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ORIGINAL RESEARCH COMMUNICATIONS

Insufficient S-sulfhydration of Methylenetetrahydrofolate Reductase Contribute to the Progress of Hyperhomocysteinemia

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ABSTRACT

Aims: Hyperhomocysteinemia (HHcy) has been considered as a risk factor for cardiovascular disease (CVD), Alzheimer's disease (AD), nonalcoholic fatty liver (NAFL) and many other pathological conditions. Vitamin B6, Vitamin B12, and folate have been used to treat HHcy in clinic. However, at present clinical therapies of HHcy display unsatisfactory effects. Here, we would like to explore a new mechanism involved in Hcy metabolic disorders and a novel target for HHcy treatment. The key enzymes involved in Hcy metabolism deserve more insightful investigation. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme regulating the intracellular Hcy metabolism. Until now, the effect of post-translational modification on bioactivity of MTHFR still remains unclear. This study aimed to explore the relationship between MTHFR S-sulfhydration and its bioactivity, and identify the contribution of elevated Hcy level on MTHFR bioactivity.

Results: By both in vivo and in vitro studies, we observed the following results: (1) The bioactivity of MTHFR was positively associated with its S-sulfhydration level; (2) MTHFR was modified at Cys32, Cys130, Cys131, Cys193, and Cys306 by S-sulfhydration under physiological conditions; (3) Hydrogen sulfide (H_2S) deficiency caused the decrease of MTHFR S-sulfhydration level and bioactivity in HHcy, which resulted in further aggravation of HHcy; (4) H_2S donors reversed the decreased bioactivity of MTHFR in HHcy, thus reduced the excessive Hcy level.

Innovation and Conclusion: Our study suggested that H_2S could improve MTHFR bioactivity by S-sulfhydration, which might provide a candidate therapeutic strategy for HHcy.

INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid derived from methionine (Met). The pathological elevation of serum Hcy is clinically called hyperhomocysteinemia (HHcy)[27]. Quite a few diseases, such as cardiovascular disease[39], dementia[33], schizophrenia[28], cancer[12],liver injury[10, 43], and osteoporosis[11] are associated with HHcy. Therefore, keeping the Hcy in the normal range is particularly important for the body's health. Vitamin B6, Vitamin B12, and folate supplementation are conventionally used to decrease the total Hcy level in clinic [14,3,37]. However, the therapeutic effect of HHcy sometimes falls short of expectations. *Zhang W et al.* [36]adopted a prospective method to observe the efficacy of folate therapy and found that 374 of 858 patients with HHcy (43.59%) still failed to reach the normal range with treatment of folate (5mg/d) for three months. Continuing to explore the novel mechanisms involved in Hcy metabolism are essential for HHcy patients who fail to respond to conventional treatment. Therefore, further study on how to reduce Hcy level effectively will have great clinical significance.

The liver is a major organ for Hcy metabolism. Hcy is metabolized by two major pathways: remethylation and transsulfuration. In remethylation pathway, Hcy acquires a methyl group from N-5-methyltetrahydrofolate produced by folate to form methionine[30]. As a key enzyme in folate metabolism, methylenetetrahydrofolate reductase (MTHFR) plays a crucial role in keeping the normal Hcy level in serum[19]. Previous studies have shown that MTHFR expression or bioactivity deficiency can cause abnormal utilization of folate and inhibit metabolism of Hcy by remethylation pathway, which leads to the occurrence and development of HHcy[22, 25]. Nevertheless, the impact of HHcy itself on the bioactivity of MTHFR remains largely unexplored.

Hydrogen sulfide (H_2S), ever regarded as a toxic gas, has recently been classified as a gasotransmitter that can modify proteins by S-sulfhydration[24,40,41]. Proteins S-sulfhydration is a kind of post-translational modification of reactive cysteine residues, which modulates proteins structure or function. *Snyder S et al.*[29]report that S-sulfhydration typically increases the reactivity of the cysteine residue being modified. It has been reported that HHcy can cause H_2S deficiency[7,32], which reduces the level of proteins S-sulfhydration. However, whether H_2S deficiency can affect MTHFR is

completely unknown yet. Thus, this study was designed to identify the relationship between MTHFR S-sulfhydration and its bioactivity under physiological conditions and observed the change of MTHFR in HHcy. In this way, we intended to explore a novel mechanism that might be involved in the disorder of Hcy metabolism, and therefore, provided possible new therapeutic targets for HHcy.

RESULTS

MTHFR was modified by S-sulfhydration under physiological conditions

Until now, there's no study showing whether MTHFR can be modified by S-sulfhydration. To explore the relationship between MTHFR and S-sulfhydrlation, two distinct cells models (HL-7702 cells and QSG-7701 cells) were used. Previous studies have shown that NaHS (a rapid release H_2S donor) or GYY4137 (a slow release H_2S donor) can enhance the proteins S-sulfhydration level while DTT or TCEP hydrochloride (selectively cleaves disulfide bonds) are used to reduce S-sulfhydrlation formation[5,35,46]. First, in this study, red maleimide assay **(Fig S1A)** was employed to verify whether these drugs could change proteins S-sulfhydration level. We treated HL-7702 cells with NaHS or GYY4137 **(Fig S1B)** to detect the total proteins S-sulfhydration level. Maleimide assay **(Fig 1A, 1B)** showed that total proteins S-sulfhydration level was increased. To the contrary, treating HL-7702 cells with DTT or TCEP hydrochloride decreased S-sulfhydration level **(Fig 1A, 1B)**. These results suggested that the drugs we used were effective and could be used in further experiments. Then, we continued to detect the level of MTHFR S-sulfhydration. Both the maleimide assay **(Fig 1E, 1F)** and modified biotin switch assay **(Fig 1G, 1H)** showed that the variation trend of MTHFR S-sulfhydration level was the same as the total proteins S-sulfhydration level. To further verify this finding, QSG-7701 cells and liver lysates were also treated with these drugs (NaHS, GYY4137, DTT, and TCEP hydrochloride. **Fig S1B**), and the same variation trend as HL-7702 cells were observed **(Fig 1C, 1D, Fig 1I-1N)**. This evidence indicated that MTHFR could be S-sulfhydrated under physiological conditions.

The bioactivity of MTHFR was positively correlated with its S-sulfhydration level

Next, we would like to determine the relationship between change in MTHFR S-sulfhydration level and its bioactivity. HL-7702 cells or QSG-7701 cells were treated with DTT or NaHS for three hours respectively. In order to increase accuracy of the experiment, the cells (HL-7702 cells, QSG-7701 cells) were also treated with TCEP or GYY4137 for six hours respectively **(Fig S2A)**. As for HL-7702 cells, after treating with NaHS or GYY4137, enzyme linked chemiluminescent assay **(Fig 2A, 2B)** or NADPH-Menadione Oxidoreductase Assay **(Fig 2C, 2D)** showed that MTHFR bioactivity was enhanced. By contrast, DTT or TCEP decreased MTHFR bioactivity **(Fig 2A-2D)**. Similarly, the same trend occurred when QSG-7701 cells or liver lysates were treated in the same way **(Fig 2E-2H, Fig 2N, 2O)**. Furthermore, different concentrations of DTT or NaHS were used to treat HL-7702 cells or QSG-7701 cells. As shown in **Fig 2I (Fig S2B, S2C)**, **2J (Fig S2D, S2E)**, these data indicated that MTHFR bioactivity was positively correlated with its S-sulfhydration level. In order to identify the modification sites of MTHFR, we constructed the human MTHFR site-directed mutation plasmids. Mutation of cysteine residue in human MTHFR to alanine (C14A, C32A, C120A, C130A, C131A, C193A, C243A, C250A, and C306A) together with the wild-type MTHFR (WT) plasmids were constructed and transiently transfected into HEK 293 cells respectively **(Fig S2F)**. The maleimide assay **(Fig 2K)** and modified biotin switch assay **(Fig 2L)** were used to evaluate MTHFR S-sulfhydration level. The sites of C32A, C130A, C131A, C193A, and C306A mutation significantly decreased the level of MTHFR S-sulfhydration. Next, we detected the enzymatic bioactivity of MTHFR. As shown in **Fig 2M**, mutant C32A, C130A, C131A, C193A, and C306A exhibited a significant decrease in enzymatic bioactivity, meanwhile, there was no significant decrease in enzymatic bioactivity of mutant C14A, C120A, C243A, and C250A. These data suggested that the cysteine residues at positions 32, 130, 131, 193, and 306 were sites at which MTHFR was S-sulfhydrated, and that these residues might be essential for MTHFR bioactivity.

HHcy inhibited the bioactivity of MTHFR

To confirm the effects of Hcy on MTHFR, we fed mice with high Met diet. After 17 weeks, the serum Hcy level was increased significantly **(Fig 3A)**, indicating the HHcy mice model was established successfully. In the liver of mice model, enzyme linked

chemiluminescent assay **(Fig 3B)** and NADPH-Menadione Oxidoreductase Assay **(Fig 3C)** indicated that MTHFR bioactivity was significant decreased in HHcy mice model. Meanwhile, in vitro, we treated HL-7702 cells with Hcy and detected a decline of MTHFR bioactivity as well **(Fig 3D, 3E)**. To further verify these results, QSG-7701 cells were treated with Hcy, and the results of decreased MTHFR bioactivity were gotten **(Fig 3F, 3G)**. These results indicated that MTHFR bioactivity was inhibited in HHcy. Why did this happen? Next, we will further explore the underlying mechanism.

Hydrogen sulfide level was inhibited in HHcy

In view of the influence of S-sulfhydration on MTHFR bioactivity, firstly we observed the impact of HHcy on H_2S level. The 64 serum samples of coronary heart patients were divided into the control group (Hcy ≤10 μmol/L, n=17) and HHcy group (Hcy >10 μmol/L, n=47) according to their Hcy level. The level of H_2S in HHcy group were significantly lower than control group **(Fig 4A)**, and Hcy level was negatively correlated with H_2S level **(Fig 4B)**. HHcy mice also exhibited a significant decrease of serum H_2S level **(Fig 4C)**. In vitro, we detect the intracellular H_2S level with methylene blue and H_2S fluorescence probe. We found that HL-7702 cells **(Fig 4D, 4E)** or QSG-7701 cells **(Fig 4F, 4G)** treated with Hcy produced less H_2S compared to control. Similar with previous findings[1,3,21], our results also confirmed that HHcy could reduce H_2S level.

HHcy reduced the level of MTHFR S-sulfhydration

In view of H_2S can S-sulfhydrate proteins and affect its bioactivity, next, we would like to prove whether HHcy had an impact on the level of proteins S-sulfhydration level. In vivo, a large number of red bands were detected in normal mice liver **(Fig 5A)**, representing proteins with -SH as well as -SSH substitutes were marked. The red bands were reduced about 50% after DTT treatment **(Fig 5A)**. However, in HHcy mice liver, after DTT treatment the red bands were reduced about 30% compared with before DTT processing **(Fig 5A)**. In HL-7702 cells **(Fig 5B)** and QSG-7701 cells **(Fig 5C)** with or without Hcy treatment, the variation trend of red bands was consistent with the animal model. These results indicated that HHcy could reduce total proteins S-sulfhydration level.

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Further, to verify the impact of HHcy on MTHFR S-sulfhydration level, both maleimide assay and modified biotin switch assay were conducted. As shown in **Fig 5D** and **5E**, in the liver of HHcy mice, the S-sulfhydration level of MTHFR was decreased significantly compared to normal mice. In vitro, after treated with Hcy, similar phenomenon was observed in HL-7702 cells **(Fig 5F, 5G)** and QSG-7701 cells **(Fig 5H, 5I)**. All of the above results confirmed that HHcy could reduce MTHFR bioactivity due to the decrease of MTHFR S-sulfhydration level.

H_2S donor rescued the bioactivity of MTHFR and reduced Hcy level

As H_2S donors, NaHS was used to further reversal experiments. C57BL/6J mice were fed with high Met diet for 17 weeks to establish the HHcy model. Meanwhile, NaHS was administered by intraperitoneal injection to generate the rescue group. Compared with the HHcy mice model, mice treated with H_2S donor NaHS showed a significantly increased serum H_2S level **(Fig 6A)**, and elevated total proteins S-sulfhydration level **(Fig 6B)**. Both maleimide assay **(Fig 6C)** and modified biotin switch assay **(Fig 6D)** were conducted to detect MTHFR S-sulfhydration level. We discovered that the supplementation of H_2S significantly restored the MTHFR S-sulfhydration level in HHcy mice. Our previous data manifested that MTHFR bioactivity was positively correlated with its S-sulfhydration level under physiological conditions. However, whether the same trend would occur in HHcy still need further verification. As shown in **Fig 6E** and **6F**, the supplement of H_2S increased MTHFR bioactivity in HHcy mice. These in vivo data indicated that H_2S donors could rescue the deficiency of MTHFR bioactivity by enhancing its S-sulfhydration level in HHcy.

In vitro, our results indicated that the level of H_2S **(Fig 6G)**, total proteins S-sulfhydration level **(Fig 6H)**, MTHFR S-sulfhydration level **(Fig 6I, 6J)**, and MTHFR bioactivity **(Fig 6K, 6L)** were restored after treating with NaHS in HL-7702 cells. Consistent with these data, the level of H_2S **(Fig 6M)**, total proteins S-sulfhydration **(Fig 6N)**, MTHFR S-sulfhydration level **(Fig 6O, 6P)**, and MTHFR bioactivity **(Fig 6Q, 6R)** were restored as well after NaHS treatment in QSG-7701 cells.

In order to further explore the effect of exogenous H_2S on Hcy metabolism in vivo, we measured the serum Hcy level in the NaHS rescue group and found that the total serum

Hcy level was significantly lower than that in the HHcy group **(Fig 6S)**. These data confirmed that treatment with H_2S donors rescued MTHFR bioactivity in HHcy, thereby increasing the metabolic capacity of Hcy and reducing Hcy level.

Discussion

Our results demonstrated a novel mechanism connecting insufficiency MTHFR S-sulfhydration with HHcy. In this study, we, for the first time, revealed that MTHFR bioactivity was positively correlated with its S-sulfhydration level under physiological conditions, which was occurred at 32,130,131,193, and 306 cysteine sites. On this basis, by both in vivo and in vitro studies, we indicated that MTHFR bioactivity was inhibited in HHcy due to reduced S-sulfhydration level, which could be rescued by H_2S donors. This study represented a new direction in our understanding of HHcy progress and treatment.

HHcy is related to many diseases, for instance, cardiovascular disease and stroke[39], hyperglycemia associated atherogenesis[6], schizophrenia[28] and so on. Vitamin B6, Vitamin B12, and folate serve as cofactors[44] are conventionally used to treat HHcy in clinic. However, clinical trials of these therapies display unsatisfactory effect. According to current reports, there are three main reasons for the unsatisfactory effect: (1) MTHFR polymorphisms: two common non-synonymous variants, the C677T (rs1801133) and A1298C (rs1801131), are described for the MTHFR gene and associated with a decreased enzymatic activity. Other MTHFR polymorphisms with marginal impact on enzymatic bioactivity are also reported[4,26]; (2) Abnormality of key enzymes in Hcy metabolism: rare genetic defects resulting in deficiencies of cystathionine beta synthase (CBS), cystathionine-γ-lyase (CSE)[13,30]; (3) Serum Hcy level: the higher Hcy level or the longer onset time may lead to a low effective rate of folate treatment for these patients with HHcy[36]. However, those reasons fail to entirely explain the unsatisfactory effect in treating HHcy. Here, we would like to explore a new mechanism involved in Hcy metabolic disorders and a novel target for HHcy treatment. In order to improve the accuracy of experimental results, we used two kinds of cells (HL-7702 cells, QSG-7701 cells) to detect and verify various indicators. In the liver of HHcy mice, we showed that MTHFR bioactivity was significant decreased. Meanwhile, a slight upregulation of MTHFR protein level **(Fig S3A)** and mRNA level **(Fig S3B)** in the liver of the HHcy mice model were detected, as

similar to HL-7702 cells **(Fig S3C, S3D)** with Hcy treatment. Based on these results, we guessed that: (1) Decrease of MTHFR bioactivity in HHcy should be one of the reasons for unsatisfactory effects of HHcy treatment; (2) The decline in MTHFR bioactivity contributed to the progress of HHcy; (3) The increased MTHFR expression might be a compensatory response to its decreased activity. In addition, the upregulation of MTHFR was slight and did not contribute to the interpretation of HHcy unsatisfactory therapeutic effect. Therefore, we considered that further exploring the reasons for MTHFR decreased bioactivity would afford a new mechanism involved in Hcy metabolism disorders and provide a new idea for clinical treatment of HHcy.

As we know, there are two ways to regulate the MTHFR bioactivity. First, as shown in figure **S3E**, MTHFR is allosterically inhibited by S-adenosylmethionine (SAM). This inhibition could be relieved by S-adenosylhomocystein (SAH) in a competitive manner[2,18]. Second, MTHFR is found to be phosphorylated, which is regulated by the SAM/SAH ratio in the cell. The phosphorylation of MTHFR leads to decrease MTHFR bioactivity and increase sensitivity to allosteric inhibition by SAM[8,42,45]. We examined the phosphorylation levels of MTHFR both in animal and cells models and found that there was no change in phosphorylation level with Hcy treatment **(Fig S3F, S3G)**. Therefore, we wonder whether there's a novel mechanism involved in regulation of MTHFR bioactivity in HHcy. In our previous study, we have discovered reduced S-sulfhydration level in HHcy model mice and Hcy treated cells[20]. Hence, we hypothesized that HHcy might reduce MTHFR S-sulfhydration level and then affect MTHFR enzymatic bioactivity. In HL-7702 cells and QSG-7701 cells, we found that MTHFR was modified by S-sulfhydration and MTHFR bioactivity was positively correlated with its S-sulfhydration level under physiological conditions, which was one of the innovations of our study. Furthermore, S-sulfhydrated MTHFR level was decreased, in keeping with reduced MTHFR bioactivity in HHcy mice, which could be reversed with H_2S donor treatment. HL-7702 cells and QSG-7701 cells treated with Hcy exhibited similar phenotypes. We speculated that the decrease of MTHFR bioactivity was caused by the reduction of MTHFR S-sulfhydration in HHcy. Thus, our findings identified a novel regulation model for MTHFR bioactivity. It is worth mentioning that H_2S does not S-sulfurate cysteine residues directly. Catalase oxidizes H_2S to generate H_2Sn in the presence

of oxygen. H_2Sn can S-sulfurate cysteine residues of proteins[17]. Alternatively, under oxidative conditions some cysteine residues (R-Cys-SH) are oxidized to become R-Cys-SOH, and at the exposure to NO, R-Cys-SH is oxidized to R-Cys-SNO. Both R-Cys-SOH and R-Cys-SNO can be S-sulfurated by H_2S to R-Cys-SSH[15,16]. In order to further explore the effect of exogenous H_2S on Hcy metabolism in vivo, we measured the serum Hcy level in the NaHS rescue group and found that the total serum Hcy level was significantly lower than that in the HHcy group. This result showed that H_2S donors might be a potential target for effective treatment of HHcy.

Human MTHFR protein is a homodimer with each subunit consisting of an N-terminal catalytic domain (amino acids, 1–356 aa) and a C-terminal regulatory domain (363–656 aa), connected by a short linker region (357–362 aa)[9]. The site of protein S-sulfhydration is cysteine residue. There are eleven cysteine sites in amino acid sequence of MTHFR, among which nine are located in the N-terminal catalytic domain. The catalytic domain appears to be sufficient to carry out the entire enzymatic reaction. Therefore, the nine cysteine sites located in the catalytic region were mainly focused next. In our study, the WT MTHFR plasmid and nine mutated plasmids (cysteine residues to alanine) were constructed. After transfection of HEK293 cells with either WT or mutated plasmid, we discovered it was C32A, C130A, C131A, C193A, and C306A which reduced S-sulfhydration level and bioactivity of MTHFR, but not C14A, C120A, C243A, C250A. In this study, we found five possible MTHFR S-sulfhydration sites under the physiological condition. In theory, S-sulfhydration level or bioactivity of MTHFR should decrease around 20% after each site mutation, but our results had shown a disproportionate reduction. This disproportionate decline has also been observed by others. *Snyder S et al.*[38] have demonstrated that Cys59, Cys95 and Cys182 in Parkin are targets for S-sulfhydration by Mass spectrometric analysis. In their study, when singly mutating Cys95, enhanced ubiquitination bioactivity of Parkin in response to GYY4137 is abolished. However, when mutating Cys59 or Cys182, the enhancement of ubiquitination is partial diminution. In another report, *Sun H et al.*[34] have also observed a similar phenomenon. They have found that two zinc finger domains in Sirt1 can be S-sulfhydrated. While mutating any of the two domains, S-sulfhydration of Sirt1 can be completely abolished. Thus, the number of

mutations were not strictly positive correlation with the MTHFR S-sulfhydration level or bioactivity, which might be related to the synergistic, antagonistic or complementary roles of the remaining amino acid residues in the protein. Our present study was for the first time to illustrate that MTHFR could be S-sulfhydrated and this kind of post-translational modification resulted in change of its bioactivity. Of cause, this was only the rather preliminary discussion about S-sulfhydration sites of MTHFR, and the specific effect of each mutation or synergistic effects after multiple site mutations on MTHFR need further effort to be clarified in the future.

In summary, the work presented here suggested an overarching idea that MTHFR bioactivity was positively correlated with its S-sulfhydration level. Here we highlighted that MTHFR inactivation was not only due to polymorphisms or phosphorylation, but also an outcome of insufficient S-sulfhydration. It was also worth noting that we had uncovered an important role of MTHFR S-sulfhydration' decline in the progress of HHcy. Further, our work indicated that H_2S donors could protect MTHFR bioactivity, thus might be a potential target for effective treatment of HHcy in clinic.

Innovation

MTHFR, a key enzyme involved in Hcy metabolism, was closely related the occurrence and development of HHcy. Our study for the first time demonstrated that bioactivity of MTHFR was positively correlated with its S-sulfhydration level. NaHS, an H_2S donor, improved Hcy metabolic capacity via S-sulfhydration of MTHFR, and thus reduced Hcy level in HHcy mice model **(Fig 7)**. These findings imply that activation of MTHFR by exogenous H_2S donor may be a potential therapeutic strategy against HHcy.

MATERIALS AND METHODS

Materials

Homocysteine (Hcy), sodium hydrosulfide (NaHS), GYY4137 Dichloromethane complex (GYY4137), dithiothreitol (DTT), L-cysteine (Cys) were purchased from Sigma-Aldrich. TCEP hydrochloride (TCEP) were purchased from MedChemExpress, Alexa Fluor 680 conjugated C2 maleimide was purchased from Invitrogen. The following antibodies were used in this

work: anti-MTHFR antibody (Abcam, ab203786), anti-β-tubulin antibody **(**ABclonal, AC031), Normal Rabbit IgG (Cell Signaling Technology, 2729)

Ethics and clinical experiment

The clinical trial was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The 64 serum samples of coronary heart patients were collected from Xuanwu Hospital, Capital Medical University. The study protocol was approved by the institutional ethics committee of Capital Medical University. All subjects have given written informed consent after being informed of the purpose and nature of this study. All serum samples were collected from the fasting 8 h research objects for the detection total Hcy levels by automatic biochemical analyzer. The H_2S level was measured with methylene blue assay.

Animals and cell culture

Healthy male C57BL/6J mice (8-week-old, SPF grade, n=10 each group) were provided by the Animal Center, Capital Medical University. Mice fed on common diet were used as control (Normal). HHcy models were established by feeding mice with high methionine diet (2.5% Met diet) for 17 weeks (Met). The NaHS rescue group mice were intraperitoneal injected with NaHS (diluted in normal saline, 10mg/Kg) every other day (Met+NaHS). All animals received human care in compliance with institutional guideline and the “Guide for the Care and Use of Laboratory Animals” prepared by the “Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council”.

HL-7702 cells and QSG-7701 cells were used for in vitro research and were maintained at 37℃ with 5% CO_2 in DMEM/HIGH GLUCOSE media (Hyclone, Logan, UT) containing phenol red supplemented with 10% fetal calf serum and 100 U/mL penicillin/1000 μg/mL streptomycin. Cells were used for the following treatment: (1) Control group; (2) HHcy group: 500 μmol/L Hcy, 6 days; (3) NaHS reverse group: 500 μmol/L Hcy+300 μmol/L NaHS, 6 days; (4) DTT group: 1 mmol /L DTT, 3 h; (5) TCEP group: 1 mmol /L TCEP hydrochloride, 6 h; (6) NaHS group: 1 mmol /L NaHS, 3 h; (7) GYY4137 group: 200 μmol/L GYY4137, 6 h.

Western Blot analysis

Liver tissue was isolated and lysed with Cell lysis buffer for Western (100 mg/mL, Beyotime) on ice. Liver homogenates were centrifuged at 4°C, 12000 g, for 10 min. The total proteins concentration was determined by the BCA Protein Assay kit (Thermo Scientific, Massachusetts, USA). Equal amounts of proteins from the liver were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a PVDF membrane. Nonspecific sites were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween (TBST) for 1 h at room temperature. The blots were incubated overnight at 4°C with appropriate primary antibodies (anti-MTHFR 1:1000, anti-actin 1:10000), then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. After washing, ECL Plus substrate (Thermo Scientific, Inc.) was applied to the blots, and images were captured in a gel documentation system. Relative optical density of protein bands was analyzed using gel software Image lab 3.0.

Quantitative real-time PCR

Total RNA was extracted from liver tissues or cells and reverse transcribed for quantitative RT-PCR analysis with SYBR green I dye in the ABI7500 Quantitative PCR System. The expression of MTHFR was normalized to that of β-actin and analysed by the 2−CT method. The primers were in Table 1.

Measurement of Hcy

After the mice were anaesthetized, blood samples were collected from carotid arterial cannulation containing 7.5% EDTA-Na_2 (15 ml/L), and immediately centrifuged at 3000 g, 4°C for 10 min. The level of total Hcy was determined by commercial ELISA kit (CUSABIO, CSB-E08896r).

Measurement of H_2S level

The following two methods were used to detect H_2S level:

Method one: the H_2S level was determined by methylene blue kit (Jiancheng Bioengineering Institute, Nanjing, China, A146-1-1) according to the instructions provided by the manufacturer.

Method two: After removal of cell medium, the cells were incubated with 100 μM H_2S fluorescence probe (Sigma, 802409) in PBS buffer for 2h min at 37℃, followed by washing the cells with PBS buffer. The treated cells were further incubated with blank phenol red free media. Fluorescence images were acquired by confocal microscopy.

Measurement of MTHFR bioactivity

The following two methods were used to detect MTHFR activity:

Method one: MTHFR bioactivity in livers and cells were determined by commercial enzyme linked chemiluminescent kit (ShenZhen Ziker Biological Technology Co. Ltd, ZK-M5940) according to the instructions provided by the manufacturer. In brief, after adding separate solutions to the samples as instructed, the samples were read at 450 nm.

Method two: This assay was designed based on the principles described previously[23,42]. MTHFR bioactivity was determined by using NADPH-Menadione Oxidoreductase Assay. In brief, the reaction mixture in a total volume of 2 ml consisted of 1.0 ml phosphate buffer (100 mM, pH =7.2), 0.025 ml NADPH (10 mM NADPH in 0.02 M unneutralized Tris), and enzyme. This mixture was incubated at 25℃ for 5 min, and then the reaction was initiated by addition of 0.2 ml menadione (Saturated solution of menadione in 20% methanol/80% H_2O). The reaction was monitored by measuring the change in absorbance at 343 nm. The extinction coefficient was associated with the oxidation of NADPH at 343 nm.

Detection of protein S-sulfhydration

The following two methods were used to detect the level of proteins S-sulfhydration

Maleimide assay: This assay was designed based on the principles described before[31]. Briefly, tissue and cells proteins were enriched by immunoprecipitation assay. Next incubated with Alexa Fluor 680 conjugated C2 maleimide (red maleimide, 1 mM) and kept for 2 h at 4°C. Then the beads were re-suspended with 1 ml lysis buffer and divided equally into two tubes, among which one was treated

with DTT (1 mM), another without DTT, both were incubated for 1 h at 4°C. Lastly, added with 10 µl 5×SDS-PAGE buffer, and then the beads were boiled at 99°C for 10 min followed by gel electrophoresis. The gel was scanned with the Li-COR Odyssey system (Li-Cor Biosciences, Lincoln, NE, USA), the intensity of red fluorescence of MTHFR was quantified using software attached to the Odyssey system. These gel were employed for Western Blot with anti-MTHFR antibody to detect the loaded MTHFR.

Modified biotin switch assay: Briefly, cells were homogenized in HEN buffer (composition: 100 mM HEPES (pH 7.8), 1 mM EDTA, 0.1 mM Neocuproine). Homogenates were centrifuged at 14,000 g (4°C) for 15 min. The supernatant was collected and blocked with HEN buffer (containing 2.5% SDS and 20 mM methyl methanethiosulfonate) at 50°C for 20 min. Methyl methanethiosulfonate was removed by precipitating proteins with acetone at −20°C for 20 min. After acetone removal, proteins were resuspended in HEN buffer containing 1% SDS, and 4 mM biotin-HPDP was added. Samples were then incubated for 4 h at room temperature. Biotinylated proteins were pulled down by streptavidin magnet beads and eluted by SDS-PAGE loading buffer for analysis by Western Blot.

Site directed mutation and cell transfection

The mutation plasmids of human MTHFR were generated commercially by Hanbio Biotechnology (Shanghai, China). HEK293 cells were dissociated with trypsin, quantified and plated in 10 cm plates. The next day, cells were transfected with 15 µg of each vector using Lipo6000™ reagent (Beyotime, Shanghai, China) and Opti-MEM® Medium (Gibico) according to the manufacturer's instructions. Six hours after transfection, the transfected medium was replaced with complete DMEM/HIGH GLUCOSE media (Hyclone, Logan, UT). Cultured for another 48 h, then the cells were collected with 300 µl lysis buffer (Tris-HCl 20 mM, Triton 0.1%, NaCl 100 mM, and PMSF 100 µM) for detecting the S-sulfhydration level of MTHFR.

Statistical analyses

Data were represented as the means ± SEM. Unpaired Student's t test was used to analyze data with only two sets. One-way analysis of variance (ANOVA) was performed to determine whether there was a significant difference between more than two data sets, followed by Bonferroni's post hoc test, using Graph Pad Prism 7.0. Group differences at the level of $P < 0.05$ were considered statistically significant.

Acknowledgements

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Author Contribution

Wen Wang designed the experiments; Dengyu Ji performed most of the experimental analysis and wrote the manuscript; Chenghua Luo, Jing Liu, Yan Cao, Jiangxu Wu, Wenjing Yan, Ke Xue, Jiayin Chai, Xinyu Zhu performed some of the experiments; Ye Wu and Huirong Liu provided technical support. All authors commented on and approved the manuscript.

Competing Interest

The authors declare that they have no competing interests.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon reasonable request.

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Abbreviations

A: alanine

AD: Alzheimer's disease

BCA: bicinchoninic acid

CBS: cystathionine beta synthase

CSE: cystathionine-γ-lyase

CVD: cardiovascular disease

Cys(C): cysteine

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assay

GYY4137: GYY4137 dichloromethane complex

Hcy: homocysteine

HEK 293 cells: Human embryonic kidney 293 cells

HHcy: hyperhomocysteinemia

HL-7702 cells: human normal liver cells

H_2S: hydrogen sulfide

Met: methionine

MTHFR: methylenetetrahydrofolate reductase

NADPH: nicotinamide adenine dinucleotide phosphate

NAFL: nonalcoholic fatty liver

NaHS: sodium hydrosulfide

PMSF: phenylmethylsulfonyl fluoride

QSG-7701 cells: human hepatocyte

Red maleimide: Alexa Fluor 680 conjugated C2 maleimide

SAH: S-adenosylhomocystein

SAM: S-adenosylmethionine

SDS–PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBST: tris-buffered saline with 0.1% Tween

TCEP: TCEP hydrochloride

WT: wild-type

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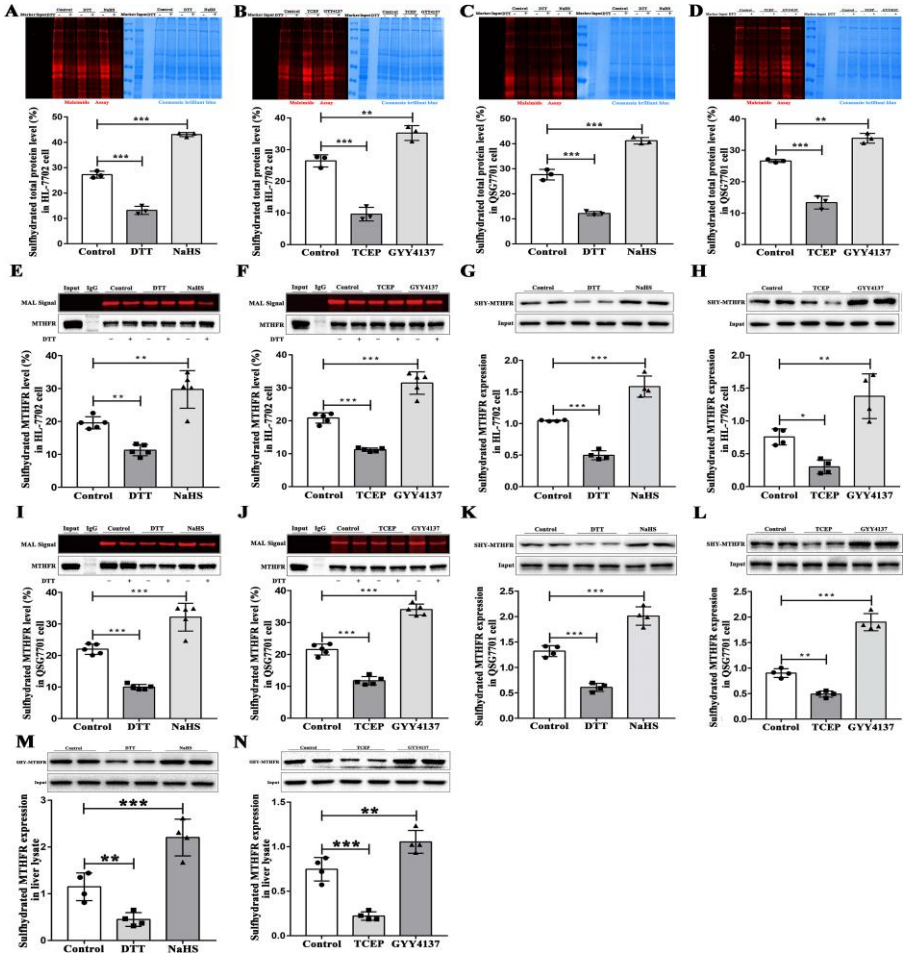

Fig. 1. MTHFR could be S-sulfhydrated under physiological conditions.

The HL-7702 cells or QSG-7701 cells were treated with DTT (1 mmol /L) or NaHS (1 mmol /L) for three hours respectively. In addition, the cells (HL-7702 cells, QSG-7701 cells) were treated with TCEP (1 mmol /L) or GYY4137 (200 μmol/L) for six hours respectively. The drugs were effective to change the total proteins S-sulfhydration level (A-D, n=3). Both maleimide assay (E, F, I, J) and modified biotin switch assay (G, H, K, L) showed that MTHFR could be modified by S-sulfhydration (n=4-5). Liver lysates were treated with 1M NaHS or 1M DTT for 60 min at 37°C respectively. In addition, liver lysates were treated with 2M GYY4137 or 2M TCEP for 8h at 37°C respectively. MTHFR S-sulfhydration level (M, N, n=4) was detected with modified biotin switch assay.

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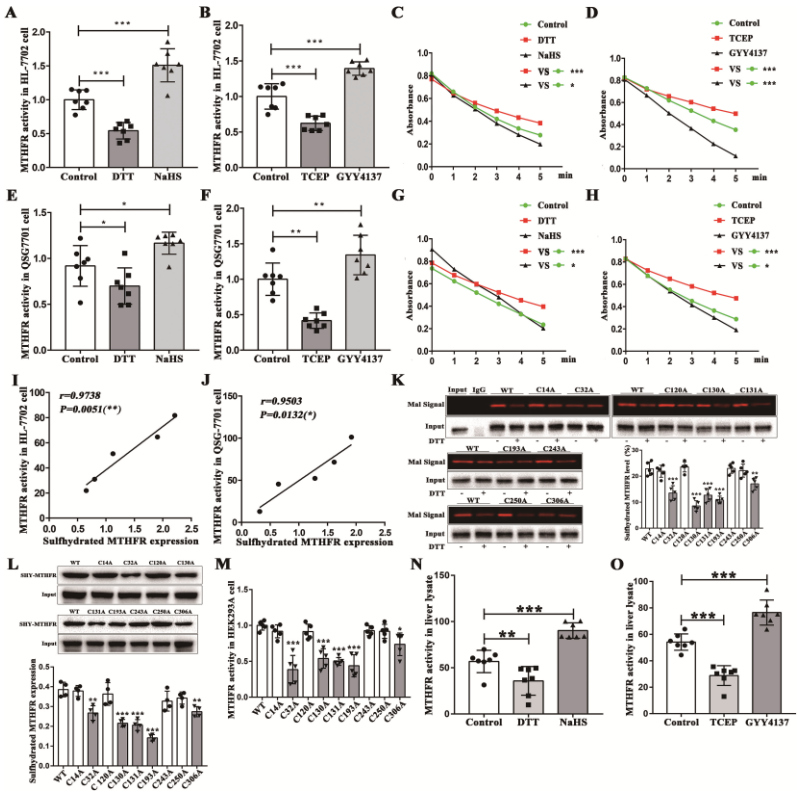

Fig. 2. MTHFR bioactivity was positively correlated with its S-sulfhydration level. (A-H) The HL-7702 cells or QSG-7701 cells were treated with DTT (1 mmol /L) or NaHS (1 mmol /L) for three hours respectively. In addition, the cells (HL-7702 cells, QSG-7701 cells) were treated with TCEP (1 mmol /L) or GYY4137 (200 μmol/L) for six hours respectively, enzyme linked chemiluminescent kit (A, B, E, F, n=7) or NADPH-Menadione Oxidoreductase Assay (C, D, G, H, n=6, the greater the slope of the curve, the higher the activity) were used to detect MTHFR bioactivity. (I-J) HL-7702 cells or QSG-7701cells were treated with different concentrations DTT (1 mmol /L, 500μmol/L) or NaHS (1 mmol /L, 500μmol/L) for three hours. MTHFR bioactivity was detected with enzyme linked chemiluminescent kit, and MTHFR S-sulfhydration level was detected with modified biotin switch assay. Pearson correlation analysis was used to analyze the correlation between the two factors (n=3). (K-L) Maleimide assay (K) and modified biotin switch assay (L) of MTHFR S-sulfhydration, with mutated MTHFR cysteine residues (n=4-5). (M) Changes in MTHFR bioactivity with mutated S-sulfhydration MTHFR sites (n=5). Liver lysates were treated with 1M NaHS or 1M DTT for 60 min at 37°C respectively. In addition, liver lysates were treated with 2M GYY4137 or 2M TCEP for 8h at 37°C respectively. MTHFR bioactivity (N, O, n=7) was detected with enzyme linked chemiluminescent kit.

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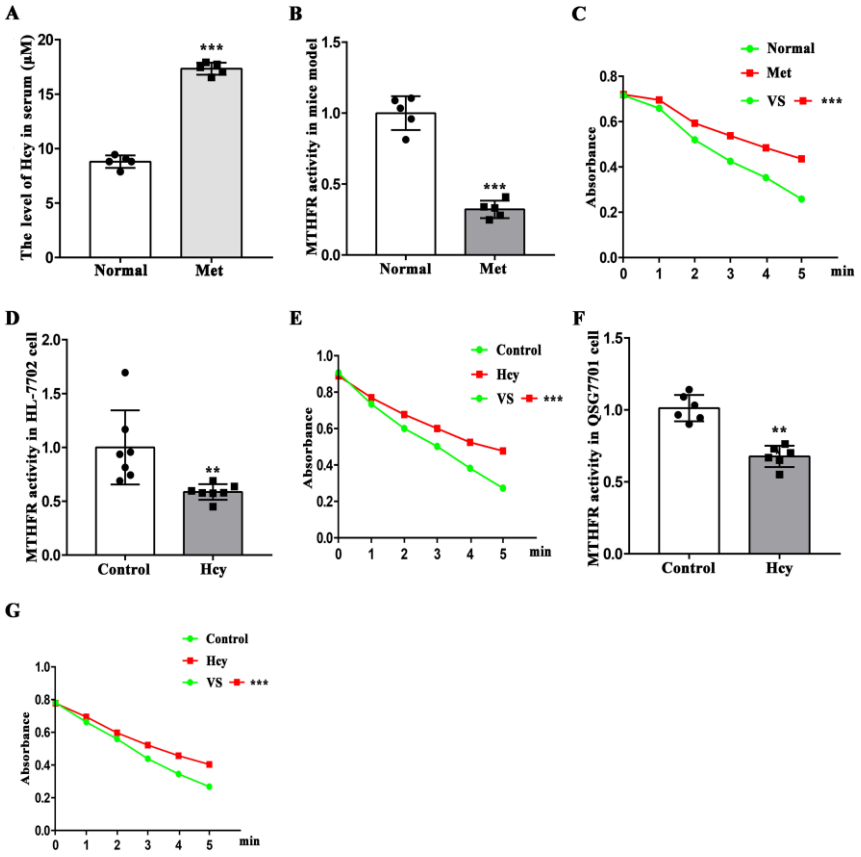

Fig. 3. HHcy inhibited the bioactivity of MTHFR. (A) The serum Hcy level was significantly increased after 17 weeks of 2.5% methionine feeding in c57BL/6J mice (n=5). Measurement of MTHFR bioactivity in c57BL/6J mice liver with enzyme linked chemiluminescent kit (B) or NADPH-Menadione Oxidoreductase Assay (C) (n=5). MTHFR bioactivity of HL-7702 cells (D-E) or QSG-7701 cells (F-G) after challenged with Hcy (500μmol/L) (n=4-7).

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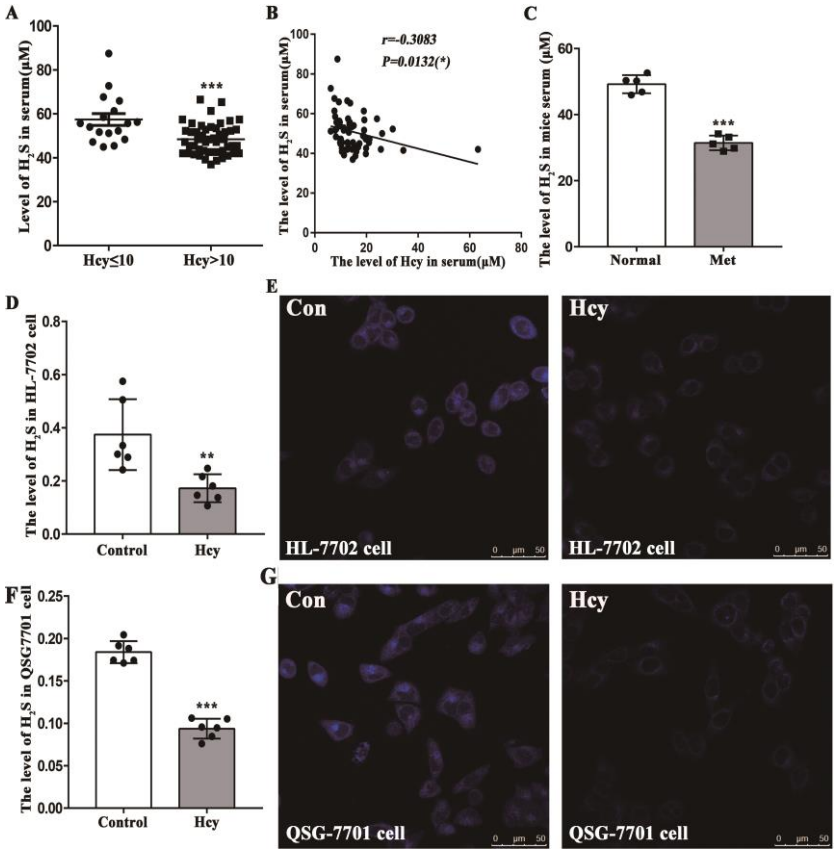

Fig. 4. H_2S level were inhibited in HHcy. Methylene blue assay was used to detect H_2S level. (A) The serum H_2S level in 64 coronary heart patients (Hcy ≤10 μmol/L, n=17; Hcy >10 μmol/L, n=47). (B) Pearson correlation analysis of the correlation between the serum Hcy level and H_2S level in coronary heart patients. H_2S level was detected with methylene blue (C, D, F, n=5-6) and H_2S fluorescence probe (E, G, n=4) in mice model, HL-7702 cells, and QSG-7701 cells respectively.

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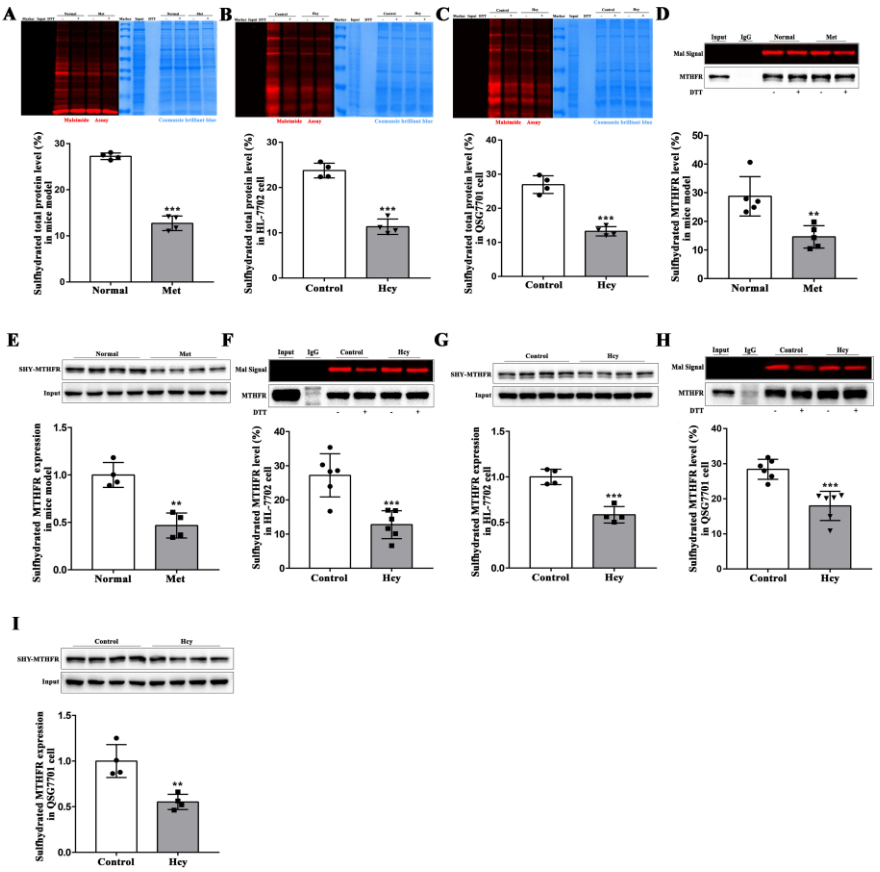

Fig. 5. HHcy reduced the level of MTHFR S-sulfhydration. (A-C) HHcy changed the proteins S-sulfhydration level both in mice model and cells model (n=4). (D-I) Maleimide assay and modified biotin switch assay proved that HHcy reduced MTHFR S-sulfhydration level both in mice model (D-E, n=4-5), HL-7702 cells (F-G, n=4-6), and QSG-7701 cells (H-I, n=4-6).

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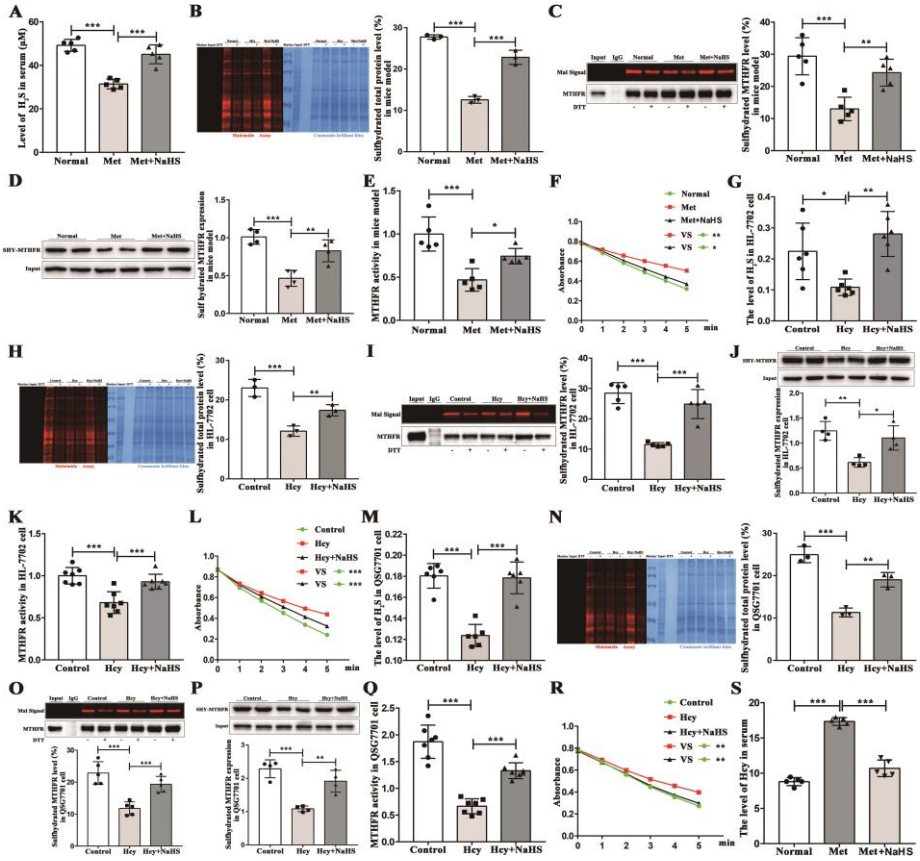

Fig. 6. H_2S donor rescued the bioactivity of MTHFR in HHcy and reduced Hcy level.

c57BL/6J mice given a Met diet and intraperitoneal injection of NaHS for 17 weeks were used as the rescue mice model. HL-7702 cells and QSG-7701 cells were treated with Hcy and NaHS to establish rescue cells model. After H_2S donor treatment, H_2S level in mice serum (A, n=5), HL-7702 cells (G, n=6), QSG-7701 cells (M, n=6), total protein S-sulfhydration level (B, H, N, n=3 for each of three groups) were measured. In mice model (C-F, n=4-5) and cells model (I-R, n=4-7), MTHFR S-sulfhydration level and bioactivity were detected. (S) The serum level of total Hcy in mice model were detected (n=5).

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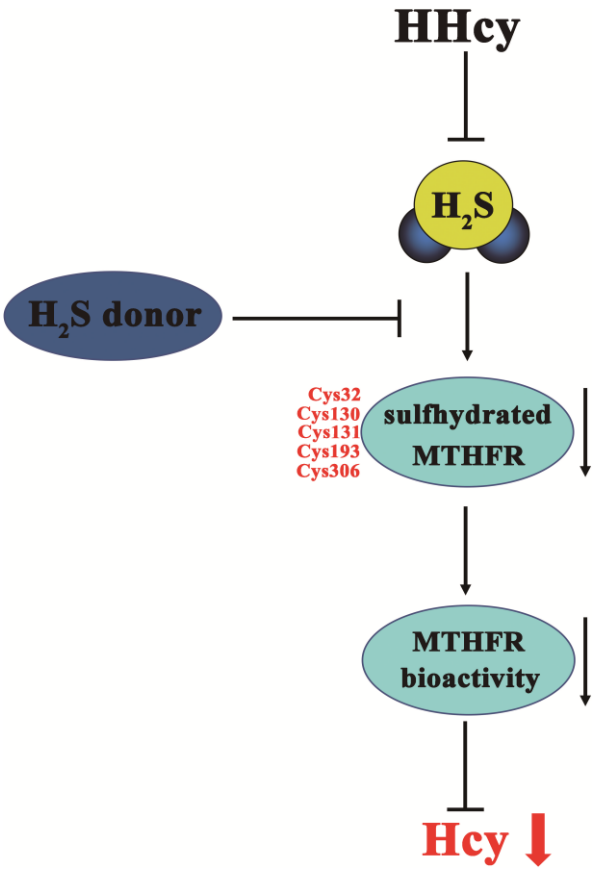

Fig. 7. Summary of our findings in the present study. MTHFR bioactivity was positively correlated with its S-sulfhydration level under physiological conditions, which was occurred at 32,130,131,193, and 306 cysteine sites. MTHFR bioactivity was inhibited in HHcy due to reduced S-sulfhydration level, which could be rescued by H_2S donors.

Table 1

Primers sequence of PCR

Genes	Primers sequence
MTHFR(Human)	Forward:CTGTGCCACCTTCCATCAGT Reverse:CTTTGGAGCTCTCACTGCCA
MTHFR(Mouse)	Forward:GAAACCATCCTGCATATGACCT Reverse:CAAAATAGTCAGCAAACTCGGT
Actin(Human)	Forward:CCTGGCACCCAGCACAAT Reverse:GGGCCGGACTCGTTCATAC
Actin(Mouse)	Forward:GTGCTATGTTGCTCTAGACTTCG Reverse:ATGCCACACAGGATTCCATACC

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