin (1 µmol/L), FCCP (0,25 µmol/L) and a mixture of antimycin (1 µmol/L), rotenone (1 µmol/L), 2-Deoxy-D-glucose (100 mmol/L). Results were normalized for the protein concentration of each sample.

2.9. Bile duct ligation

Male Wistar rats were anaesthetized with halothane/O_2/N_2O and subjected to bile duct ligation (BDL) as described by Kountouras J et al. [27]. At the indicated times after bile duct ligation (BDL), the rats (n = 4 per group) were sacrificed, livers were perfused with saline and removed. Control rats received a sham operation (SHAM). Specimens of these livers were snap-frozen in liquid nitrogen for isolation of mRNA and protein.

2.10. Statistical analysis

Results are presented as mean ± standard deviation (mean ± SD). Every experiment was repeated at least 3 times. Statistical significance was analyzed by Mann-Whitney test between the two groups and Kruskal-Wallis followed by post-hoc Dunn's test for multiple comparison test. Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Hydrogen sulfide production is increased upon activation of hepatic stellate cells

In order to determine the dynamics of H_2S production and H_2S producing enzymes during HSC activation, mRNA expression of H_2S synthesizing enzymes was measured in quiescent (q) and activated (a) HSCs and compared to the expression of these enzymes in hepatocytes and Kupffer cells. As shown in Fig. 1, the H_2S producing enzymes *Cth*, *Cbs* and *Mpst* were expressed at low levels in qHSCs compared to hepatocytes and Kupffer cells. Upon activation, *Cth* gene expression increased in HSCs (Fig. 1A) while *Cbs* and *Mpst* mRNA levels were not changed (Fig. 1B and C). In line with this, the accumulation of H_2S in culture medium was increased during HSC activation. In Fig. 1D, values are normalized for cell number since the morphology and proliferation rate of quiescent and activated stellate cells are very different (Fig. 1D). Western blotting results showed similar trend as observed for the mRNA expression data. Protein expression of CTH was increased during HSCs activation (Fig. 1E).

Cth, *Cbs* and *Mpst* mRNA expression was determined in HSCs at day 1, 3, and 7 and compared to primary rat hepatocytes and Kupffer cells (**A-C**). The cytosolic enzymes *Cth* and *Cbs* were abundantly expressed in hepatocytes, while their expression was relatively low in HSCs. Upon HSCs activation, *Cth* expression was induced 7-fold, and *Mpst* slightly upregulated, whereas expression of *Cbs* was downregulated. Expression levels are relative to *18S* expression. D. Production of H_2S in activated and quiescent HSCs. The production of H_2S was increased upon activation of HSCs. Results were normalized with respect to the number of cells. **E.** Protein expressions of CTH, CBS, MPST of HSCs at different time point and hepatocytes and Kupffer cells. Equal protein loading was confirmed by Ponceau S staining and Western blot for GAPDH.

3.2. Effect of H_2S on activation markers in hepatic stellate cells

In order to avoid confounding effects of cell toxicity, we optimized the concentration of H_2S donors and inhibitors by Sytox green staining. At concentrations twice as high as used in the experiments, none of the donors or inhibitors were toxic to HSCs (Fig. 2).

Toxicity of the compounds was checked by Sytox Green staining. Hydrogen peroxide (1 mmol/L; 6 h exposure) was used as a positive control. The compounds DL-PAG (Cth inhibitor), AOAA (Cbs inhibitor), GYY4137 (slow releasing donor) and NaHS (fast releasing donor) were not toxic for HSCs. Duration of the treatment was 24hrs.

We next evaluated the effect of H_2S on activation markers in aHSCs. Inhibitors of H_2S producing enzymes (DL-PAG, AOAA) decreased the expression of the fibrogenic markers *Col1α1* and *Acta2* (Fig. 3A). The

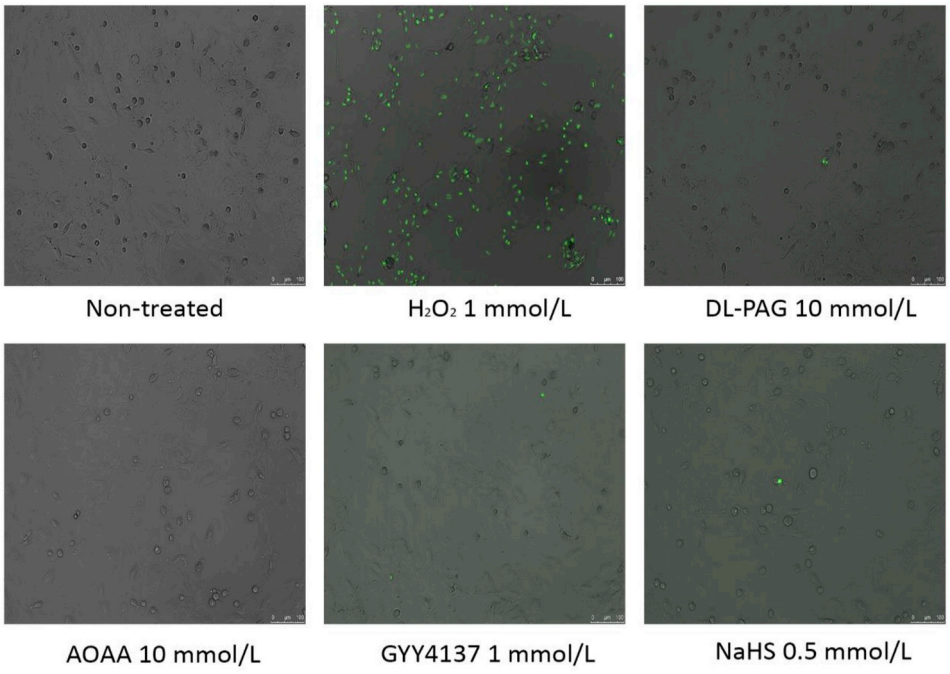

Fig. 2. H_2S releasing donors and enzyme inhibitors are not toxic for hepatic stellate cells.

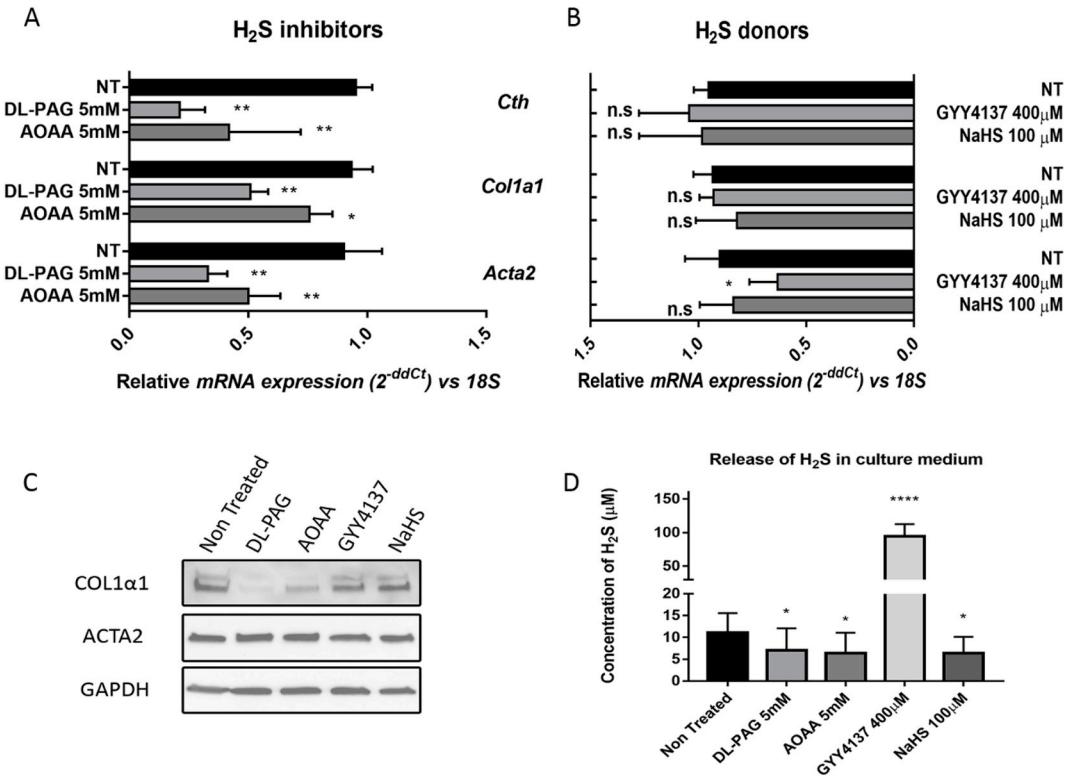

Fig. 3. mRNA and protein expression of HSC activation markers in response to H_2S donors and enzyme inhibitors.

H_2S donors GYY4137 and NaHS did not affect the expression of *Col1α1*. However, GYY4137 slightly, but significantly, reduced *Acta2* mRNA expression (Fig. 3B). Interestingly, both of the two enzyme inhibitors also downregulated the expression of *Cth* mRNA. The changes in mRNA expression were reflected in similar changes in protein expression of COL1α1 but not ACTA2 (Fig. 3C). Accumulation of H_2S in culture medium was reduced by inhibitors, whereas GYY4137 increased H_2S accumulation. Because of the fast release, no accumulation of H_2S was measured in NaHS-treated group. In Fig. 3D, we did not normalize values to the number of cells (in contrast to Fig. 1), because experiments were performed with only activated stellate cells over a limited time span, in which it can be assumed that cell numbers will not differ significantly (Fig. 3D).

The H_2S synthesizing enzyme inhibitors DL-PAG and AOAA downregulated *Col1α1*, *Acta2* and *Cth* mRNA expression while the H_2S donors GYY4137 and NaHS did not affect *Cth* and *Col1α1* mRNA expression (**A, B**). In contrast, GYY4137, but not NaHS reduced *Acta2* mRNA expression slightly. *18S* was used as a housekeeping gene. The inhibitors also reduced COL1α1 protein level but not ACTA2 protein level (**C**). GAPDH was used as loading control for protein analysis. The accumulation over 72 h of H_2S in culture medium was measured in the experimental groups (**D**). DL-PAG and AOAA significantly reduced the accumulation of H_2S. Because of its fast release, no accumulation of H_2S was measured in the NaHS-treated group. Accumulation of H_2S was detected with the slow releasing donor GYY4137.

3.3. H_2S promotes hepatic stellate cell proliferation

The effect of H_2S on rat HSC proliferation was assessed using real-time cell analyzing xCelligence and BrdU incorporation ELISA assays. H_2S donors promote, whereas H_2S synthesizing enzyme inhibitors inhibit aHSCs proliferation, indicating a stimulatory effect of H_2S on HSC

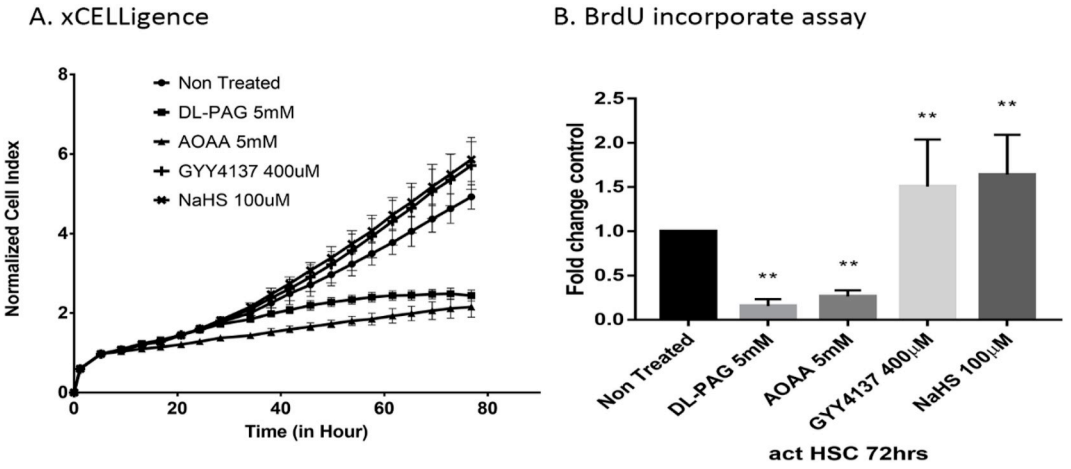

Fig. 4. H_2S promotes the proliferation of activated hepatic stellate cells.

proliferation (Fig. 4).

Culture-activated HSCs were treated with H_2S donors and enzyme inhibitors over period of 72 h. Cell proliferation was monitored by real-time xCELLigence system (**A**) and confirmed with BrdU incorporation ELISA assay (**B**). Inhibition of endogenous production of H_2S suppressed cell proliferation, whereas H_2S donors increased aHSCs proliferation. Data are presented ± SD.

3.4. H_2S increases cell metabolic activity

H_2S at low concentrations can increase cellular bioenergetics as an electron donor in mitochondrial oxidative phosphorylation [20,28]. Since enhanced bioenergetics is associated with HSC activation, we investigated the effect of H_2S on the bioenergetics of aHSCs. Two parameters of cellular metabolic activity, oxygen consumption rate (OCR) for mitochondrial oxidative phosphorylation and extracellular acidification rate (ECAR) for glycolysis, were determined using the Seahorse Extracellular Flux analyzer (Fig. 5). The H_2S donors GYY4137 and NaHS increased both the OCR and ECAR and ATP production, whereas the enzyme inhibitors DL-PAG and AOAA decreased metabolic activity of HSCs and ATP production.

Effect of H_2S donors and enzyme inhibitors on bioenergetics of aHSCs. Treatments with donors and inhibitors was for 48hrs. OCR and ECAR are represented as mean ± SEM of a representative experiment (**A, B**). Results were normalized with respect to the total amount of protein. Fold change of normalized maximal and basal level of OCR and ECAR between conditions were analyzed in 3 different experiments. For each experiment, every condition was repeated at least two times (**C, D**). Production of ATP was calculated using Seahorse XF Cell Mito Stress Test Report Generator software. Fold change of ATP production in experimental groups was calculated in 3 independent experiments (**E**).

3.5. *Cth* is specifically induced in hepatic stellate cells during fibrogenesis

We next evaluated the expression of H_2S synthesizing enzymes in the bile duct ligation model, an experimental model of chronic inflammation leading to fibrosis [29]. mRNA levels of all H_2S synthesizing enzymes, Cth, Cbs and Mpst, decreased progressively in the bile duct ligation model (Fig. 6A–C). As expected, expression of the profibrogenic cytokine TGFβ1 increased progressively in the bile duct ligation model (Fig. 6D). We next evaluated the effect of TGFβ1 on the mRNA expression of H_2S synthesizing enzymes in different liver cell populations. TGFβ1 decreased mRNA expression of all H_2S synthesizing enzymes in hepatocytes. In contrast, TGFβ1 increased mRNA expression of *Cth* in HSCs and did not change the mRNA expression of *Cbs* and *Mpst* in HSCs (Fig. 6E–G).

Comparison of H_2S synthesizing enzymes mRNA levels during fibrosis *in vivo* and *in vitro*. *Cth, Cbs, Mpst* were downregulated in total liver in the BDL model of liver fibrosis (**A,B,C**). *Tgfβ1* expression is increased in fibrosis (**D**). *36b4* was used as a housekeeping gene. TGFβ1 reduced the expression of H_2S synthesizing enzymes in hepatocytes (**F,G**), but it specifically induced *Cth* mRNA expression in HSCs *in vitro* (**E**).

4. Discussion

The main message of our study is that H_2S promotes hepatic stellate cell activation. This conclusion is based on the fact that production of H_2S and expression its producing enzyme cystathionine γ-lyase (*Cth*) expression are increased during hepatic stellate cell activation and on the fact that exogenous H_2S increased HSC proliferation while inhibitors of endogenous H_2S production reduce proliferation of HSCs. Although the inhibitors we used are not completely specific for one of the H_2S producing enzymes, e.g. the CBS inhibitor AOAA is also a potent inhibitor of CTH [30] it is important to note that reducing H_2S production leads to reduced stellate cell activation. In addition, since

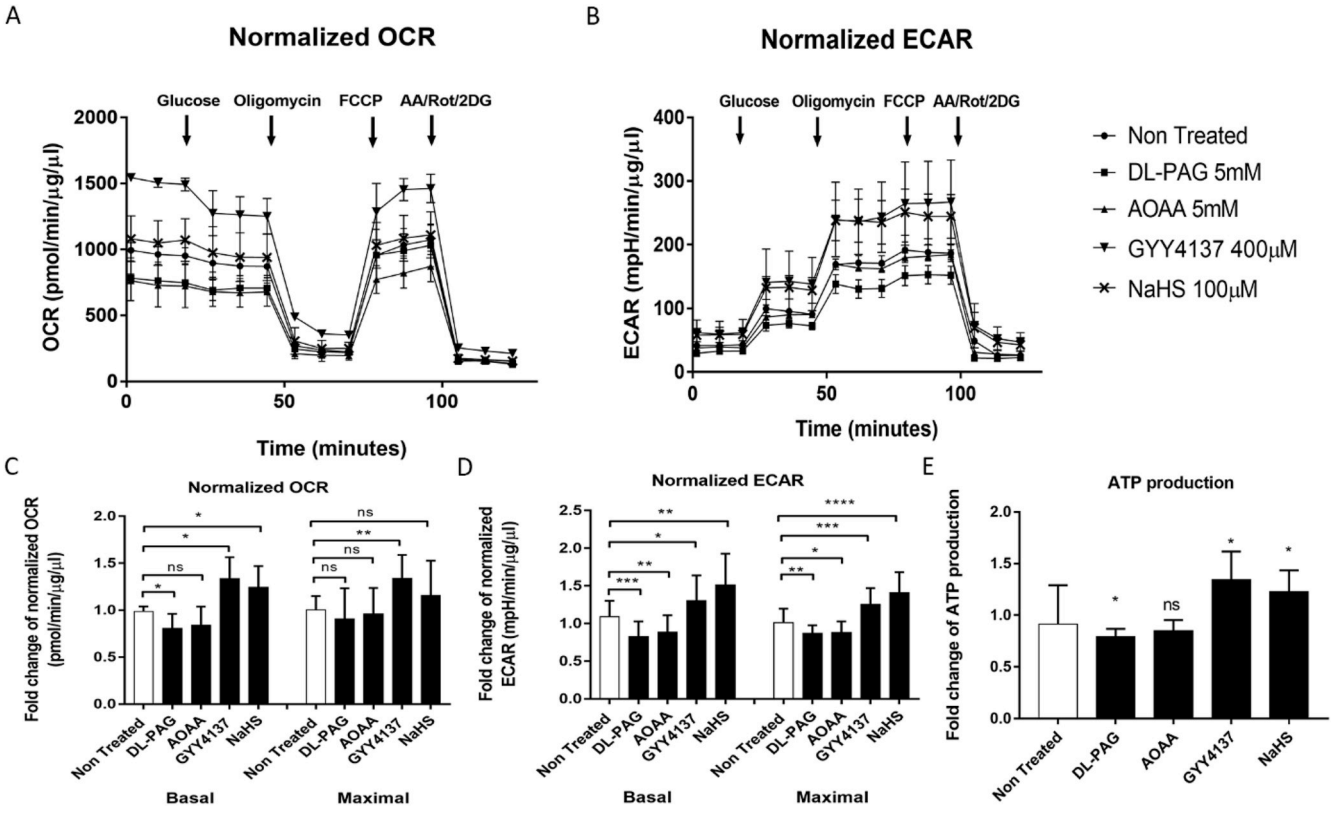

Fig. 5. H_2S increases mitochondrial oxidative phosphorylation and glycolysis in aHSCs.

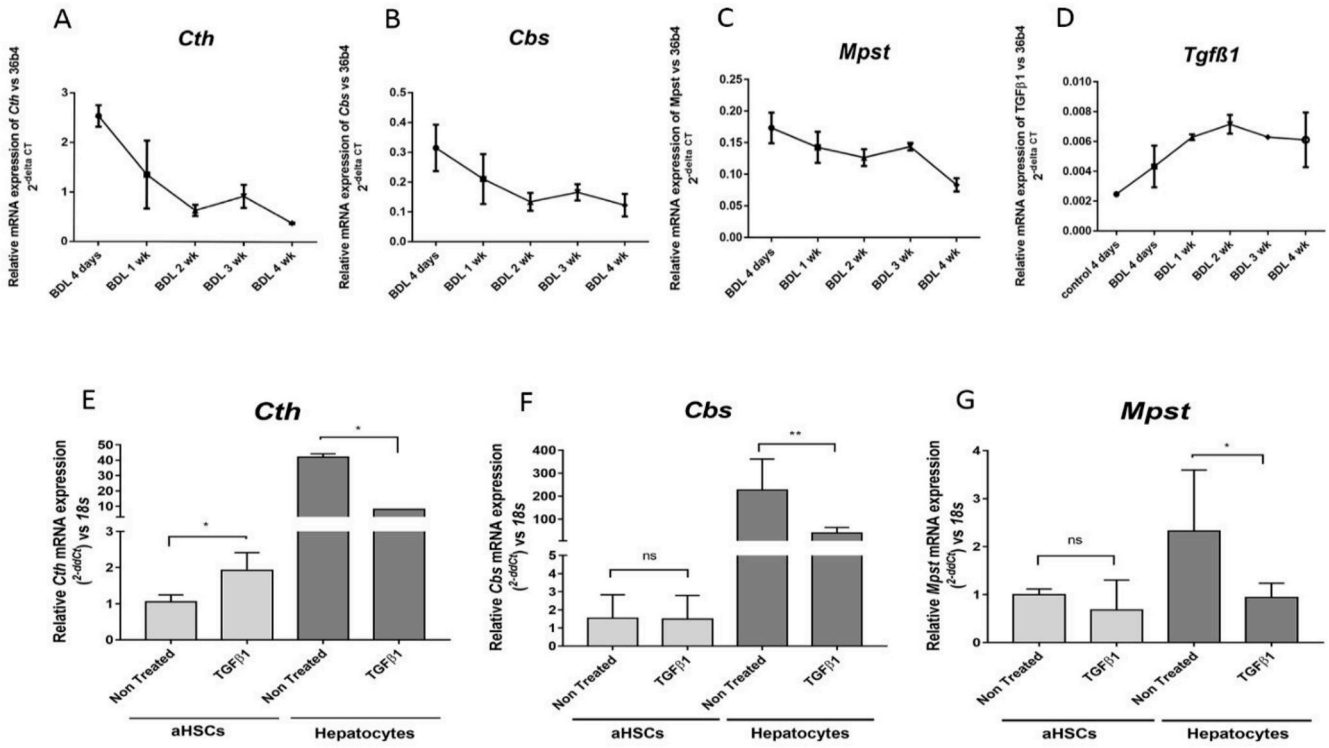

Fig. 6. mRNA expression of *Cth*, *Cbs* and *Mpst* in the bile duct ligation model of liver fibrosis and their regulation by TGFβ1 in different liver cell populations.

CTH is the sole enzyme to upregulated during HSCs activation, it is likely that the effect of AOAA is mediated via inhibition of CTH.

The effect of H_2S on stellate cell activation correlated with increased cellular bioenergetics. Previous *in vivo* studies reported that H_2S has anti-fibrotic properties due to its antioxidant and/or anti-inflammatory actions and its ability to reduce portal hypertension in the liver. In models of (experimental) fibrosis and cirrhosis, reduced expression of H_2S producing enzymes are observed and an anti-fibrotic effect as well as reduction of portal hypertension of systemically administered H_2S donors has been reported [7,13–15]. In line with this, *in vitro* studies, using the fast-releasing H_2S donor NaHS have demonstrated that H_2S inhibits stellate cell proliferation, possibly via decreasing the phosphorylation of p38 MAP-Kinase and increasing the phosphorylation of Akt [9,15]. In another study, the natural H_2S donor diallyl trisulfide suppressed activation of HSCs through cell cycle arrest at the G2/M checkpoint associated with downregulation of cyclin B1 and cyclin-dependent kinase 1 in primary rat HSCs [8]. However, the results described above were obtained using potentially toxic, fast-releasing H_2S donors, which is not representative of the continuous production of low levels of H_2S by cells. Furthermore, the use of systemically administered donors or inhibitors does not allow to distinguish effects of H_2S on different cell types present within one organ. Therefore, we applied 2 different H_2S releasing donors, GYY4137 and NaHS at concentrations 5 times as lower as in some *in vitro* studies. Furthermore, most studies used exogenous H_2S donors to study the role of H_2S in stellate cell activation and fibrogenesis and the importance of endogenous production of H_2S in HSC activation has not been properly addressed. Therefore, we also used 2 inhibitors of H_2S synthesizing enzymes (DL-PAG and AOAA) and we determined H_2S production by HSCs during the process of activation [8,9].

Our observations of increased expression of H_2S synthesizing enzyme *CTH* and increased H_2S production during HSC activation indicates a role for H_2S in HSC activation and fibrogenesis. Indeed, inhibition of endogenous H_2S production in HSCs reduced proliferation and expression of activation markers. These results are in line with the observation that platelet-derived growth factor BB (PDGF-BB) induced proliferation of rat mesangial cells via induction of CTH [31] and the observation that homocysteine, a precursor in H_2S synthesis, enhances activation of rat HSCs via activation of the PI3K/Akt pathway [32]. In contrast, an anti-fibrotic role has been proposed for cystathionine-β-synthase (CBS), another PLP-dependent enzyme which is involved in H_2S synthesis in the liver [33,34].

Since our results demonstrated a pro-fibrogenic effect of H_2S on HSCs, whereas most *in vivo* studies reported an anti-fibrotic role for H_2S, we investigated in more detail the H_2S generating capacity in different liver cell types. First, we determined that expression of H_2S-synthesizing enzymes in hepatocytes and Kupffer cells is much higher than in HSCs. Next, we determined the expression of H_2S-synthesizing enzymes in the bile duct ligation model of liver fibrosis. We observed a down-regulation of total hepatic expression of both *Cth* and *Cbs* in our bile duct ligation model. As expected, the pro-fibrogenic cytokine *Tgfβ1* was increased in the bile duct ligation model. Finally, we studied the effect of TGFβ1 on the expression of H_2S-synthesizing enzymes in different liver cell types. Of note, we observed that TGFβ1 decreases *Cth* and *Cbs* mRNA expression in hepatocytes, but increased *Cth* mRNA expression in stellate cells. These findings could explain the contradictory results between *in vivo* and *in vitro* studies with regard to the role of H_2S in fibrogenesis: since hepatocytes are the major source of H_2S in total liver, the increased expression of Tgfβ1 will lead to an overall reduction in the hepatic expression of Cth and Cbs and H_2S production, whereas at the same time it will increase expression of Cth and H_2S production in hepatic stellate cells. The cell-specific and local increase in H_2S generation also explains the effect of H_2S donors and inhibitors of H_2S-synthesizing enzymes on HSC proliferation and activation. Recently, Szabo et al. reported that a low exogenous dose of H_2S or endogenously produced H_2S increases mitochondrial oxidative phosphorylation [35,36]. In accordance, Katalin et al. described that low concentrations of H_2S stimulates mitochondrial bio-energetics via S-sulfhydration of ATP- synthase in HepG2 and HEK293 cell lines [20]. Activation of stellate cells is also accompanied by increased bioenergetics [37]. We have extended these findings by demonstrating that H_2S increases cellular bioenergetics in hepatic stellate cells.

In summary, we demonstrate that stellate cell activation is accompanied by increased generation of H_2S via induction of the H_2S-synthesizing enzyme CTH, leading to increased cellular bioenergetics and proliferation of HSCs. In addition, the response of H_2S-synthesizing enzymes to the fibrogenic cytokine Tgfβ1 is liver cell-type specific. Our results indicate that the H_2S generation in hepatic stellate cells is a target for anti-fibrotic intervention and that systemic interventions with H_2S should take into account cell-specific responses to H_2S.

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