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...
Among these, PPARγ and CEBPα are particularly important in the early stages of adipogenesis since they stimulate FABP4/aP2 thereby activating FABP4/aP2 to trigger downstream FAS, ChREPB 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 sulfur-transferase (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 μg/ml) and differentiation medium consisting of DMEM containing 15% w/v FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml), 1-methyl-3-isobutylxanthine (MIX, 0.5 mM), dexamethasone (DEX, 0.5 μM) and insulin (1.7 μM) 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 μg/ml), aprotinin (10 μg/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 (β3-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 μM) 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 Lipofectamine™ 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 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 H<sub>2</sub>S concentration**

The H<sub>2</sub>S 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 (Na<sub>2</sub>S) 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 Na<sub>2</sub>S 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<sup>-ΔΔCT</sup> 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
manufacturer’s instructions, 250 μl of Lipofectamine™ 3000 only or siCSE (5 μg) or siCBS (5 μg):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 μM), NaHS (50 μM), 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,
hematoxylin (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 H$_2$S 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).

H$_2$S 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 H$_2$S 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 H$_2$S generated by the activity of either CBS, CSE or 3-MST or a combination thereof, may have functional role(s) to play in adipocytes.
H2S Up-regulates 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 μM) or NaHS (50 μM) were incubated for 48 h. PPARγ and CEBPα, 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 μM) or ZYJ1122 (50 μM; a GYY4137 analogue lacking sulfur and hence incapable of releasing H2S (for chemical structure, see [37]) during adipocyte differentiation (Fig 4).

Expression of PPARγ, 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 PPARγ and ChREBP mRNA (Fig 4). Taken together, these results imply that
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 AOAA (CBS inhibitor) and PAG (CSE inhibitor) (Fig 5).

Expression of CEBPα, ChREBP, SREBP1, HSL and perilipin A mRNA expressions were significantly reduced by both AOAA and PAG. However, TIP47 mRNA expression was not altered by either AOAA 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, AOAA or PAG for 5 days and processed for Western blotting (Figs 6 and 7).

Fig 3. H2S upregulates adipogenesis-related genes. 3T3L1 cells were co-incubated with GYY4137 (50 μM) or NaHS (50 μM) 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±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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GYY4137, 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.
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
enzymes (i.e. CBS, CSE and 3-MST) was upregulated in a time-dependent manner during 3T3L1 cell differentiation. Thus, we propose firstly that H\textsubscript{2}S, generated by one or more of these enzymes, plays a part in the process of adipocyte differentiation. To investigate the role of exogenous H\textsubscript{2}S in adipogenesis we evaluated the effect of the H\textsubscript{2}S donor agents, GYY4137 and NaHS, as well time-expired NaHS and ZYJ1122 as controls. The role of endogenous H\textsubscript{2}S 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 \cite{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, PPAR\textsubscript{γ}, CEBP\textsubscript{α}, FAS, ChREPB, SREBP1, HSL, perilipin A and TIP47 \cite{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 \cite{7,43}. Interestingly, GYY4137 (but not NaHS) promoted adipogenesis-related and lipolysis-related enzyme gene up-regulation (Fig 3). Moreover, compounds without H\textsubscript{2}S-releasing activity (time-expired NaHS and ZYJ1122) did not affect 3T3L1 differentiation-induced adipogenesis genes expression in 3T3L1 cells suggesting that H\textsubscript{2}S is indeed responsible for the effect of H\textsubscript{2}S donors on adipogenesis. GYY4137 was more effective than NaHS in these experiments presumably due to ability to release H\textsubscript{2}S slowly over a long period of time \cite{37}. Intriguingly, both AOAA and PAG diminished adipogenesis-related genes expression during adipocytes.

\begin{figure}
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\includegraphics[width=0.5\textwidth]{h2s-upregulates-fabp4-ap2-expression-in-3t3l1-cells.png}
\caption{H\textsubscript{2}S upregulates FABP4/aP2 expression in 3T3L1 cells. 3T3L1 cells were incubated with either NaHS (50 \textmu M) or GYY4137 (50 \textmu M) for 5 days during differentiation, FABP4/aP2 expression was determined by Western Blotting. Data shows protein expression compared to the control group (normalized to \textbeta -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. doi:10.1371/journal.pone.0119511.g006}
\end{figure}
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.
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 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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