

RESEARCH ARTICLE

Hydrogen Sulfide Promotes Adipogenesis in 3T3L1 Cells

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Abstract

The effect of hydrogen sulfide (H₂S) on differentiation of 3T3L1-derived adipocytes was examined. Endogenous H₂S was increased after 3T3L1 differentiation. The expression of the H₂S-synthesising enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST), was increased in a time-dependent manner during 3T3L1 differentiation. Expression of genes associated with adipogenesis related genes including fatty acid binding protein 4 (FABP4/aP2), a key regulator of this process, was increased by GYY4137 (a slow-releasing H₂S donor compound) and sodium hydrosulfide (NaHS, a classical H₂S donor) but not by ZYJ1122 or time-expired NaHS. Furthermore expression of these genes were reduced by aminooxyacetic acid (AOAA, CBS inhibitor), DL-propargylglycine (PAG, CSE inhibitor) as well as by CSE small interference RNA (siCSE) and siCBS. The size and number of lipid droplets in mature adipocytes was significantly increased by both GYY4137 and NaHS, which also impaired the ability of CL316,243 (β₃-agonist) to promote lipolysis in these cells. In contrast, AOAA and PAG had the opposite effect. Taken together, we show that the H₂S-synthesising enzymes CBS, CSE and 3-MST are endogenously expressed during adipogenesis and that both endogenous and exogenous H₂S modulate adipogenesis and adipocyte maturation.

Introduction

Obesity, a major health issue in developed countries, is now widely regarded as a chronic inflammatory state which contributes to numerous pathologies including dyslipidemia, coronary heart disease, non-alcoholic fatty liver, insulin resistance and type II diabetes [1–4]. Obesity is associated with accumulation of excess triacylglyceride (TG) in adipocytes either due to innate hyperadipogenesis or to lipid overloading in adipose tissue [5–7]. Lipid accumulation in adipose tissue is tightly controlled by a range of adipogenesis-related molecules including fatty acids binding protein 4 (FABP4/aP2), peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (CEBPα), sterol regulatory element binding protein-1 (SREBP1), carbohydrate responsive element binding protein (ChREBP), fatty acid synthase (FAS), hormone-sensitive lipase (HSL), perilipin A and a 47 kDa tail interacting protein

(TIP47) [8–12]. Among these, PPAR γ and CEBP α are particularly important in the early stages of adipogenesis since they stimulate FABP4/aP2 thereby activating FABP4/aP2 to trigger downstream FAS, ChREBP and SREBP1 mRNA activation and thus promote adipocyte maturation [8,13,14]. Moreover, HSL, perilipin A and TIP47, enzymes which bind intracellular lipid droplets, serve to regulate TG breakdown and glycerol release from mature adipocytes [13,15].

Hydrogen sulfide (H₂S) is generated from L-cysteine by cystathionine γ lyase (CSE) or cystathionine β -synthase (CBS) and from 3-mercaptopyruvate by 3-mercaptopyruvate sulfurtransferase (3-MST) [16,17]. These enzymes occur widely in mammalian cells and tissues and produce H₂S which, in turn, plays multiple roles in regulating cardiovascular function, inflammation, insulin resistance and glucose metabolism [18–23]. It has recently been reported that H₂S is also formed in fat tissues [21,24] and that H₂S can impair insulin signaling and glucose uptake into adipocytes [21–24]. Moreover, H₂S reportedly reduces insulin resistance in adipocytes from obese mice fed a high fat diet [24]. Together, these studies suggest a role for adipose H₂S in insulin resistance and glucose homeostasis [24]. However, the precise biological effect of either endogenous or exogenous H₂S on adipocytes and the contribution which this gas makes on adipogenesis are not clear. With this in mind, we have now used both fast- (NaHS) and slow-releasing (GYY4137) H₂S donors, and for comparison, time-expired NaHS and ZYJ1122 (a structural analogue of GYY4137 lacking sulfur and thence unable to release H₂S), drugs which inhibit endogenous H₂S biosynthesis (AOAA, PAG) as well as siCBS and siCSE as tools to assess the effect of H₂S on adipocyte biology *in vitro*.

Materials and Methods

Reagents, drugs and antibodies

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco[®] (Grand Island, NY). Growth medium comprising DMEM containing 15% w/v FBS and penicillin (100 U/ml)/streptomycin (100 μ g/ml) and differentiation medium consisting of DMEM containing 15% w/v FBS, penicillin (100 U/ml)/streptomycin (100 μ g/ml), 1-methyl-3-isobutylxanthine (MIX, 0.5 mM), dexamethasone (DEX, 0.5 μ M) and insulin (1.7 μ M) were prepared. Phosphate buffered saline (PBS) containing 1% w/v Triton X-100 (1% v/v), sodium chloride (NaCl, 250 mM), Tris hydrochloride (50 mM, pH7.5), ethylenediaminetetraacetic acid (EDTA, 5 mM), leupeptin (1 μ g/ml), aprotinin (10 μ g/ml) and phenylmethylsulfonyl fluoride (PMSF, 1 mM) was used to lyse cells. Lipid droplets in cells were stained using Oil red O (Cayman chemicals, USA). Adipocyte lipolysis was stimulated with CL-316243 (β 3-adrenoceptor agonist, 5 nM). NaHS, AOAA and PAG were purchased from Sigma Aldrich (St. Louis, MO). Time-expired NaHS was prepared by exposing a solution of NaHS (50 μ M) to the air for 18 h. ZYJ1122 and GYY4137 were provided by Professor Tan Choon-Hong (Department of Chemistry, Nanyang Technological University, Singapore). For siRNA knockdown experiments, CSE siRNA (siCSE), CBS siRNA (siCBS) and Lipofectamine³⁰⁰⁰ were purchased from Life Technologies (Paisley, UK). For western blotting, goat anti-mouse FABP4/aP2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-mouse CBS, β -actin and mouse anti-mouse CSE were obtained from Abcam (Cambridge, MA), and rabbit anti-mouse 3-MST was obtained from Sigma-Aldrich (St. Louis, MO). Downstream metabolites of lipolysis and the released glycerol were detected by colorimetry using a lipase-based adipolysis assay kit was purchased from Cayman chemicals (Ann Arbor, MI, USA).

Cell Culture

3T3L1, a fibroblast-like mouse preadipocyte cell line, was purchased from the American Type Culture Collection (ATCC). Frozen 3T3L1 cells were recovered and incubated (37°C, 5% CO₂)

in DMEM containing 10% w/v FBS until confluency. Confluent 3T3L1 cells were then incubated in differentiation medium for 3 days after which medium was aspirated and growth medium added for an additional 4 days to allow 3T3L1 cells to differentiate into mature adipocytes. The medium was changed every 2 days until cells were fully differentiated.

Measurement of H₂S concentration

The H₂S concentration in cultured medium was assessed using a sulfonyl azide-based fluorescent probe, 2,6-dansyl azide, as described elsewhere [25]. Briefly, confluent 3T3L1 cells were cultured in differentiating medium and incubated with or without GYY4137 (50 μM) or NaHS (50 μM). After incubation (48 h), cells were collected and medium mixed (50% v/v) with 2,6-dansyl azide probe solution (0.5 ml of 0.4 mM in 90% v/v MeCN in pH 7.4 PBS mixture ('buffer solution')) in 4 ml screw-cap glass vials. Fluorescence was read in a SpectraMax M3 Microplate reader at EX325\EM450 in triplicate. The samples were kept at 37°C in the dark. Readings were compared with a standard curve generated from a 400 μM stock solution of probe in buffer solution, dispensed into 15 x 4 ml screw-cap glass vials (1.5 ml stock solution per vial). A 400 μM solution of sodium sulfide (Na₂S) in buffer solution was added to the probe solution and the total volume made up to 3 ml with buffer solution so that the final probe concentration was 200 μM, and the final Na₂S concentrations were 0, 50, 100, 150 and 200 μM. Fluorescence readings (gain = 80) were recorded for 3 samples from each vial.

Western Blotting

Adipocytes were rinsed once with ice-cold PBS, lysed with lysis buffer on ice (10 min) and the lysate centrifuged (12000g, 5 min, 4°C). Protein concentration was then determined by the Bradford reaction (Bio-Rad Ltd., California, USA). Aliquots (50 μg) of cell suspension were resolved in 8% or 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Ltd., California, USA). After blocking (1 h) with 5% w/v skimmed milk in 0.1% w/v Tween/PBS, blots were incubated with the appropriate primary antibodies and then with HRP-conjugated secondary antibodies. Blots were detected using enhanced chemiluminescent reagent (Merck Millipore Ltd., USA) and quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

mRNA extraction and quantitative real-time PCR

Genomic mRNA was collected from 3T3L1 cells which had been incubated with or without GYY4137, NaHS, AOAA or PAG for 48 h. Cultured cells were washed with ice cold PBS and incubated with TRIzol[®] reagent (Invitrogen, Carlsbad, CA) for 1 min. RNA was extracted using Aurum Total RNA Mini Kits (Bio-Rad, Hercules, CA) and 1 μg of total RNA was transcribed into cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's protocols. Relative quantitative real-time PCR was performed by administering 3 μl of cDNA, 2 μl of primers to 5 μl of the reaction mix buffer from the Power SYBR Green PCR master mix kit (Life Technologies, Paisley, UK), the amplification reaction was monitored using a ViiA7 qPCR thermal cycler (Applied Biosystem, Paisley, UK). Expression values were determined by 2^{-ΔΔCT} equation and normalized with 18S housekeeping gene. The specific primers for representative genes are listed in [Table 1](#).

Transfection of adipocytes by small interference RNA (si-RNA)

3T3L1 cells were cultured in differentiation medium and transfected with either siCSE or siCBS siRNAs using Lipofectamine[™] 3000 for 72 h. Briefly, and according to the

Table 1. Primer sequences of adipogenesis-related genes.

Category	Genes name		Sequence	References
Adipocyte differentiation factors	FABP4/aP2	-F	5'-TGGAAGCTTGTCTCCAGTGA-3'	[26]
		-R	5'-AATCCCCATTTACGCTGATG-3'	
	PPAR γ	-F	5'-CAAGAATACCAAAGTGCATCAA-3'	[26]
		-R	5'-GAGCAGGGTCTTTTCAGAATAATAAG-3'	
	CEBP α	-F	5'-TGGACAAGAACAGCAACGAGTA-3'	[27]
		-R	5'-GCAGTTGCCATGGCCTTGA-3'	
Adipogenesis transcription factors	FAS	-F	5'-GGTCGT TTCTCCATTAATTCTCA T-3'	[28]
		-R	5'-CCT TCTAAAGACCCCTTTCAAAGAT C-3'	
	ChREBP	-F	5'-GTCCGATATCTCCGACACACTCTT-3'	[29]
		-R	5'-CATTGCCAACATAAGCATCTTCTG-3'	
	SREBP1	-F	5'-GGAGCCA-TGGATTGCACATT-3'	[30]
		-R	5'-AGGCCAGGGAAGT-CACTGTCT-3'	
Lipolysis related enzymes	HSL	-F	5'-GCTGGGCTGTCAAGCACTGT-3'	[28]
		-R	5'-TACCGTCGGATGGGTCAATG-3'	
	Perilipin A	-F	5'-GGCCTGGACGACAAAACC-3'	[31]
		-R	5'-CAGGATGGGCTCCATGAC-3'	
	TIP47	-F	5'-GGAAGTGGTGCATCAACAG-3'	[32]
		-R	5'-GGT CAC ATC CAC TGC TCC TG-3'	
Internal control	18S	-F	5'-TAAGTCCCTGCCCTTGGTACACA-3'	[33]
		-R	5'-GATCCGAGGGCCTCACTAAAC-3'	

-F for forward primer sequence

-R for reverse primer sequence.

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manufacturer's instructions, 250 μ l of LipofectamineTM 3000 only or siCSE (5 μ g) or siCBS (5 μ g):Lipofectamine 3000 complexes were mixed at room temperature for 5 min and then added to 50% confluent 3T3L1 cells for 72 h prior to Western Blotting of CSE, CBS and FABP4/aP2 as described above.

Measurement of cell lipolysis and release of glycerol in mature 3T3L1 derived adipocytes

Mature adipocytes were pre-treated with GYY4137 (50 μ M), NaHS (50 μ M), AOAA (1 mM), PAG (10 mM) for 2 h. After washing with PBS, cells were treated with 5 nM CL316,243 (to stimulate adipocytes releasing glycerol) and co-incubated with or without GYY4137, NaHS, AOAA or PAG in Krebs Ringer Buffer (KRB) (13 mM NaCl, 4.7 mM KCl, 2.5 mM MgSO₄, 3.3 mM CaCl₂, 24.5 mM NaHCO₃, 1 mM KH₂PO₄, 5 mM glucose, 3% w/v bovine serum albumin) for 1 h. Buffer was then collected and the release of glycerol determined using an Adipolysis assay kit (Cayman Chemicals, MI, USA).

Oil-Red O Staining

Mature adipocytes were fixed in 24 well plates with 4% w/v paraformaldehyde (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. Fixed cells were washed with PBS and then stained (15 min) with Oil Red O (stock solution: 3 mg/ml dissolved in isopropanol; working solution: 60% Oil Red O stock solution and 40% distilled water) and the counterstain,

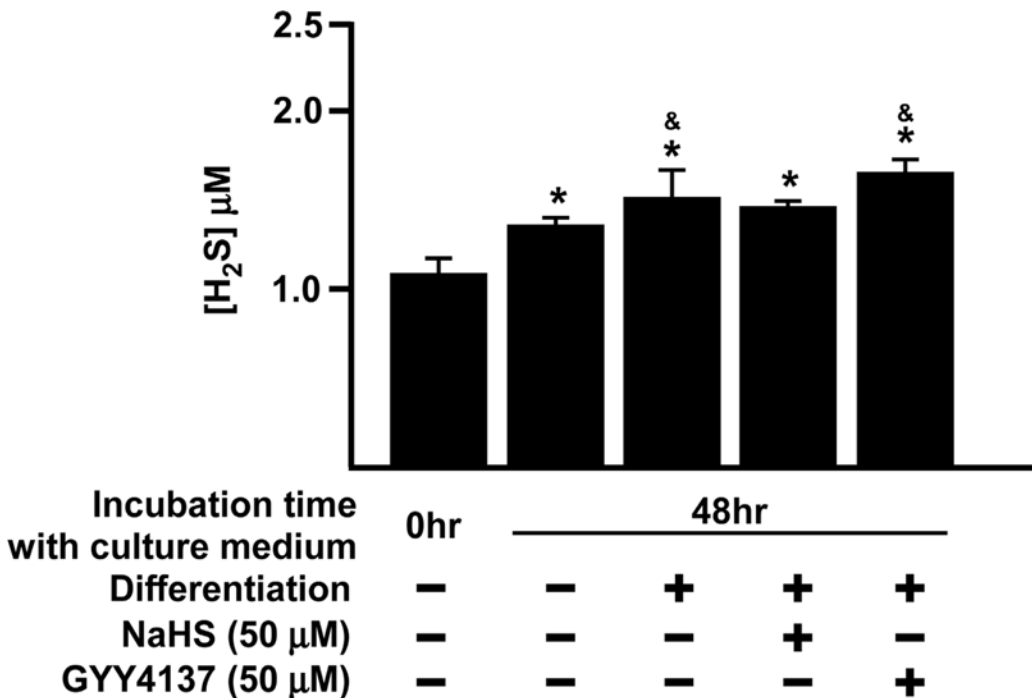


Fig 1. Increase in H₂S concentration in culture medium after differentiation. Confluent 3T3L1 cells were washed with PBS (37°C) and resuspended in normal growth DMEM for 10 min. Aliquots of medium were collected ('baseline' group). 3T3L1 cells were then cultured for 2 days either in DMEM ('negative control' group) or differentiation medium ('positive' control group). To test the effect of H₂S releasing drugs, 3T3L1 cells were co-incubated with GYY4137 (50 μM) or NaHS (50 μM) for 2 days. H₂S concentration in the medium was detected using a 2,6-dansyl azide fluorescent probe. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean±SEM of 4 independent experiments. *P < 0.05 vs. baseline group; &P < 0.05 vs. 48 h negative control group.

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hemotoxylin (Sigma-Aldrich, St. Louis, MO). Lipid density was analyzed at 540 nm using a Bio-Tek plate reader (BioTek. Instruments Inc., Winooski, VT, USA).

Statistical analysis

All experiments were performed on at least four separate occasions and quantitative data is expressed as mean±SEM. Statistical significance was determined by One-Way ANOVA followed by Fisher's least significant difference (LSD) posthoc analysis. SPSS version 21 (SPSS Inc., Chicago, IL) was used for analysis. Statistical significance was set at P < 0.05.

Results

Expression of CBS, CSE and 3-MST during adipocyte differentiation

We first determined whether H₂S was generated naturally during adipocyte differentiation (Fig 1) and whether the expression of CBS, CSE and 3-MST was altered during the process (Fig 2).

H₂S concentration in the culture medium was significantly increased after 48 h of differentiation whilst inclusion of GYY4137, but not NaHS, into the medium caused a small, but statistically significant, increase in H₂S concentration (Fig 1). CBS expression increased after differentiation for 1 day and plateaued at day 5–7 just prior to cells reaching full maturity (Fig 2). CSE and 3-MST expression increased steadily from day 1 to day 7 (Fig 2). These data suggest that H₂S, generated by the activity of either CBS, CSE or 3-MST or a combination thereof, may have functional role(s) to play in adipocytes.

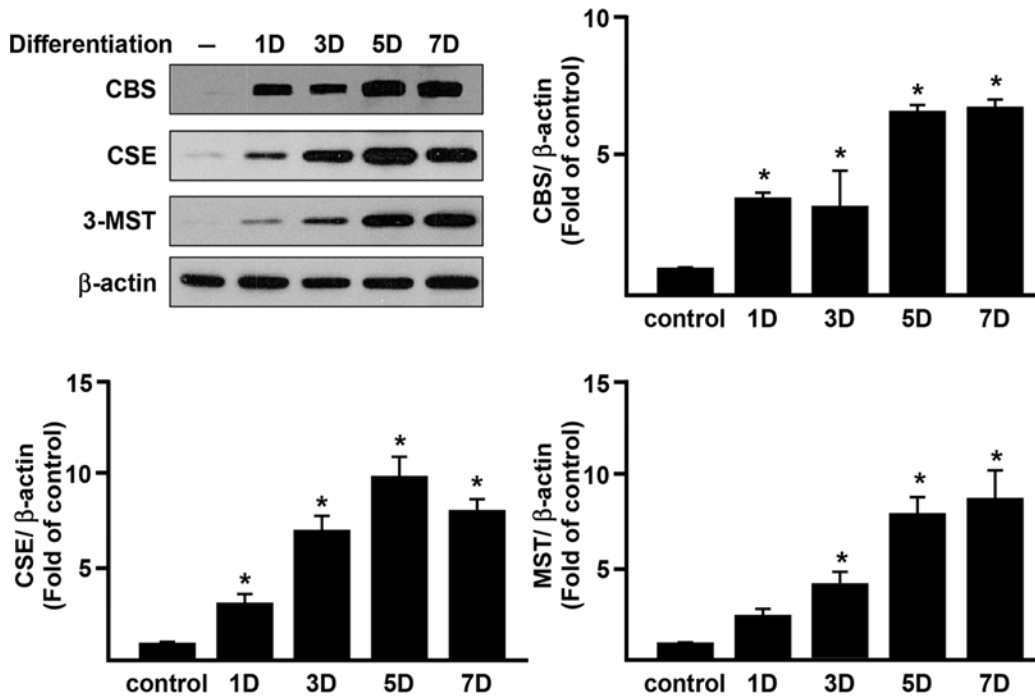


Fig 2. Upregulation of CBS, CSE and 3-MST during differentiation of 3T3L1 cells. H₂S-synthesising enzyme (CBS, CSE and 3-MST) expression during adipocyte differentiation was determined by Western Blotting. Data shows protein expression compared to the negative control group (normalized to β -actin) set as 1. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean \pm SEM from 4 independent experiments. *P < 0.05 vs. negative control group.

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H₂S upregulates adipogenesis-related genes

The maturation of adipocytes is tightly regulated by adipocyte differentiation factors, adipogenesis transcription factors and lipolysis related enzymes [7–9,14]. To determine the effect of H₂S on adipogenesis, confluent adipocytes in normal growth medium (i.e. negative control group), cells with differentiation medium (i.e positive control group) and cells co-incubated with differentiation medium containing either GYY4137 (50 μ M) or NaHS (50 μ M) were incubated for 48 h. PPAR γ and CEBP α , mRNA expression was induced by both GYY4137 and NaHS (Fig 3).

GYY4137 (but not NaHS) also induced expression of FABP4/aP2, ChREBP, SREBP1, HSL and perilipin A mRNA expression after 48 h incubation. Among the H₂S responsive genes, adipogenesis transcription factors (FAS, ChREBP and SREBP1c), are responsible for promoting adipocyte formation [12,14,34], whilst the lipolysis related enzyme genes (HSL, TIP47 and perilipin A), play significant roles in facilitating TG breakdown into glycerol and free fatty acids (FAA) in mature adipocytes [6,7,35,36]. Expression of FAS and TIP47 mRNA was significantly enhanced in both GYY4137- and NaHS-treated groups (c.f. positive control group, P < 0.05). To determine whether these effects of GYY4137 were indeed due to H₂S release we conducted control experiments in which 3T3L1 cells were treated with either time-expired NaHS (50 μ M) or ZYJ1122 (50 μ M; a GYY4137 analogue lacking sulfur and hence incapable of releasing H₂S (for chemical structure, see [37]) during adipocyte differentiation (Fig 4).

Expression of PPAR γ , ChREBP and Perilipin A mRNA was increased by GYY4137 and NaHS (Fig 4) but not time-expired NaHS or ZYJ1122. GYY4137 (but not NaHS) also induced expression of PPAR γ and ChREBP mRNA (Fig 4). Taken together, these results imply that

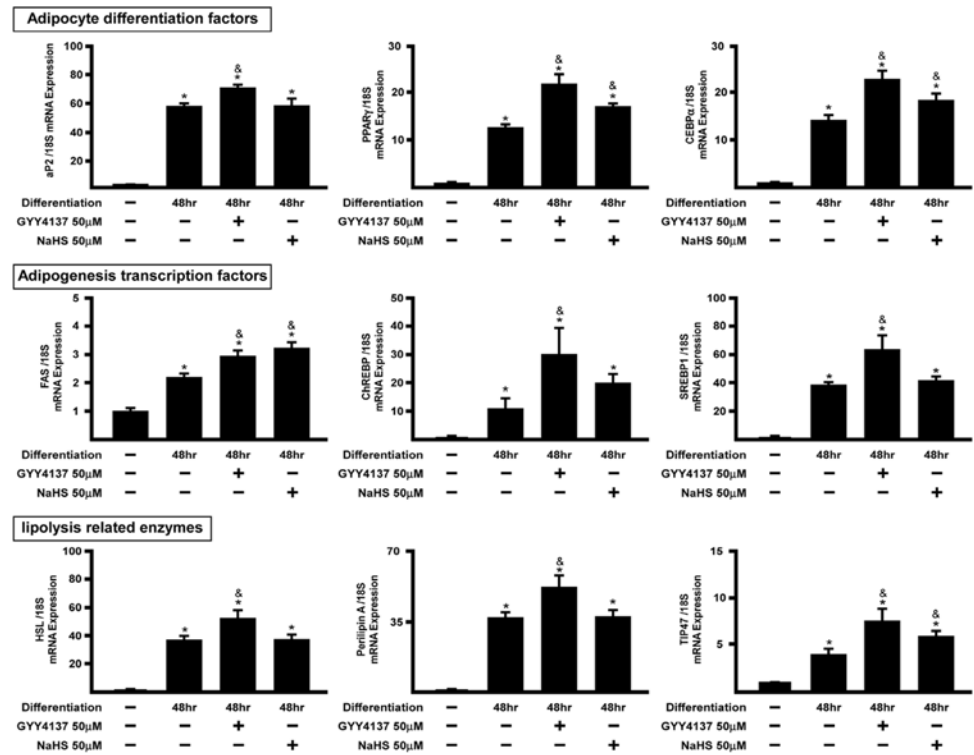


Fig 3. H₂S upregulates adipogenesis-related genes. 3T3L1 cells were co-incubated with GYY4137 (50 μ M) or NaHS (50 μ M) for 2 days and subjected to real-time PCR assay of genes relevant to lipid metabolism. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean \pm SEM of 6 independent experiments. *P < 0.05 vs. negative control group; [§]P < 0.05 vs. 48 h positive control group.

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exogenous H₂S released from either GYY4137 or NaHS has a role to play in adipocyte differentiation.

Inhibition of H₂S production impairs adipogenesis-related gene activation.

Since H₂S donors promoted adipocyte differentiation we then determined whether endogenous H₂S may also regulate adipogenesis and/or adipocyte maturation. To this end, we evaluated the effect of two pharmacological inhibitors of H₂S-synthesizing enzymes i.e AOA (CBS inhibitor) and PAG (CSE inhibitor) (Fig 5).

Expression of CEBP α , ChREBP, SREBP1, HSL and perilipin A mRNA expressions were significantly reduced by both AOA and PAG. However, TIP47 mRNA expression was not altered by either AOA or PAG suggesting that TIP47 gene is likely not a target for H₂S (Fig 5).

H₂S regulates adipocyte differentiation by modulating FABP4/aP2 expression.

FABP4/aP2 is a key transcription factor in adipocyte differentiation [10,13,38] and a marker of adipogenesis [9,38]. To assess the effect of H₂S on adipogenesis, 3T3L1 cells were incubated with or without GYY4137, NaHS, AOA or PAG for 5 days and processed for Western blotting (Figs 6 and 7).

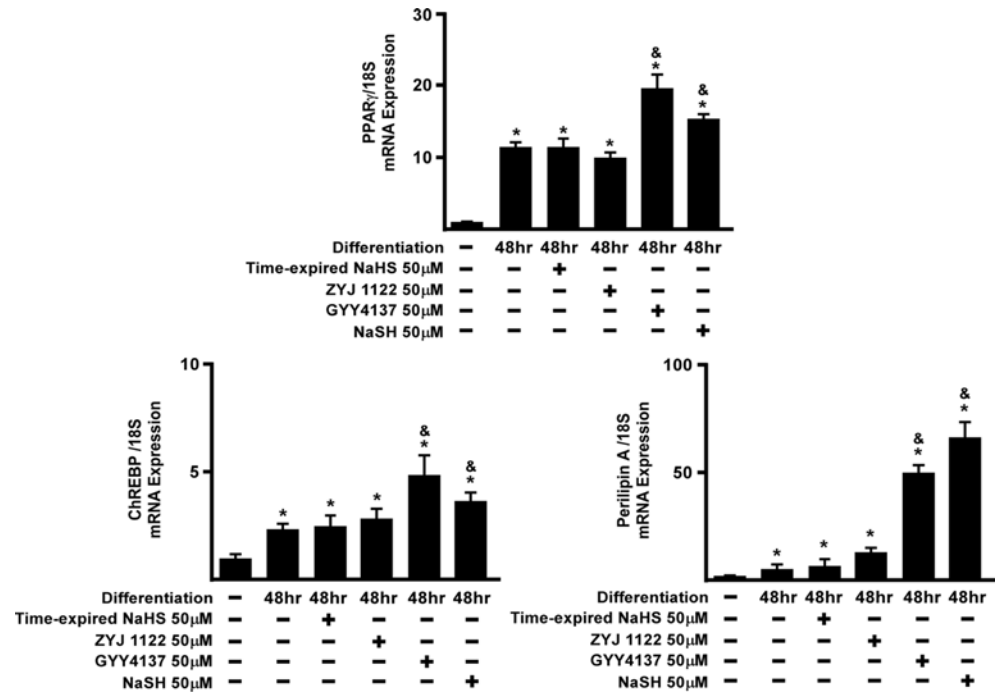


Fig 4. Effect of H₂S releasing drugs on expression of markers of adipogenesis. 3T3L1 cells were co-cultured with GYY4137 (50 μ M), NaHS (50 μ M), time-expired NaHS (50 μ M note: which was left at room temperature for 18 h and without H₂S-releasing activity) or ZYJ1122 (50 μ M), for 2 days and subject to real-time PCR assay of genes relevant to adipogenesis. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean \pm SEM of 4 independent experiments. *P < 0.05 vs. negative control (non-differentiated) group; &P < 0.05 vs. 48 h positive control group.

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GY4137, but not NaHS, increased FABP4/aP2 protein expression (Fig 6). Moreover, treatment of 3T3L1 cells with either AOAA or PAG suppressed FABP4/aP2 protein expression (Fig 7). Experiments were also conducted using siCSE or siCBS in adipocytes to knock down endogenous CSE or CBS and the expression of FABP4/aP2 was then determined 72 h after transfection (Fig 8).

In these experiments, siCBS or siCSE selectively reduced the expression of each enzyme respectively. Both treatments, like AOAA and PAG, suppressed FABP4/aP2 protein (Fig 8). Thus, endogenous H₂S likely promotes adipogenesis by increasing FABP4/aP2 protein expression.

H₂S upregulates lipid accumulation by inhibiting lipolysis

To explore whether H₂S affected the function of mature (c.f. differentiating) adipocytes, cells were first differentiated to adipocytes in the presence or absence of GYY4137, NaHS, AOAA or PAG for 7 days until the cells reached full maturation. Next, lipid droplets in mature adipocytes as well as glycerol content (an index of lipolysis), were examined by Oil Red O staining (Fig 9) and glycerol measurement (Fig 10) respectively.

As shown by Oil Red O staining, both GYY4137 and NaHS treatment significantly increased lipid accumulated in the cell bodies of mature adipocytes (Fig 9). In contrast, PAG reduced lipid accumulation in cells whilst, AOAA was without effect (Fig 9). These data suggest that change in endogenous H₂S concentration contributes to modulation of adipocyte formation.

In order to probe further the effect of H₂S in regulating adipocyte function, we assessed the effect of H₂S donors and H₂S synthesis inhibitors on glycerol release triggered by CL-361,234

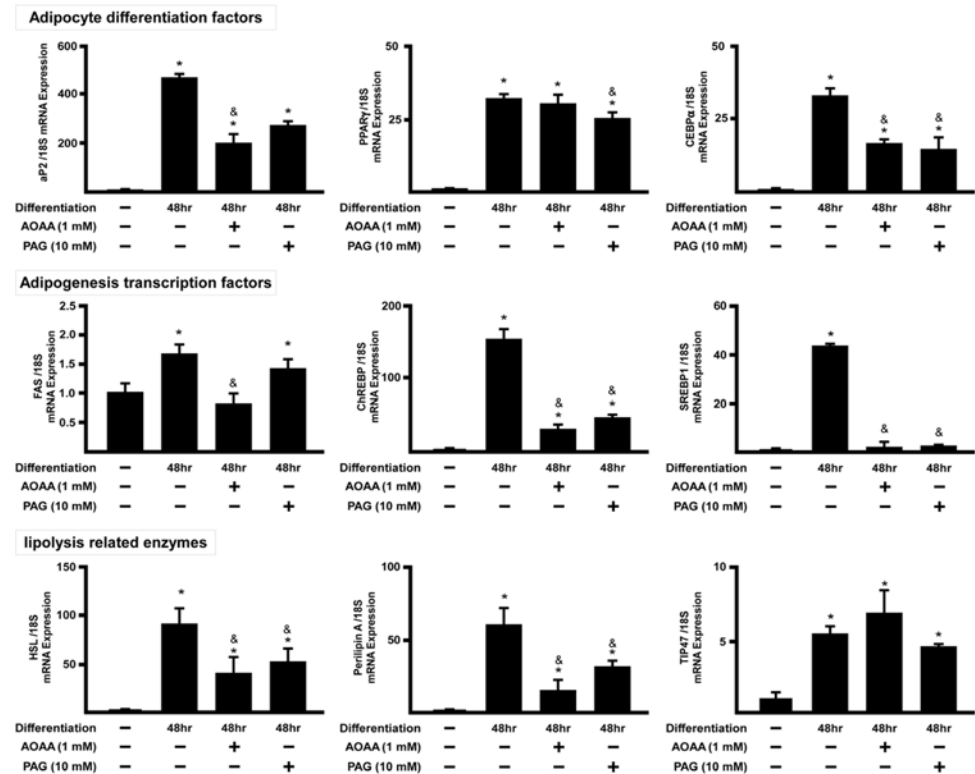


Fig 5. Inhibition of endogenous H₂S synthesis with AOAA/PAG downregulates adipogenesis-related genes expression in 3T3L1 cells. 3T3L1 cells were co-incubated either with the AOAA (a CBS inhibitor) or PAG (a CSE inhibitor), for 2 days followed by real-time PCR assay of genes relevant to lipid metabolism. The relative mRNA expression levels were compared with the control group (normalized to 18S gene expression level) set as 1. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean±SEM from 6 independent experiments. *P < 0.05 vs. negative control group; &P < 0.05 vs. 48 h positive control group.

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(β-adrenoceptor agonist) incubation. During lipolysis, glycerol is released due to the breakdown of TG (Fig 10A). Exposure to CL-316,243 significantly increased glycerol concentration which effect was inhibited by treating cells with either GYY4137 or NaHS. In contrast, both AOAA and PAG augmented the ability of CL-316,243 to promote glycerol release. These results suggest that both exogenous and endogenous H₂S modulates lipolysis in mature adipocytes.

Discussion

Dysregulation of adipocyte proliferation, differentiation as well as disrupted adipocyte lipolysis contribute to obesity [5–8,14] but the role of H₂S in this process is not clear. It has recently been shown that CSE is expressed in rat adipose tissues [21] and that H₂S affects isoproterenol-stimulated lipolysis [24]. Moreover, CSE-knockout mice exhibit lower plasma cysteine and H₂S concentrations as well as reduced body weight and white adipose tissue mass when compared with wild-type mice [39]. Each of these studies therefore point to a role for H₂S in regulating adipocyte function. However, the detailed mechanism of action of H₂S in adipogenesis and its effect on lipid homeostasis are not clear.

In the present study, we utilized 3T3L-1 cells, a well-established mouse preadipocyte cell-line, as a cellular model of adipogenesis [40]. Our results demonstrate that H₂S is produced naturally during 3T3L1 differentiation. Moreover the expression of all three H₂S-synthesizing

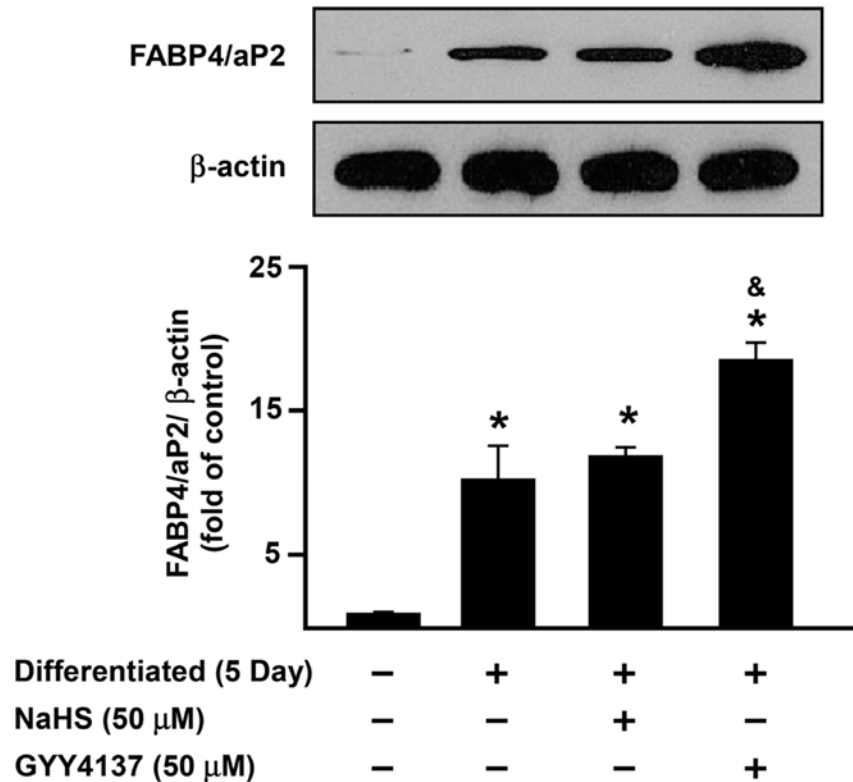


Fig 6. H₂S upregulates FABP4/aP2 expression in 3T3L1 cells. 3T3L1 cells were incubated with either NaHS (50 μM) or GYY4137 (50 μM) for 5 days during differentiation, FABP4/aP2 expression was determined by Western Blotting. Data shows protein expression compared to the control group (normalized to β-actin) set as 1. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean±SEM of 4 independent experiments. *P < 0.05 vs. negative control group; &P < 0.05 vs. 5 days positive control group.

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enzymes (i.e. CBS, CSE and 3-MST) was upregulated in a time-dependent manner during 3T3L1 cell differentiation. Thus, we propose firstly that H₂S, generated by one or more of these enzymes, plays a part in the process of adipocyte differentiation. To investigate the role of exogenous H₂S in adipogenesis we evaluated the effect of the H₂S donor agents, GYY4137 and NaHS, as well time-expired NaHS and ZYJ1122 as controls. The role of endogenous H₂S was assessed in cells treated with either AOAA or PAG which inhibit CBS and CSE respectively by targeting the pyridoxal 5'-phosphate (PLP) binding sites [41,42] of each enzyme and by using siRNA for CSE and CBS. A number of gene markers of adipogenesis are known. These include transcription factors such as FABP4/aP2, PPARγ, CEBPα, FAS, ChREPB, SREBP1, HSL, perilipin A and TIP47 [8–12]. Among these, HSL, perilipin A and TIP47 are largely responsible for hydrolysis of TG into glycerol/FFA in mature adipocytes and as such are markers of adipocyte function [7,43]. Interestingly, GYY4137 (but not NaHS) promoted adipogenesis-related and lipolysis-related enzyme gene up-regulation (Fig 3). Moreover, compounds without H₂S-releasing activity (time-expired NaHS and ZYJ1122) did not affect 3T3L1 differentiation-induced adipogenesis genes expression in 3T3L1 cells suggesting that H₂S is indeed responsible for the effect of H₂S donors on adipogenesis. GYY4137 was more effective than NaHS in these experiments presumably due to ability to release H₂S slowly over a long period of time [37]. Intriguingly, both AOAA and PAG diminished adipogenesis-related genes expression during adipocytes

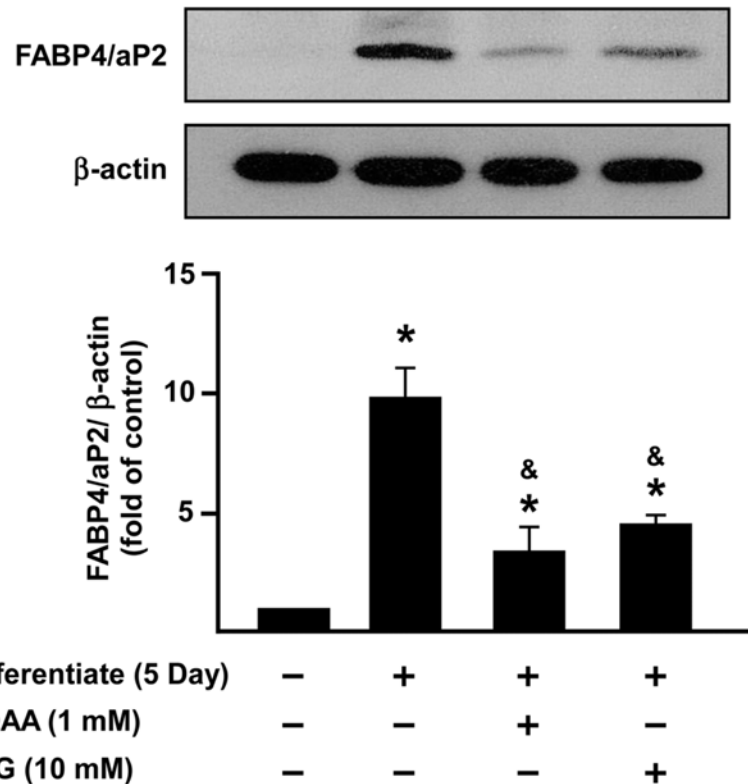


Fig 7. FABP4/aP2 protein expression is inhibited by AOAA and PAG treated 3T3L1 cells. 3T3L1 cells were incubated with either AOAA (1 mM) or PAG (10mM) for 5 days during differentiation, FABP4/aP2 expression was determined by Western Blotting. Data shows protein expression compared to the control group (normalized to β -actin) set as 1. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean \pm SEM of 4 independent experiments. *P < 0.05 vs. negative control group; &P < 0.05 vs. 5 days positive control group.

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differentiation. These results imply that both exogenous and naturally occurring H₂S regulate 3T3L1 differentiation to adipocytes.

The present results also show that GYY4137 (but not NaHS) induced, whilst treatment with either AOAA or PAG, diminished FABP4/aP2 protein expression in differentiating 3T3L1 cells. That AOAA and PAG reduce FABP4/aP2 protein expression in these cells suggests a role for endogenous H₂S in the differentiation process. However, AOAA and PAG inhibit CBS and CSE respectively by targeting the pyridoxal 5' phosphate binding site on each of these enzymes and, as such, can at best be considered as non-selective inhibitors (reviewed in [24,44–46]). In this context, transfection of adipocytes with either siCBS or siCSE reduced expression of FABP4/aP2 thereby adding weight to the possibility that endogenous H₂S regulates adipocyte function. That both exogenous and endogenous H₂S regulates FABP4/aP2 expression is important since this protein is a fatty-acid transporter and binding protein critical for facilitating fatty acid uptake [10,38]. Indeed, deletion of FABP4/aP2 leads to embryonic lethality [47,48] whilst diminished FABP4/aP4 gene expression has been shown to protect animals from multiple metabolic syndromes including obesity, insulin resistance, hepatosteatosis and atherosclerosis [49–52]. That both endogenous and exogenous H₂S promotes FABP4/aP2 expression supports a regulatory role for this gas in regulating adipocyte differentiation and raises the possibility that H₂S donors may be of interest in the treatment of a range of metabolic diseases.

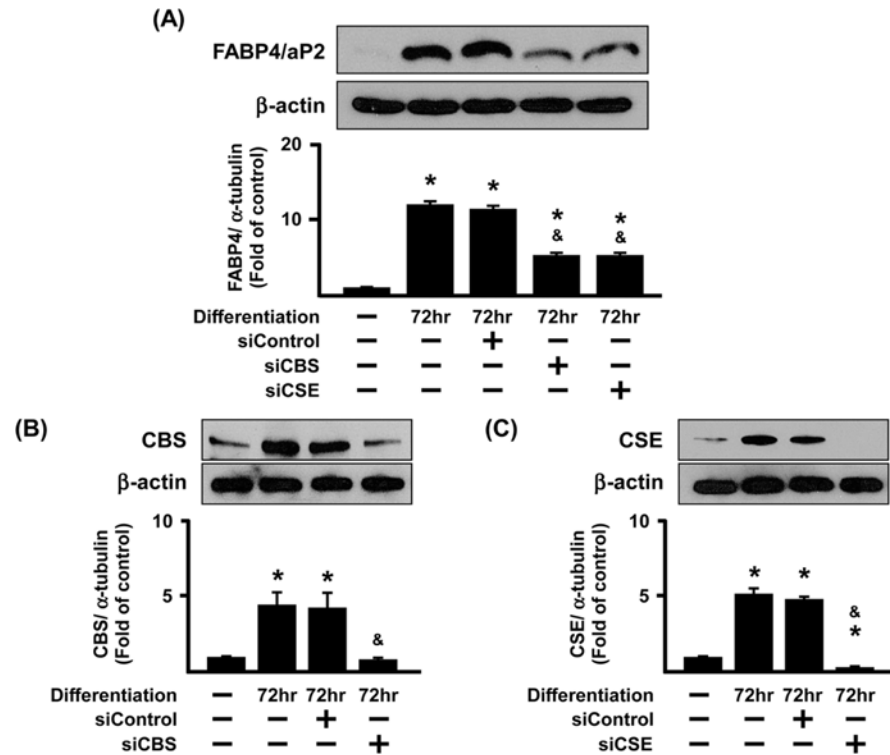


Fig 8. Effect of CBS and CSE knockdown on FABP4/aP2 protein expression in differentiated 3T3L1 cells. 3T3L1 cells were incubated with empty lipofectamine (siControl), siCBS (5 µg) or siCSE (5 µg) for 3 days during differentiation. Expression of FABP4/aP2, CBS and CSE protein expression was determined by Western Blotting. Data shows a representative blot (A) as well as protein expression compared to the control group and normalized to β-actin as 1 (B-D). Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean±SEM of 4 independent experiments. *P < 0.05 vs. negative control group; &P < 0.05 vs. 3 days positive control group.

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Lipid accumulation results illustrate that treatment with GYY4137 promoted adipogenesis by causing adipocyte hypertrophy, whilst NaHS upregulated lipid accumulation in mature adipocytes mainly by enhancing adipocyte maturation rate and stimulating adipocyte lipid droplet formation. These results suggest exogenously manipulating H₂S levels directly enhance lipid accumulation in mature adipocytes. In contrast, diminished H₂S production following treatment with either AOAA or PAG attenuates both the size and number of lipid droplets in mature adipocytes. Interestingly, PAG prevented lipid accumulation in lipid droplet suggesting that CSE plays a particularly important role in lipid droplets formation.

Mammalian cells store TG in lipid droplets to be hydrolyzed into fatty acids and glycerol when energetically desirable [6,7,13,35]. Any dysregulation of lipid synthesis and lipolysis contribute to accumulation of lipid droplets and promotes the development of obesity [5–8]. Moreover, the size of lipid droplets within the adipocyte is strongly correlated with the efficiency of adipolysis [5,6,35]. Since H₂S plays a regulatory role in lipid droplet formation we next probed whether the H₂S-regulated lipid accumulation is due to an effect on lipolysis rate. In the present study, we confirmed that treatment of mature adipocytes with either GYY4137 or NaHS significantly inhibited CL-316,243-induced adipolysis and that this was significantly enhanced by either AOAA or PAG. These data suggest that H₂S directly regulates adipolysis in adipocytes.

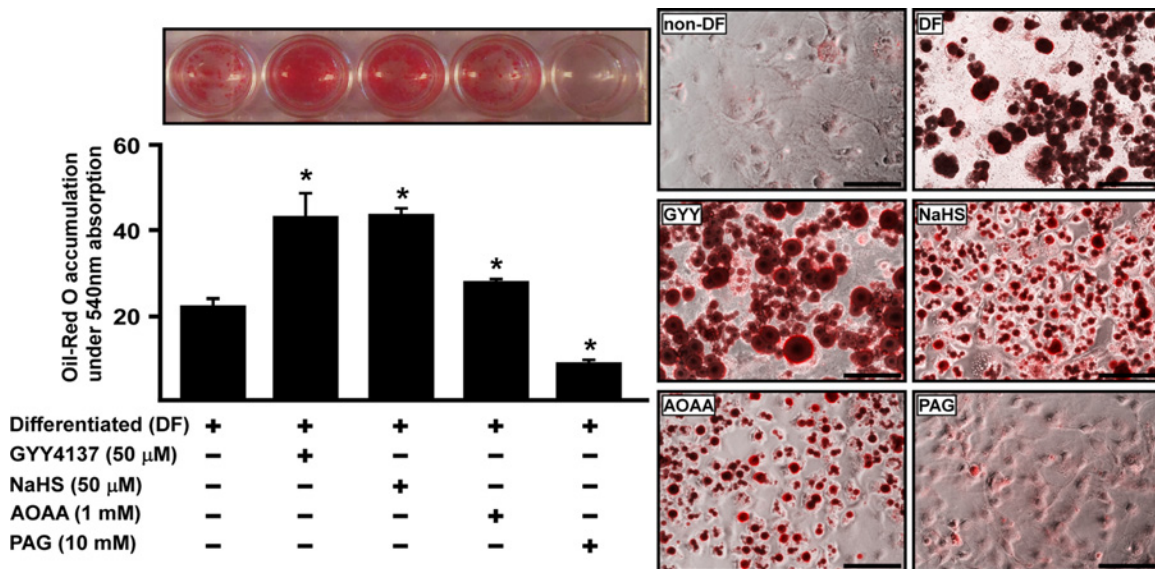


Fig 9. H₂S promotes lipid accumulated in mature 3T3L1 adipocytes. 3T3L1 cells were incubated with either GYY4137 (50 μM), NaHS (50 μM), AOAA (1 mM) or PAG (10 mM) for 7 days of differentiation, lipid accumulation was determined by Oil-red O staining and well scanning (under 540 nm). Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean±SEM OF 6 independent experiments. *P < 0.05 vs. negative control group (non-DF); &P < 0.05 vs. positive control group (DF).

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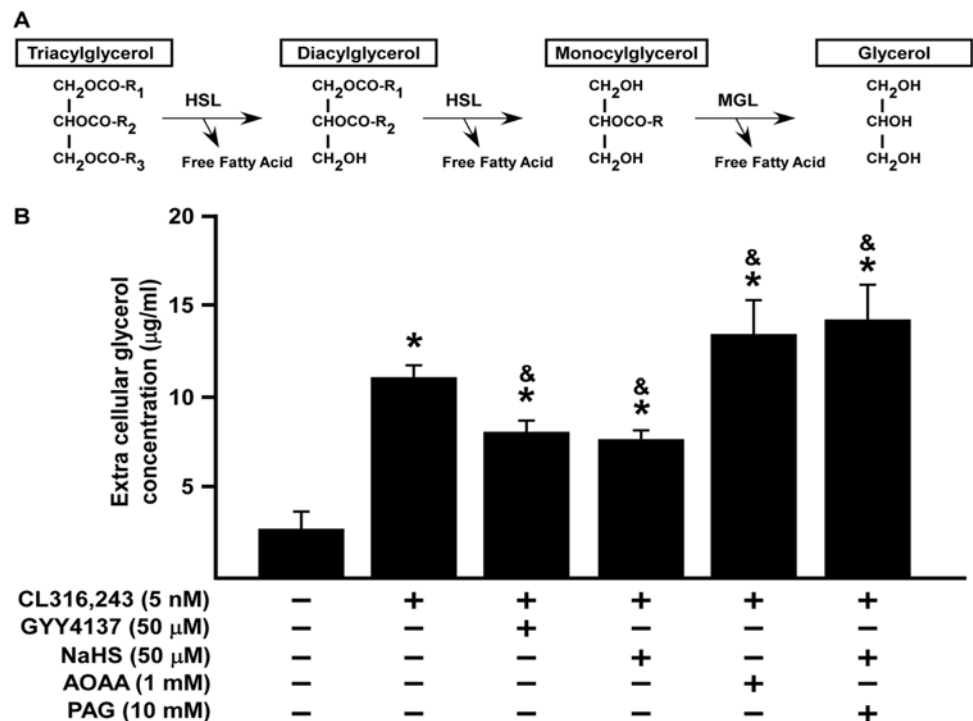


Fig 10. H₂S inhibits lipolysis in matured 3T3L1 adipocyte. (A) The process of lipolysis. (B) Fully differentiated 3T3L1 cells were co-incubated with the β₃-agonist, CL316,243 (5 nM) in the presence of absence of either GYY4137 (50 μM), NaHS (50 μM), AOAA (1 mM) or PAG (10 mM) for 1 h. Statistical significance was determined by ANOVA followed by Fisher's LSD posthoc analysis. Data shown are mean±SEM of 6 independent experiments. *P < 0.05 vs. negative control group; &P < 0.05 vs. CL-316,243-treated.

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Thus, we show here that that not only CSE but also CBS and 3-MST are highly expressed during adipogenesis. Moreover, these enzymes and H₂S derived from their activity increase expression of adipogenesis-related genes and reduce adipolysis leading to accumulation of lipid droplets and triggering adipocyte hypertrophy. These data shed new light on the complex role of H₂S in adipocyte biology and raise the possibility that drugs which manipulate endogenous H₂S levels may have a role to play in metabolic disorders such as obesity.

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Author Contributions

Conceived and designed the experiments: CYT PKM. Performed the experiments: CYT MTP WF BWD. Analyzed the data: CYT MTP WF. Contributed reagents/materials/analysis tools: CYT MTP WF BWD. Wrote the paper: CYT MTP PKM.

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