Food and Chemical Toxicology 131 (2019) 110543

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Hydrogen sulfide impacts on inflammation-induced adipocyte dysfunction

Ferran Comas[a], Jèssica Latorre[a], Olaf Cussó[b], Francisco Ortega[a], Aina Lluch[a], Mònica Sabater[a], Anna Castells-Nobau[a], Wifredo Ricart[a,c], Xavier Ribas[b], Miquel Costas[b], José Manuel Fernández-Real[a,c,**], José María Moreno-Navarrete[a,c,*]

[a] Department of Diabetes, Endocrinology and Nutrition, Hospital of Girona 'Dr Josep Trueta'Institut d'Investigació Biomèdica de Girona (IdIBGi), CIBEROBN (CB06/03/010) and Instituto de Salud Carlos III (ISCIII), Girona, Spain

[b] Grup de Química Bioinspirada, Supramolecular i Catàlisi (QBIS-CAT), Institut de Química Computacional i Catàlisi (IQCC), Departament de Química, Universitat de Girona, C/M. Aurèlia Capmany 69, 17003, Girona, Catalonia, Spain

[c] Department of Medicine, Universitat de Girona, Girona, Spain

ARTICLE INFO

Keywords:
Hydrogen sulfide
3T3-L1
Adipocyte
Inflammation
GYY4137
Na_2S*Keywords:*
Hydrogen sulfide
3T3-L1 cell differentiation
Inflammation
GYY4137
Na_2S

ABSTRACT

A dual role of hydrogen sulfide (H_2S) in inflammation is well-reported and recent studies demonstrated adipogenic effects of H_2S in 3T3-L1 cells. Here, we aimed to investigate the effects of H_2S on adipocyte differentiation and inflammation. H_2S concentration in 3T3-L1 culture media was increased during adipocyte differentiation in parallel to adipogenic and *Cth* gene expression, and its inhibition using DL-Propargyl Glycine (PPG) impaired 3T3-L1 differentiation. GYY4137 and Na_2S administration only in the first or in the last stage of adipocyte differentiation resulted in a significant increased expression of adipogenic genes. However, when GYY4137 or Na_2S were administrated during all process no significant effects on adipogenic gene expression were found, suggesting that excessive H_2S administration might exert negative effects on adipogenesis. In fact, continuous addition of Na_2S, which resulted in Na_2S excess, inhibited adipogenesis, whereas time-expired Na_2S had no effect. In inflammatory conditions, GYY4137, but not Na_2S, administration attenuated the negative effects of inflammation on adipogenesis and insulin signaling-related gene expression during adipocyte differentiation. In inflamed adipocytes, Na_2S administration enhanced the negative effects of inflammatory process. Altogether these data showed that slow-releasing H_2S improved adipocyte differentiation in inflammatory conditions, and that H_2S proadipogenic effects depend on dose, donor and exposure time.

1. Introduction

Hydrogen sulfide (H_2S) biosynthesis has been identified in a variety of mammalian tissues, notably in the brain, heart, and the gastrointestinal tract, (Stipanuk, 2004; Yang et al., 2008), with a number of possible physiologic and pathophysiologic roles and a range of potential therapeutic uses (Li et al., 2009; Szabó, 2007; Whiteman and Moore, 2009). There is emerging evidence supporting the importance of H_2S in adipocytes, offering new insight into their role in the pathogenesis of obesity. Recent studies showed a possible role of H_2S in adipogenesis, enhancing PPARγ activity and stability and increasing glucose uptake and lipid storage (Cai et al., 2016). In fact, the CSE/H_2S system has been demonstrated to promote adipogenesis and fat mass accumulation in mice (Yang et al., 2018). However, opposite findings have been reported in other in vitro studies (Kim et al., 2012; Lii et al., 2012).

H_2S has recently gained significant attention as inflammation biological mediator. Antiinflammatory effect of H_2S has been reported in acute lung injury (Tokuda et al., 2012; Ang et al., 2011; Chen et al., 2009), and in kidney injury caused by urinary-derived sepsis (Chen et al., 2014). In vitro studies confirmed this antiinflammatory role (Yang et al., 2011) through the modulation of NFκB activity (Du et al., 2014). Antiinflammatory effect of H_2S is supported by the fact that H_2S deficiency contributes to switch of adipose tissue macrophages antiinflammatory M2 phenotype to proinflammatory M1 phenotype associated with obesity (Velmurugan et al., 2015). However, other studies reported that H_2S has proinflammatory effect in liver and aggravated LPS-induced liver damage (Yan et al., 2013; Zhang et al., 2007; Collin et al., 2005; Badiei et al., 2016).

Taking together these results it is evident that H_2S is implicated in the regulation of adipocyte differentiation and inflammation. Here, we hypothesize that a possible role of H_2S in the modulation of adipocyte inflammation might underlie its adipogenic effects. To test this

* Corresponding author. Department of Medicine, Universitat de Girona, Institut d'Investigació Biomèdica de Girona (IdIBGi), Girona, Spain.
** Corresponding author. Section of Diabetes, Endocrinology and Nutrition Hospital of Girona "Dr Josep Trueta" Carretera de França s/n, 17007, Girona, Spain.
E-mail addresses: jmfreal@idibgi.org (J.M. Fernández-Real), jmoreno@idibgi.org (J.M. Moreno-Navarrete).

https://doi.org/10.1016/j.fct.2019.05.051
Received 16 January 2019; Received in revised form 9 May 2019; Accepted 29 May 2019
Available online 30 May 2019

hypothesis, we aimed to investigate the possible role of GYY4137, a long-acting H_2S releasing donor, and sodium sulfide (Na_2S), a fast-releasing H_2S donor on adipocyte differentiation and inflammation.

2. Materials and methods

2.1. 3T3-L1 cell culture and differentiation

The embryonic fibroblast mouse cell line 3T3-L1 (American Type Culture Collection) was cultured in DMEM containing 4.5 g/L glucose, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. At 2 days after confluence, insulin (5 μg/ml), dexamethasone (0.25μM), and isobutylmethylxanthine (0.25 mM) mixture was added for 2 days, followed by 5 days with insulin (5 μg/ml) alone. DL-Propargyl Glycine (PPG, 0.25 and 1 mM) and H_2S donors [Na_2S (50 μM), GYY4137 (50 μM)] and time-expired Na_2S (50 μM) were directly added into the differentiation or adipocyte maintenance media. For hydrogen sulfide excess experiments, Na_2S and time-expired Na_2S were added every 12 h in first two days of adipocyte differentiation process and then every 24 h from day 2–7. Time-expired Na_2S, which was administrated to control the effects of sodium and oxidized sulfur species accumulation in the medium, was obtained as previously described by Tsai et al. (2015). Briefly, time-expired Na_2S was prepared in Dulbecco's phosphate buffered saline (pH = 7.4) (D-PBS, Sigma Chemical) at room temperature, and was obtained leaving Na_2S (50 μM) solution in an opened falcon 50 mL centrifuge tube in aseptic conditions during ~30 h.

Cells were then considered mature adipocytes, harvested, and stored at −80 °C for RNA extraction to study adipogenic and H_2S biosynthesis-related gene expression levels during 3T3-L1 differentiation.

Inflammatory conditions during 3T3-L1 differentiation and in mature adipocytes were induced by macrophage-conditioned media (MCM, 2%) and lipopolysaccharide (1 μg/ml) administration. During 3T3-L1 differentiation, MCM (2%) and LPS (1 μg/ml) was added in two steps of the differentiation process. To obtain inflamed adipocytes, MCM (2%) and LPS (1 μg/ml) administration during 24 h on fully 3T3-L1 differentiated adipocytes was performed.

Macrophage-conditioned medium was obtained as previously described (Moreno-Navarrete et al., 2009). Briefly, the human monocyte cell line THP-1 (American Type Culture Collection, Barcelona, Spain) was cultured in Rosswell Park Memmorial Institute (RPMI media 1640; Cat. No. 21870–076) 1640 medium containing 10% fetal bovine serum, 5 mM glucose, 2 mM L-glutamine, 50 μg/ml gentamicine and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 37 °C in a humidified 5%CO_2 per 95 °C air atmosphere. The mature macrophage-like state was induced by treating THP-1 cells ($1{,}2 \times 10^6$ cells) with 0.162 μM phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical, Madrid, Spain) in 6-well culture dishes for 24 h. Differentiated, plastic-adherent cells were washed with cold Dulbecco's phosphate buffered saline (D-PBS, Sigma Chemical) and then incubated with fresh medium without phorbol 12-myristate 13-acetate during additional 24 h. The supernatants (macrophage-conditioned media (MCM)) were collected, centrifuged at 900 *g* for 5 min, aliquoted and stored at −80 °C until testing.

All in vitro experiments were performed in four independent replicates.

2.2. Quantification of H_2S media concentration

H_2S concentration in cultured media was assessed using a naphthalimide-based fluorescent sensor 6-Azido-2-(2-(2-(2-hydroxyethoxy) ethoxy)ethyl)-1H-benzo[de]isoquinoline-1,3 (2H)-dione(L1), as described previously (Choi et al., 2016). To measure H_2S production, 3T3-L1 cells were incubated 24 h in DMEM media containing 10% (vol/vol) FBS and 5 μM of L1 probe, during different days of differentiation (0, 2 and 7). After incubation, media were transferred to new eppendorf tubes to be homogenized. To measure H_2S levels, a standard curve was generated from a 10 mM stock solution of sodium sulfide (Na_2S) in DMEM containing 10% (vol/vol) and 5 μM of L1 at various concentrations (0, 7.8, 15.6, 31.25, 62.5, 125, 250 and 500 μM Na_2S). Before reading standard curve was incubated during 90 min at 37 °C. After incubation, fluorescence was read in a BiotekCytation 5 reader at λ ex = 435 ± 10 nm and λ em = 550 ± 10 nm in duplicate.

2.3. RNA expression

RNA purification and gene expression procedures and analyses were performed as previously described (Moreno-Navarrete et al., 2014). Briefly, RNA purification was performed using an RNeasy Lipid Tissue Mini kit (QIAgen, Izasa S.A., Barcelona, Spain), and the integrity was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was assessed by real time PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Barcelona, Spain), using TaqMan technology suitable for relative genetic expression quantification. The RT-PCR reaction was performed in a final volume of 12 μl. The cycle program consisted of an initial denaturing of 10 min at 95 °C then 40 cycles of 15 s denaturizing phase at 95 °C and 1 min annealing and extension phase at 60 °C. A threshold cycle (Ct value) was obtained for each amplification curve and then a ΔΔCt value was calculated as follows: (Ct, target gene - Ct, endogenous control) treatment - (Ct, target gene - Ct, endogenous control) control or vehicle. Eukaryotic 18S rRNA was used as endogenous control. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta\Delta Ct}$, so that gene expression results are expressed as expression ratio relative to *18S* gene expression according to the manufacturer's guidelines. The following primer/probe sets were used: Adiponectin (Adipoq, Mm00456425_m1), peroxisome proliferator-activated receptor gamma (Pparg, Mm0000440940_m1), glucose transporter type 4 (Slc2a4, Mm00436615_m1), perlipin 1 (Plin1, Mm00558672_m1), CCAAT/enhancer-binding protein alpha (Cebpa, Mm00514283_s1), fatty acid synthase (Fasn, Mm00662319_m1), interleukin 6 (Il6, Mm00446190_m1), fatty acid binding protein 4 (Fabp4, Mm00445880_m1), tumor necrosis factor alpha (Tnf, Mm99999068_m1), diacylglycerol O-acyltransferase 1 (Dgat1, Mm00515643_m1), patatin Like Phospholipase Domain Containing 2 (Pnpla2, Mm00503040_m1), lipase E (Lipe, Mm00495359_m1), cystathionine gamma-lyase (Cth, Mm00461247) and cystathionine beta-synthase (Cbs, Mm00460654_m1).

2.4. Oil Red O staining

Intracellular lipid accumulation was assessed by Oil Red O staining. Cells were washed twice with PBS, fixed in 4% formaldehyde for 1 h, and stained for 30 min with 0.2% Oil Red O solution in 60% isopropanol. Cells were then washed several times with water, and excess water was evaporated by placing the stained cultures at 32 °C. Oil-red staining was quantified, as previously described (Lee et al., 2018) with Fiji software (Schindelin et al., 2012). The area occupied by lipid droplets stained by Oil Red O was selected and quantified using color threshold plugin (*Hue*: exclusion criteria 41–211) to identify red color regions. Then, Oil Red O staining was represented as % of selected stained area in comparison with image total area.

2.5. Statistical analysis

Statistical analyses were performed using SPSS statistical software (SPSS v21.0; IBM, Chicago, IL, USA). The non-parametric Mann–Whitney test was used. Levels of statistical significance were set at $p < 0.05$.

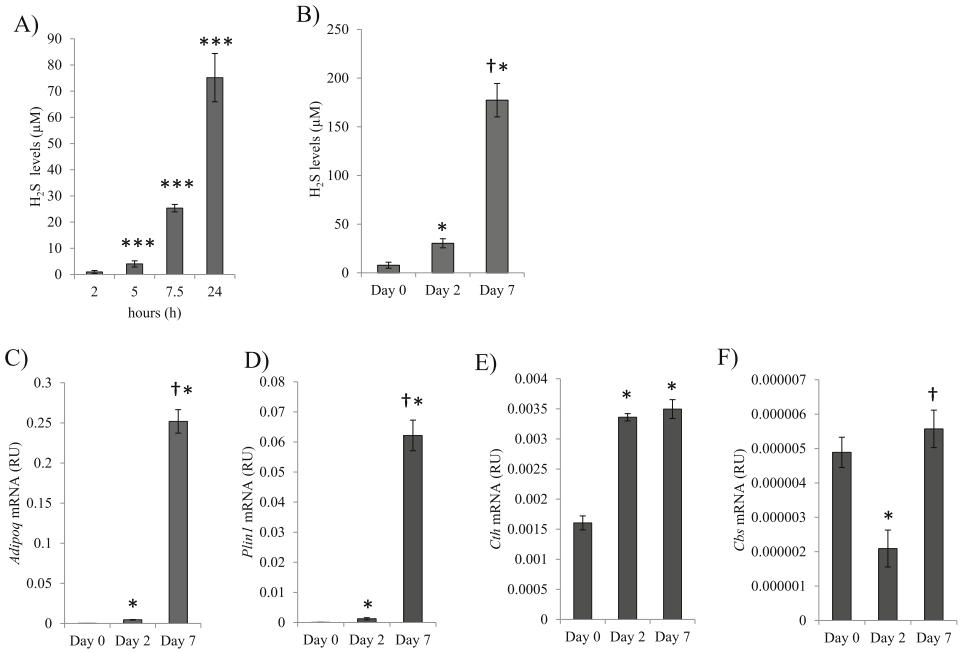

Fig. 1. A) Effects of L1 incubation (for 2, 5, 7.5 and 24 h) in 3T3-L1 cells (day 3 of adipocyte differentiation) on 3T3-L1-conditioned media H_2S concentration measurement. ***p < 0.001 compared to 2 h. **B–F)** H_2S levels **(B)**, *Adipoq* **(C)**, *Plin1* **(D)**, *Cth* **(E)** and *Cbs* **(F)** gene expression at days 0, 2 and 7 during adipocyte differentiation. *p < 0.05 compared with day 0 and †p < 0.05 compared with day 2.

3. Results

3.1. H_2S production during adipogenesis

Given the marked effects of hydrogen sulfide on adipocytes differentiation, we decided to explore H_2S synthesis in 3T3-L1 during adipogenesis. First, cell culture media from day 0, 2 or 7 plus L1 probe (5 μM) were incubated for 1 h, but H_2S levels was undetectable at any day. Then, to increase the performance and taking advantage of solubility and non-toxicity of L1 probe, 3T3-L1 cells were incubated with L1 probe (5 μM) for 2, 5, 7.5 and 24 h. A significant and cumulative increase in H_2S concentration was found, achieving the maximum levels at 24 h (Fig. 1A). Interestingly, 24 h H_2S (μM) accumulation in media increased significantly from day 0 to day 2 and from day 2 to day 7, confirming that H_2S is generated during adipogenesis (Fig. 1B). The maximum levels of H_2S accumulation in the media were associated with the higher adipogenic gene expression (Fig. 1C and D). Then, expression of H_2S-generating genes Cth (also named CSE) and Cbs were analysed. *Cth* was highly expressed compared to *Cbs* gene [0.0034962 ± 0.0001561 (Ct value 25.49 ± 0.21) *vs* 0.0000055 ± 0.0000005 (Ct value 34.81 ± 0.36) RU, p < 0.0001], supporting Cth as the main source of adipogenesis-associated H_2S biosynthesis (Yang et al., 2018). In fact, *Cth* gene expression was increased in the first stage of adipocyte differentiation process (from day 0–2), and then these levels were maintained until the end of the process (day 7) (Fig. 1E), whereas *Cbs* mRNA decreased at day 2, and recovered at day 7 (Fig. 1F).

3.2. Effects of PPG in 3T3-L1 during adipogenesis

We decided to study the effect of CTH inhibitor (PPG, 0.25 and 1 mM) on adipogenic related gene expression, as a way to identify the effect of decreased H_2S synthesis during adipogenesis. During adipogenesis, 0.25 and 1 mM PPG administration during adipogenesis led to decreased adipogenic (*Pparg, Adipoq, Glut4, Plin1* and *Cebpa*), lipogenic (*Fasn* and *Dgat1*) and lipolytic (*Pnpla2* and *Lipe*) gene expression at dose dependent-manner, and increased expression of inflammatory related gene *Il6* (Fig. 2A). As shown by Red Oil O staining, PPG (1 mM) treatment significantly decreased adipogenesis and adipocytes lipid accumulation in concordance with gene expression results (Fig. 2B). Even though, no significant effects of PPG on hydrogen sulfide synthesis related genes (*Cth* and *Cbs*) were observed (Fig. 1B), PPG administration led to decreased H_2S accumulation at day 7 (Fig. 2C).

These findings reveal that certain levels of hydrogen sulfide are necessary for adipocyte differentiation.

3.3. Effects of Na_2S and GYY4137 in 3T3-L1 during adipogenesis

Treatment with GYY4137 and Na_2S during all the differentiation process of 3T3-L1 (Day 0–7) had no significant effects (Fig. 3A). At day 7, increased H_2S accumulation in Na_2S, but not GYY4137, was observed (Fig. 3B). Interestingly, administration of GYY4137 and Na_2S in the first stage (Day 0–2) and late stage (Day 2–7) of adipocyte differentiation resulted in a significant increase of adipogenic (*Adipoq, Pparg, Glut4* and *Fabp4*) and lipogenic (*Fasn*) genes (Fig. 3C and D).

3.4. Effects of Na_2S excess and time-expired Na_2S in 3T3-L1 during adipogenesis

First, to evaluate the stability and the effects of one single dose of Na_2S (50 μM) on H_2S levels, the loss of H_2S concentration in phosphate-buffered saline (PBS) was measured in cell culture conditions (without cells) during 4 days at several points (0, 24, 48, 72 and 96 h, Fig. 4A). This experiment showed a 60% reduction of H_2S levels at 24 h. This information led to estimate excess H_2S levels in daily Na_2S administration experiment, suggesting the following concentrations: ~35 μM at 12 h, ~59.6 μM at 24 h, ~76.6 μM at 36 h, ~88.6 μM at 48 h, ~55.4 μM at 72 h, ~42.2 μM at 96 h, ~36.9 μM at 120 h, ~34.7 μM at 144 h and ~33.9 μM at 168 h.

Next, the effect of H_2S excess on adipogenesis was assessed. Continuous addition of Na_2S (50 μM) or time-expired Na_2S administration during adipocyte differentiation process were performed as detailed in methods. Time-expired Na_2S had undetectable H_2S levels. Na_2S excess led to decreased adipogenic (*Pparg, Adipoq, Glut4* and *Fapb4*), lipogenic (*Fasn*) and lipolytic (*Pnpla2* and *Lipe*) gene expression, and tend to increase expression of *Il6* (Fig. 4B). Red Oil O staining confirmed that Na_2S daily administration inhibited adipogenesis (Fig. 4C). In contrast, time-expired Na_2S had no significant effects in the expression of adipogenic and lipogenic genes, whilst significant decreased lipolytic (*Pnpla2*) and inflammatory (*Il6*) gene expression was reported (Fig. 4B).

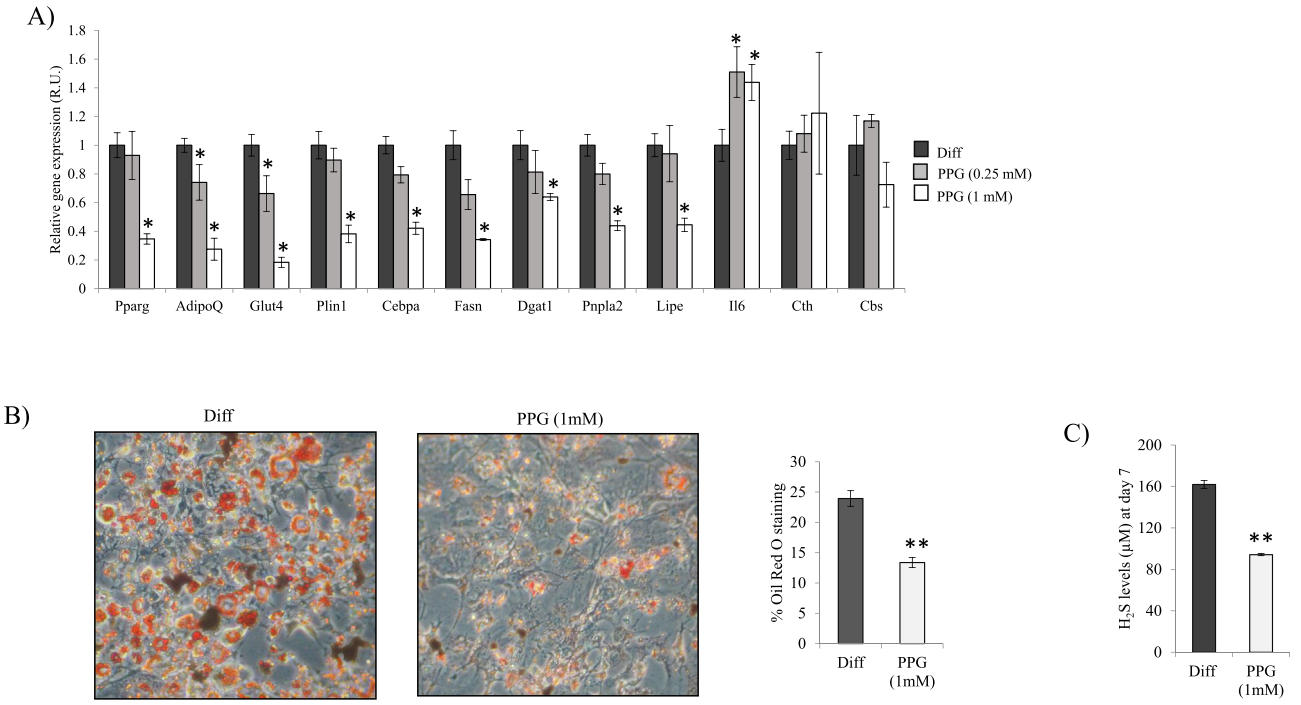

Fig. 2. A-C) Effects of PPG (0,25 and 1 mM) administration during 3T3-L1 differentiation on adipogenic, lipogenic, lipolytic, inflammatory and hydrogen sulfide synthesis gene expression (**A**), intracellular lipid accumulation using Red Oil staining (**B**) and H_2S levels at day 7 **(C)**. Control differentiated (black bars), 0.25 mM PPG (grey bars) and 1 mM PPG (white bars). *p < 0.05 and **p < 0.01 compared with control differentiated. These data are expressed as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Effects of Na_2S and GYY4137 in 3T3-L1 during adipocyte differentiation in inflammatory conditions

Inflammatory conditions (Diff + MCM) resulted in decreased adipogenesis (*Adipoq* and *Fasn*) and insulin action (*Glut4*)-related gene expression and increased expression of *Il6* gene (Fig. 5A). GYY4137, but not Na_2S, administration attenuated the negative effects of inflammation on *Adipoq*, *Fasn* and *Glut4* gene expression, but not *Il6*, during adipocyte differentiation (Fig. 5A).

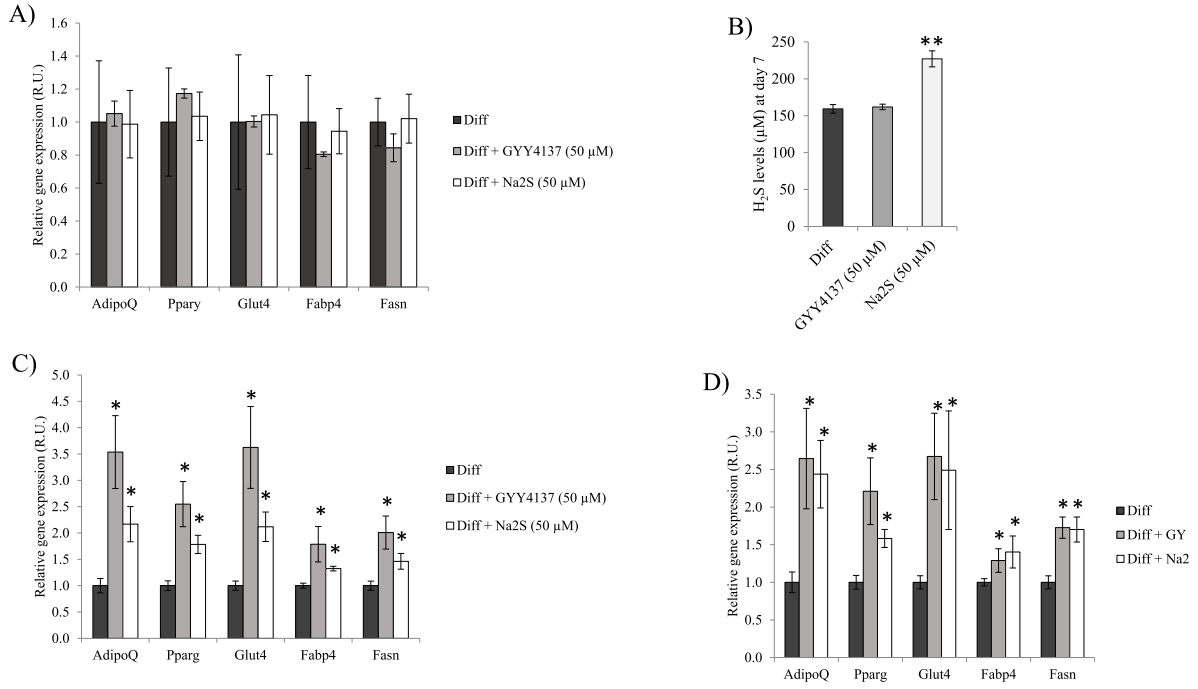

Fig. 3. A-B) Effects of GYY4137 (50 µM) and Na_2S (50 µM) treatments on expression of adipogenic and lipogenic related genes **(A)** and H_2S levels **(B)** at day 7, administrating hydrogen sulfide donors from day 0–7 during 3T3-L1 adipogenesis. **C-D)** Effects of GYY4137 (50 µM) and Na_2S (50 µM) treatments on expression of adipogenic and lipogenic related genes at day 7, administrating hydrogen sulfide donors only from day 0–2 (**C**) or from day 2–7 (**D**). Control differentiated (black bars), GYY4137 (grey bars) and Na_2S (white bars). *p < 0.05 and **p < 0.01 compared with control differentiated. These data are expressed as mean ± SEM.

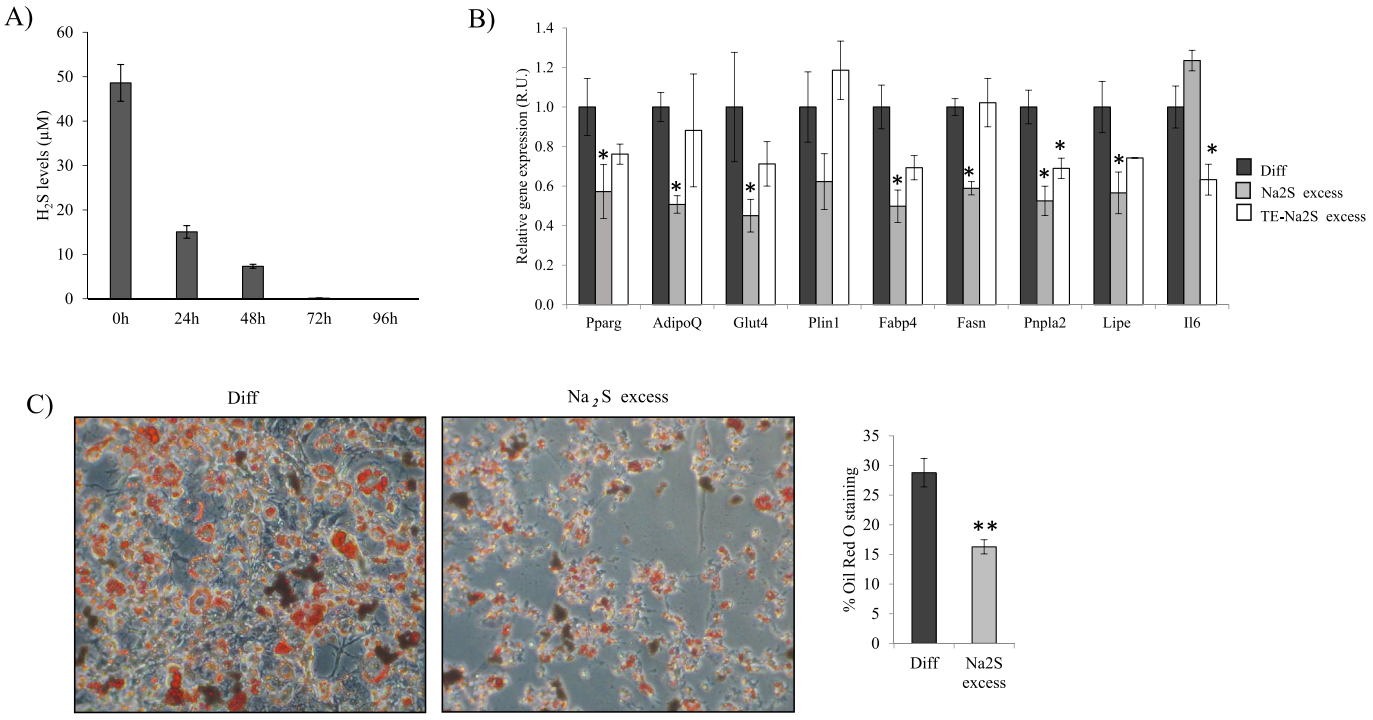

Fig. 4. A) Effects of one single dose of Na_2S (50 μM diluted in PBS) on H_2S levels in cell culture conditions (without cells) at several points (0, 24, 48, 72 and 96 h). **B–C)** Effects of Na_2S and time-expired Na_2S excess administration on expression of adipogenic, lipogenic and lipolytic related genes during 3T3-L1 adipogenesis (**B**), and intracellular lipid accumulation using Oil red staining (**C**). Control differentiated (black bars), Na_2S excess (grey bars) and time-expired Na_2S excess (white bars). *$p < 0.05$ compared with control differentiated. These data are expressed as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.6. Effects of Na_2S and GYY4137 in fully differentiated 3T3-L1 adipocytes in inflammatory conditions

Treatment of inflamed adipocytes with Na_2S led to decreased adipogenic gene expression (*Adipoq, Pparg* and *Fabp4*) and insulin action (*Glut4*) related gene expression and increases inflammatory gene expression (*Il6*), whereas GYY4137 treatment only increases significantly *Pparg*, tend to decrease the expression of *Il6*, and had no significant effect in the expression of the other adipogenic-related genes studied (Fig. 5B).

4. Discussion

To best of our knowledge this is the first study exploring the role of H_2S in 3T3-L1 preadipocytes during differentiation and in mature adipocytes in inflammatory conditions using different H_2S donors. Specifically, H_2S biosynthesis was increased during adipocyte differentiation, and H_2S donor (GYY4137 and Na_2S) administration at different stages of adipocyte differentiation process resulted in enhanced adipogenesis, whereas treatments with a specific inhibitor of endogenous H_2S biosynthesis (PPG) impaired adipogenesis and increased inflammation. Even though, several studies reported H_2S concentration in human serum in a range of 10–200 μM (Karunya et al., 2019; Chen et al., 2005; Li et al., 2005; Peng et al., 2011) or higher (Hamidi Shishavan et al., 2017), other studies (Whitfield et al., 2008; Furne et al., 2008; Olson, 2009; Shen et al., 2012) argue reduced H_2S levels in serum using sound arguments. In current study, to evaluate H_2S biosynthesis at different stage of adipocyte differentiation (day 0, 2 and 7), the 24 h cumulative H_2S concentration were measured, indicating cell capacity for H_2S production, but not its physiological levels. In line with these studies (Whitfield et al., 2008; Furne et al., 2008; Olson, 2009; Shen et al., 2012), when adipocyte conditioned medium with L1 probe was incubated for 1 h, H_2S concentration (physiological levels) were undetectable.

In addition, GYY4137 administration attenuated the negative effects of inflammation during adipogenesis (Constant et al., 2008; Yarmo et al., 2010), whereas Na_2S aggravates the negative effects of inflammation on mature inflamed adipocytes.

These adipogenic and antiinflammatory activities of GYY4137 might be explained through the sulfhydration of PPARγ and NFκB cysteine residues (Cai et al., 2016; Du et al., 2014). Sulfhydrated PPARγ increased its nuclear accumulation, DNA binding activity and adipogenesis gene expression, thereby increasing glucose uptake and lipid storage (Cai et al., 2016), whereas sulfhydrated p65 subunit of NF-κB at cysteine-38 inhibited macrophage inflammation by suppressing NFκB pathway activation (Du et al., 2014). Supporting current data, Na_2S and GYY4137 administration was found to be associated positively with adipogenic and lipogenic gene expression in 3T3-L1 cells, whereas ZYJ1122 (a structural analogue of GYY4137 lacking sulfur) had not any effects in these gene expression (Tsai et al., 2015). Furthermore, Lee and colleagues demonstrated that hydrogen sulfide donor, diallyl disulfide, promotes adipogenesis in 3T3-L1 cells (Lee et al., 2007). On the other hand, other studies reported antiadipogenic effect of hydrogen sulfide donors derived from garlic (Kim et al., 2012; Lii et al., 2012). Of note, daily administration of Na_2S during 3T3-L1 differentiation inhibited adipogenesis, suggesting that H_2S excess suppressed adipogenic differentiation. No significant effects of time-expired Na_2S on adipogenic markers were found, indicating that H_2S from donors was the responsible of adipogenic effect. Donors used in current study released H_2S at different rates, Na_2S is a fast-releasing H_2S donor and GYY4137 is a slow-releasing H_2S donor, producing in consequence different concentrations of the H_2S at several times (Whiteman et al., 2010). These differences in H_2S availability and concentration could explain why GYY4137 was more effective than Na_2S in promoting adipocyte differentiation and attenuating the negative effects of inflammation on adipogenesis.

Inflammation in adipocytes is known to decrease adipogenesis in parallel to increased insulin resistance (Gustafson and Smith, 2006), so

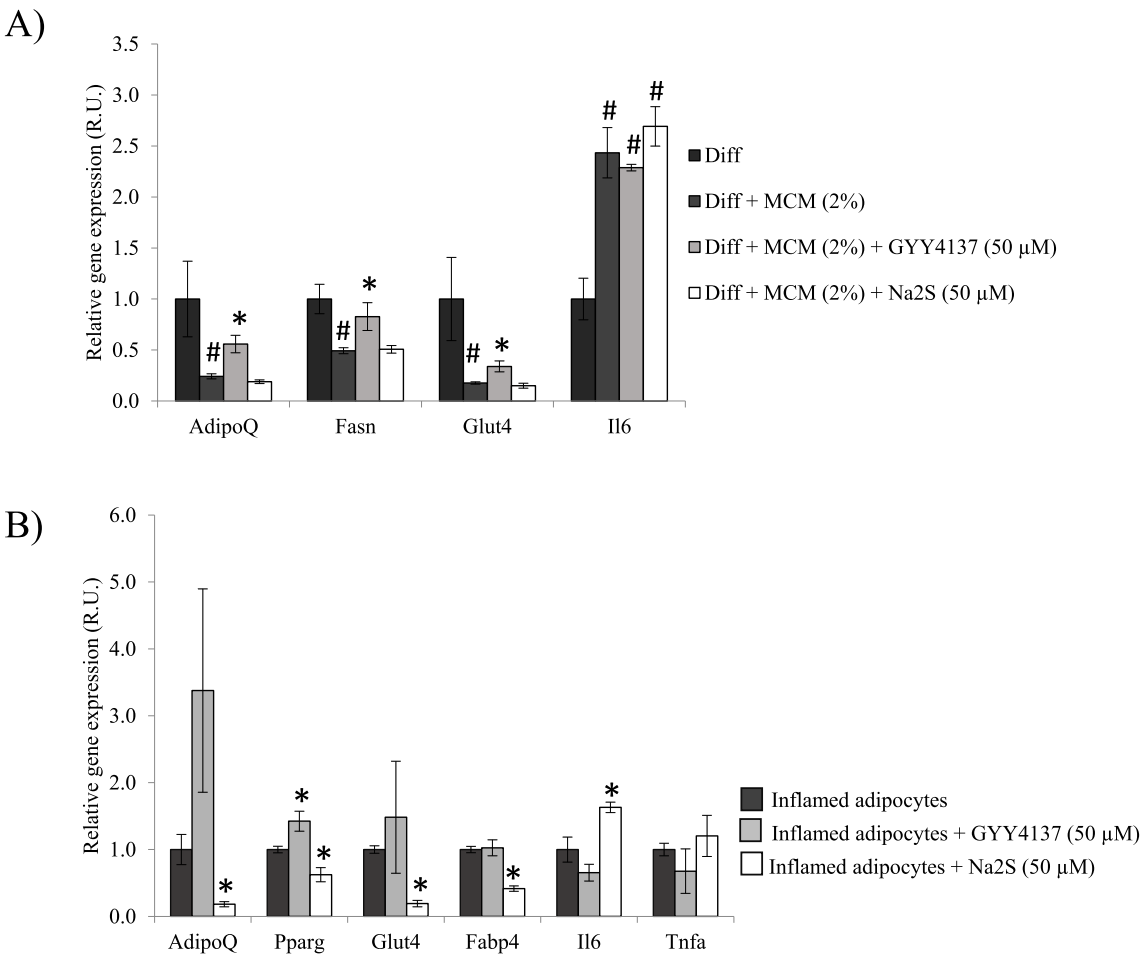

Fig. 5. A) Effects of GYY4137 (50 µM) and Na_2S (50 µM) administration on adipogenic and inflammatory gene expression during 3T3-L1 differentiation in inflammatory conditions at day 7. **B)** Effects of GYY4137 (50 µM) and Na_2S (50 µM) administration during 48 h on adipogenic and inflammatory gene expression in inflamed fully differentiated 3T3-L1 adipocytes. Control differentiated (black bars), inflamed control (dark grey bars), GYY4137 (grey bars) and Na_2S (white bars). #p < 0.05 compared with control differentiated and *p < 0.05 compared with inflamed control. These data are expressed as mean ± SEM.

novel therapeutic strategies targeting adipose tissue to mitigate inflammation are emerging (Kusminski et al., 2016). Current findings reveal that GYY4137 may represent an innovative therapeutic tool against obesity-related adipose tissue inflammation.

It is well known that GYY4137 release is slower than Na_2S (Powell et al., 2018; Rose et al., 2015). A previous study demonstrated that the rate of H_2S release from GYY4137 (1 mM, pH 7.4, 37 °C) was 4.17 ± 0.5 nmol/25 min (Li et al., 2008). In agreement with these studies, GYY4137 (50 µM) administration did not result in a significant increase in H_2S levels in adipocyte conditioned media.

In conclusion, altogether these data demonstrated that H_2S in adipocytes plays a pivotal role regulating adipogenesis under normal and pro-inflammatory conditions. Interactions between adipocytes and H_2S may represent a novel target for restoring adipocytes physiologic function.

Funding

This work was partially supported by research grants PI15/01934 and PI16/01173 from the Instituto de Salud Carlos III from Spain and VII Spanish Diabetes Association grants to Basic Diabetes Research Projects led by young researchers. CIBEROBN Fisiopatología de la Obesidad y Nutrición is an initiative from the Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional (FEDER) from Spain.

Declarations of interest

None.

Conflicts of interest

The authors declare no conflict of interest.

Authors' contributions.

FC, JMF-R and JMM-N participated in conception and design of the study, acquisition of data and analysis and interpretation of data, and drafted the article. JL, OC, FO, AL, MS, AC-N, WR, XR, MC participated in acquisition and analysis of data, and read the article critically for important intellectual content. All authors read and approved the final manuscript, and agreed to be accountable for all aspects of the work.

Acknowledgements

This work was partially supported by research grants PI15/01934 and PI16/01173 from the Instituto de Salud Carlos III from Spain and VII Spanish Diabetes Association grants to Basic Diabetes Research Projects led by young researchers. CIBEROBN Fisiopatología de la Obesidad y Nutrición is an initiative from the Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional (FEDER) from Spain.

References

Ang, S.-F., Sio, S.W.S., Moochhala, S.M., MacAry, P.A., Bhatia, M., 2011. Hydrogen sulfide upregulates cyclooxygenase-2 and prostaglandin E metabolite in sepsis-evoked acute lung injury via transient receptor potential vanilloid type 1 channel activation. J. Immunol. 187 (9), 4778–4787. https://doi.org/10.4049/jimmunol.1101559.

Badiei, A., Chambers, S.T., Gaddam, R.R., Bhatia, M., 2016. Cystathionine-γ-lyase gene silencing with siRNA in monocytes/macrophages attenuates inflammation in cecal ligation and puncture-induced sepsis in the mouse. J. Bio Sci. 41 (1), 87–95. http://www.ncbi.nlm.nih.gov/pubmed/26949091, Accessed date: 4 December 2018.

Cai, J., Shi, X., Wang, H., et al., 2016. Cystathionine γ lyase–hydrogen sulfide increases peroxisome proliferator-activated receptor γ activity by sulfhydration at C139 site thereby promoting glucose uptake and lipid storage in adipocytes. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1861 (5), 419–429. https://doi.org/10.1016/j.bbalip.2016.03.001.

Chen, Y.H., Yao, W.Z., Geng, B., Ding, Y.L., Lu, M., Zhao, M.W., Tang, C.S., 2005. Endogenous hydrogen sulfide in patients with COPD. Chest 128 (5), 3205–3211. https://doi.org/10.1378/chest.128.5.3205.

Chen, Y.-H., Wu, R., Geng, B., et al., 2009. Endogenous hydrogen sulfide reduces airway inflammation and remodeling in a rat model of asthma. Cytokine 45 (2), 117–123. https://doi.org/10.1016/j.cyto.2008.11.009.

Chen, X., Xu, W., Wang, Y., et al., 2014. Hydrogen sulfide reduces kidney injury due to urinary-derived sepsis by inhibiting NF-κB expression, decreasing TNF-α levels and increasing IL-10 levels. Exp. Ther. Med. 8 (2), 464–470. https://doi.org/10.3892/etm.2014.1781.

Choi, S.-A., Park, C.S., Kwon, O.S., et al., 2016. Structural effects of naphthalimide-based fluorescent sensor for hydrogen sulfide and imaging in live zebrafish. Sci. Rep. 6 (1), 26203. https://doi.org/10.1038/srep26203.

Collin, M., Anuar, F.B.M., Murch, O., Bhatia, M., Moore, P.K., Thiemermann, C., 2005. Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. Br. J. Pharmacol. 146 (4), 498–505. https://doi.org/10.1038/sj.bjp.0706367.

Constant, V.A., Gagnon, A., Yarmo, M., Sorisky, A., 2008. The antiadipogenic effect of macrophage-conditioned medium depends on ERK1/2 activation. Metabolism 57 (4), 465–472. https://doi.org/10.1016/j.metabol.2007.11.005.

Du, J., Huang, Y., Yan, H., Zhang, Q., Zhao, M., Zhu, M., Liu, J., Chen, S.X., Bu, D., Tang, C., Jin, H., 2014. Hydrogen sulfide suppresses oxidized low-density lipoprotein (ox-LDL)-stimulated monocyte chemoattractant protein 1 generation from macrophages via the nuclear factor κB (NF-κB) pathway. J. Biol. Chem. 289 (14), 9741–9753. https://doi.org/10.1074/jbc.M113.517995.

Furne, J., Saeed, A., Levitt, M.D., 2008. Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. Am. J. Physiol. Regul. Integr. Comp. Physiol. 295 (5), R1479–R1485. https://doi.org/10.1152/ajpregu.90566.2008.

Gustafson, B., Smith, U., 2006. Cytokines promote wnt signaling and inflammation and impair the normal differentiation and lipid accumulation in 3t3-L1 preadipocytes. J. Biol. Chem. 281 (14), 9507–9516. https://doi.org/10.1074/jbc.M512077200.

Hamidi Shishavan, M., Henning, R.H., van Buiten, A., Goris, M., Deelman, L.E., Buikema, H., 2017. Metformin improves endothelial function and reduces blood pressure in diabetic spontaneously hypertensive rats independent from glycemia control: comparison to vildagliptin. Sci. Rep. 7 (1), 10975. https://doi.org/10.1038/s41598-017-11430-7.

Karunya, R., Jayaprakash, K.S., Gaikwad, R., Sajeesh, P., Ramshad, K., Muraleedharan, K.M., Dixit, M., Thangaraj, P.R., Sen, A.K., 2019. Rapid measurement of hydrogen sulphide in human blood plasma using a microfluidic method. Sci. Rep. 9 (1), 3258. https://doi.org/10.1038/s41598-019-39389-7.

Kim, E.J., Lee, D.H., Kim, H.J., et al., 2012. Thiacremonone, a sulfur compound isolated from garlic, attenuates lipid accumulation partially mediated via AMPK activation in 3T3-L1 adipocytes. J. Nutr. Biochem. 23 (12), 1552–1558. https://doi.org/10.1016/j.jnutbio.2011.10.008.

Kusminski, C.M., Bickel, P.E., Scherer, P.E., 2016. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat. Rev. Drug Discov. 15 (9), 639–660. https://doi.org/10.1038/nrd.2016.75.

Lee, J.-H., Kim, K.-A., Kwon, K.-B., et al., 2007. Diallyl disulfide accelerates adipogenesis in 3T3-L1 cells. Int. J. Mol. Med. 20 (1), 59–64. http://www.ncbi.nlm.nih.gov/pubmed/17549389, Accessed date: 4 December 2018.

Lee, M., Katerelos, M., Gleich, K., Galic, S., Kemp, B.E., Mount, P.F., Power, D.A., 2018. Phosphorylation of acetyl-CoA carboxylase by AMPK reduces renal fibrosis and is essential for the anti-fibrotic effect of metformin. J. Am. Soc. Nephrol. 29 (9), 2326–2336. https://doi.org/10.1681/ASN.2018010050.

Li, L., Bhatia, M., Zhu, Y.Z., Zhu, Y.C., Ramnath, R.D., Wang, Z.J., Anuar, F.B., Whiteman, M., Salto-Tellez, M., Moore, P.K., 2005. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J. 19 (9), 1196–1198. https://doi.org/10.1096/fj.04-3583fje.

Li, L., Whiteman, M., Guan, Y.Y., Neo, K.L., Cheng, Y., Lee, S.W., Zhao, Y., Baskar, R., Tan, C.H., Moore, P.K., 2008. Characterization of a novel, water-soluble hydrogen sulfide-releasing molecule (GYY4137): new insights into the biology of hydrogen sulfide. Circulation 117 (18), 2351–2360. https://doi.org/10.1161/CIRCULATIONAHA.107.753467.

Li, L., Hsu, A., Moore, P.K., 2009. Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation — a tale of three gases!. Pharmacol. Ther. 123 (3), 386–400. https://doi.org/10.1016/j.pharmthera.2009.05.005.

Lii, C.-K., Huang, C.-Y., Chen, H.-W., et al., 2012. Diallyl trisulfide suppresses the adipogenesis of 3T3-L1 preadipocytes through ERK activation. Food Chem. Toxicol. 50 (3–4), 478–484. https://doi.org/10.1016/j.fct.2011.11.020.

Moreno-Navarrete, J.M., Ortega, F.J., Ricart, W., Fernandez-Real, J.M., 2009. Lactoferrin increases (172Thr)AMPK phosphorylation and insulin-induced (p473Ser)AKT while impairing adipocyte differentiation. Int. J. Obes. 33 (9), 991–1000. https://doi.org/10.1038/ijo.2009.143.

Moreno-Navarrete, J.M., Ortega, F., Moreno, M., Serrano, M., Ricart, W., Fernández-Real, J.M., 2014. Lactoferrin gene knockdown leads to similar effects to iron chelation in human adipocytes. J. Cell Mol. Med. 18 (3), 391–395. https://doi.org/10.1111/jcmm.12234.

Olson, K.R., 2009. Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? Biochim. Biophys. Acta 1787 (7), 856–863. https://doi.org/10.1016/j.bbabio.2009.03.019.

Peng, H., Cheng, Y., Dai, C., King, A.L., Predmore, B.L., Lefer, D.J., Wang, B., 2011 Oct 4. A fluorescent probe for fast and quantitative detection of hydrogen sulfide in blood. Angew Chem. Int. Ed. Engl. 50 (41), 9672–9675. https://doi.org/10.1002/anie.201104236.

Powell, C.R., Dillon, K.M., Matson, J.B., 2018. A review of hydrogen sulfide (H2S) donors: chemistry and potential therapeutic applications. Biochem. Pharmacol. 149, 110–123. https://doi.org/10.1016/j.bcp.2017.11.014.

Rose, P., Dymock, B.W., Moore, P.K., 2015. GYY4137, a novel water-soluble, H2S-releasing molecule. Methods Enzymol. 554, 143–167. https://doi.org/10.1016/bs.mie.2014.11.014.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9 (7), 676–682. https://doi.org/10.1038/nmeth.2019.

Shen, X., Peter, E.A., Bir, S., Wang, R., Kevil, C.G., 2012. Analytical measurement of discrete hydrogen sulfide pools in biological specimens. Free Radic. Biol. Med. 52 (11–12), 2276–2283. https://doi.org/10.1016/j.freeradbiomed.2012.04.007.

Stipanuk, M.H., 2004. SULFUR AMINO ACID METABOLISM: pathways for production and removal of homocysteine and cysteine. Annu. Rev. Nutr. 24 (1), 539–577. https://doi.org/10.1146/annurev.nutr.24.012003.132418.

Szabó, C., 2007. Hydrogen sulphide and its therapeutic potential. Nat. Rev. Drug Discov. 6 (11), 917–935. https://doi.org/10.1038/nrd2425.

Tokuda, K., Kida, K., Marutani, E., et al., 2012. Inhaled hydrogen sulfide prevents endotoxin-induced systemic inflammation and improves survival by altering sulfide metabolism in mice. Antioxidants Redox Signal. 17 (1), 11–21. https://doi.org/10.1089/ars.2011.4363.

Tsai, C.-Y., Peh, M.T., Feng, W., Dymock, B.W., Moore, P.K., 2015. Hydrogen sulfide promotes adipogenesis in 3T3L1 cells. PLoS One 10 (3). https://doi.org/10.1371/journal.pone.0119511. e0119511.

Velmurugan, G.V., Huang, H., Sun, H., et al., 2015. Depletion of H_2S during obesity enhances store-operated Ca2+ entry in adipose tissue macrophages to increase cytokine production. Sci. Signal. 8 (407), ra128. https://doi.org/10.1126/scisignal.aac7135.

Whiteman, M., Moore, P.K., 2009. Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability? J. Cell Mol. Med. 13 (3), 488–507. https://doi.org/10.1111/j.1582-4934.2009.00645.x.

Whiteman, M., Li, L., Rose, P., Tan, C.-H., Parkinson, D.B., Moore, P.K., 2010. The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages. Antioxidants Redox Signal. 12 (10), 1147–1154. https://doi.org/10.1089/ars.2009.2899.

Whitfield, N.L., Kreimier, E.L., Verdial, F.C., Skovgaard, N., Olson, K.R., 2008. Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294 (6), R1930–R1937. https://doi.org/10.1152/ajpregu.00025.2008.

Yan, Y., Chen, C., Zhou, H., et al., 2013. Endogenous hydrogen sulfide formation mediates the liver damage in endotoxemic rats. Res. Vet. Sci. 94 (3), 590–595. https://doi.org/10.1016/j.rvsc.2012.10.009.

Yang, G., Wu, L., Jiang, B., et al., 2008 Oct 24. H2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. Science 322 (5901), 587–590. https://doi.org/10.1126/science.1162667.

Yang, C., Yang, Z., Zhang, M., et al., 2011. Hydrogen sulfide protects against chemical hypoxia-induced cytotoxicity and inflammation in HaCaT cells through inhibition of ROS/NF-κB/COX-2 pathway. Agarwal, S. (Ed.), PLoS One 6 (7), e21971. https://doi.org/10.1371/journal.pone.0021971.

Yang, G., Ju, Y., Fu, M., et al., 2018. Cystathionine gamma-lyase/hydrogen sulfide system is essential for adipogenesis and fat mass accumulation in mice. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1863 (2), 165–176. https://doi.org/10.1016/j.bbalip.2017.11.008.

Yarmo, M.N., Gagnon, A., Sorisky, A., 2010. The anti-adipogenic effect of macrophage-conditioned medium requires the IKKβ/NF-κB pathway. Horm. Metab. Res. 42 (12), 831–836. https://doi.org/10.1055/s-0030-1263124.

Zhang, H., Zhi, L., Moochhala, S., Moore, P.K., Bhatia, M., 2007. Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-κB. Am. J. Physiol. Lung Cell Mol. Physiol 292 (4), L960–L971. https://doi.org/10.1152/ajplung.00388.2006.