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Hydrogen sulfide mitigates homocysteine mediated pathological remodeling by inducing miR-133a in cardiomyocytes

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Abstract

An elevated level of homocysteine called hyperhomocysteinemia (HHcy) is associated with pathological cardiac remodeling. Hydrogen sulfide (H₂S) acts as a cardioprotective gas, however the mechanism by which H₂S mitigates homocysteine mediated pathological remodeling in cardiomyocytes is unclear. We hypothesized that H₂S ameliorates HHcy mediated hypertrophy by inducing cardioprotective miR-133a in cardiomyocytes. To test the hypothesis, HL1 cardiomyocytes were treated with: 1) plain medium (control, CT), 2) 100μM of homocysteine (Hcy), 3) Hcy with 30μM of H₂S (Hcy+H₂S), and 4) H₂S for 24 hour. The levels of hypertrophy markers: c-fos, atrial natriuretic peptide (ANP), and beta-myosin heavy chain (β-MHC), miR-133a and its transcriptional inducer myosin enhancer factor- 2c (MEF2C) were determined by Western blotting, RT-qPCR, and immunofluorescence. The activity of MEF2C was assessed by co-immunoprecipitation of MEF2C with histone deacetylase -1(HDAC1). Our results show that H₂S ameliorates homocysteine mediated up regulation of c-fos, ANP and β-MHC, and down regulation of MEF2C and miR-133a. HHcy induces the binding of MEF2C with HDAC1, whereas H₂S releases MEF2C from MEF2C-HDAC1 complex causing activation of MEF2C. These findings elicit that HHcy induces cardiac hypertrophy by promoting MEF2C-HDAC1 complex formation that inactivates MEF2C causing suppression of anti-hypertrophy miR-133a in cardiomyocytes. H₂S mitigates hypertrophy by inducing miR-133a through activation of MEF2C in HHcy cardiomyocytes. To our knowledge this is a novel mechanism of H₂S mediated activation of MEF2C and induction of miR-133a and inhibition of hypertrophy in HHcy cardiomyocytes.

Keywords

Hyperhomocysteinemia; Hydrogen sulfide; MiR-133a; Hypertrophy; MEF2C; HDAC

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Conflict of interest

Authors declares that there are no conflicts of interest.

1. Introduction

Homocysteine (Hcy) is a sulfur containing non-protein coding amino acid that contributes to cardiovascular diseases [1, 2]. Elevated level of Hcy is called hyperhomocysteinemia (HHcy), which is associated with increased incidence of mortality [3]. It is considered that 5 μ M/L raise in plasma Hcy level increases the risk of coronary artery disease by as much as 0.5mmol/L of cholesterol [4]. HHcy differentially regulates miRNAs [5] and attenuates beta-adrenergic signaling by suppressing stimulatory G protein (Gs) [6], which lead to pathological cardiac remodeling. HHcy is caused either due to impaired re-methylation process, which involves methyl tetrahydrofolate reductase enzyme and folic acid and B vitamins co-factors, or defect in any of the three Hcy metabolic enzymes: cystathionine β -synthase (CBS), cystathionine γ -lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3MST). These three enzymes convert Hcy into hydrogen sulfide (H₂S) [7]. Contrary to HHcy, H₂S is a cardioprotective gas [8–11]. H₂S ameliorates cardiac dysfunction at several levels. It preserves mitochondrial function in myocardial ischemia-reperfusion injury [12], suppresses activation of nuclear factor kappa B and mitogen activated protein kinases in Cocksackie virus B3 infected rat cardiomyocytes [13], mitigates endoplasmic reticulum stress in high fat diet fed mice [14], induces Nrf2 transcription factor to alleviate oxidative stress in myocardial ischemia [15], and up regulates endothelial nitric oxide synthase for nitric oxide bioavailability in ischemia-reperfusion injury [16]. Although the pathological effect of HHcy and the cardioprotective role of H₂S are well documented, the mechanism by which HHcy induces pathological cardiac remodeling and H₂S mitigates that remodeling is poorly understood.

MicroRNAs (miRs) are a newly emerged tiny, non-coding, regulatory RNAs that modulate gene expression either by mRNA degradation or translational repression [17]. They are recognized as a novel therapeutic target for cardiovascular diseases [18, 19]. In human hearts, >800 miRNAs are present and >250 miRNAs are differentially regulated in pathological hearts [20]. MiR-133a is one of the most abundant miRNA in the heart [21]. It is cardioprotective and mitigates cardiac fibrosis [22, 23] and hypertrophy [24]. MiR-133a transcription is regulated by myosin enhancer factor 2C (MEF2C) [25]. Members of the MEF2 family are repressed when they make complex with histone deacetylase (HDAC) [26]. Therefore, formation of MEF2C-HDAC complex inactivates MEF2C. However, role of Hcy in formation of MEF2C-HDAC complex, and thereby transcription of miR-133a in cardiomyocytes is unclear. Also, whether the H₂S ameliorates the effect of Hcy on MEF2C-HDAC complex formation, and miR-133a deregulation is nebulous. Accordingly, we investigated the role of Hcy on MEF2C mediated attenuation of miR-133a, and H₂S in restoring the level of miR-133a in HHcy cardiomyocytes.

2. Materials and Methods

2.1. Cell culture and treatments

HL1 cardiomyocytes were cultured in Claycomb media as described elsewhere [5]. In brief, cells are treated with only incomplete (serum deprived) medium (control, CT), Hcy (100 μ M) and/or H₂S (30 μ M) in the incomplete medium for 24 hour. We selected 100 μ M dose of Hcy because at *in vitro* level, lower dose of Hcy (30 μ M) did not engender significant effect on

pathological remodeling in cardiomyocytes [5]. It is also reported that inborn errors of Hcy metabolism results into elevation of plasma Hcy level from 200–300 $\mu\text{m/L}$, which is markedly higher than the mild elevation ($>15 \mu\text{m/L}$) of Hcy in humans [27]. Therefore, 100 μm is physiologically relevant dose of Hcy. After 24 hour, cells were used for either protein extraction or imaging. Hematoxylin-Eosin (H-E) (#HAE-1, ScyTeK laboratories Inc., USA) staining of cells were performed after fixing the cells with 4% paraformaldehyde. Cells were observed under bright field color microscope. For inhibiting miR-133a, we used anti-miR-133a oligonucleotide (AMO-133a) (Catalogue # AM17000, ID: AM10413 Ambion, Life technologies Inc., USA). AMO-133a was transfected before treatment with Hcy and H_2S . In brief, 75 picomole AMO-133a was transfected with lipofectamine (6 μl per well of 6 well plate) in final volume of 300 μl optiMEM (Catalogue #31985-70, Gibco Life technologies Inc., USA) for 8 hours. Cells were then treated for Hcy + H_2S for 24 hour in antibiotic free medium.

2.2. Western Blotting

Radio-Immuno-Precipitation Assay (RIPA, catalogue # BP-115D, Boston BioProducts, USA) lysis buffer was used to isolate total protein from HL1 cells after treatment. The quantity of protein was estimated by using Pierce BCA protein Assay kit (catalogue # 23227, Thermo scientific, USA). Western blotting was performed using SDS-PAGE (10%). Protein was transferred onto nitrocellulose membrane (catalogue #162-0115, Bio-Rad Laboratories, USA) by overnight transfer at 4°C . The primary antibodies of MEF2C (catalogue # ab79436, Abcam, USA), β -MHC (catalogue # ab172967, Abcam, USA), ANP (catalogue # AB5490, Millipore, USA) and β -actin (catalogue # sc-47778, Santa Cruz Biotech, USA) were diluted in the ratio of 1:1000. The secondary antibodies, anti-mouse IgG-HRP (catalogue # sc-2005, Santa Cruz Biotech, USA) and anti-rabbit IgG-HRP (catalogue # sc -2054, Santa Cruz Biotech, USA) were diluted in the ratio of 1:2000. The membranes were developed and band intensity was analyzed by ChemiDoc software (Bio-Rad Laboratories, USA).

2.3. miRNA assay

For miRNA assay, RNA was isolated from HL1 cells using miRVana™ miRNA Isolation Kit (catalogue # AM1560 Life technologies, USA). The RNAs quality was assessed by NanoDrop 2000c (Thermo Scientific Inc., USA) and RNAs with 260/280 >1.8 and 260/230 >1.8 were used for miR-133a assay using Taqman probes. The miRNA Reverse Transcriptase-PCR followed by Taqman qPCR were performed using miR-133a (Assay ID-002246) and U6 SnRNA (Assay ID: 001973) specific primers following the manufacturer's instruction and protocol (Life technologies, USA). The details of the protocol is published elsewhere [28]. U6 SnRNA was used as an endogenous control for miR-133a. The miRNA PCR reactions mixtures were amplified in a 96 well plate in Bio-Rad CFX qPCR Instrument, and data were analyzed by integrated BioRad CFX Manager3.0 software (Bio-Rad Laboratories, USA).

2.4. Immunocytochemistry

In vitro immunocytochemistry studies on HL1 cells were performed using standard protocol. In brief, cells were cultured in 24 well plates and treated for 24 hour in incomplete medium. After treatment medium was removed and cells were washed with 1xPBS, pH 7.4 followed by fixation in 4% paraformaldehyde (catalogue #158127, Sigma, USA) for 40 minutes. Fixed cells were washed in 1xPBS for 5 minutes three times, then permeabilized in 0.01% Triton-X-100 in 1xPBS for 30 minutes, and blocked in 1% BSA in PBS for 1 hour. After blocking solution was removed, cells were washed with 1xPBS for 5 minutes three times followed by incubation in diluted primary antibody solution in 1xPBS with 0.1% BSA overnight at 4°C. The primary antibodies used were: 5µg/mL of rabbit anti-Atrial Natriuretic Peptide (catalogue # AB5490, Millipore, USA), 1:400 dilution of c-fos (9F6) Rabbit mAb (catalogue # 2250, Cell Signaling Technologies, USA), 1:500 dilution of rabbit anti-MEF2C (catalogue # ab79436, Abcam, USA), and 1:500 dilution of rabbit anti-β-MHC (catalogue # ab172967, Abcam, USA). Primary antibody was removed after 24 hour at 4°C, and cells were washed in 1xPBS for 5 minutes three times at room temperature. Cells were incubated with secondary antibody for 1 hour in dark. The secondary antibodies were: chicken anti-rabbit-IgG and/or anti-mouse Alexa Fluor 488/594 conjugated secondary antibodies (catalogue # A21441, A21200, A21201, Life Technologies, USA). Then cells were washed with 1x PBS for 5 minutes 3 times, and incubated with 1µg/ml DAPI solution in 1xPBS (catalogue # A1001, AppliChem, USA) for 1 minute. Cells were washed twice in 1xPBS for 5 minute each and observed under EVOS Cell Imaging Systems (Life Technologies, USA). The intensity of color was analyzed by Image J software (NIH, USA).

2.5. Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) was performed using dynabeads (catalogue # 10003D, Life Technologies, USA) following the protocol from the company. In brief, cells were lysed using IP lysis buffer (Thermo Scientific, USA). Diluted anti-HDAC1 antibody (catalogue # ab46985, Abcam, USA) was added to dynabeads and mixed by gentle shaking and incubated for 10 minute at room temperatures. Tubes were placed on a magnet and supernatant was removed. Beads were re-suspended in PBS and washed three times with PBS-Tween-20. Protein samples were added in the tubes and gently pipetted to re-suspend the samples. Tubes were incubated with rotation (15 rpm) for 30 minute at room temperature. After incubation beads were washed and transferred into a new tube. Elution was done with sample buffer having β-mercaptoethanol. 10% SDS-PAGE was performed and membranes were blotted with MEF2C and HDAC1 antibodies. In another gel, same protein lysates were electrophoresed for β-actin to confirm the equal loading of proteins that was used for immunoprecipitation.

2.6. Statistical Analysis

The values are represented as mean ± standard error of mean (S.E). To determine the statistical significance between the groups, one way analyses of variance (ANOVA) was performed, which was followed by Newman-Keuls method to determine differences in means between the groups. P<0.05 was considered statistically significant.

3. Results

3.1. H₂S mitigates HHcy induced hypertrophy in cardiomyocytes

To determine the hypertrophic effect of Hcy on cardiomyocytes, we treated HL1 cells with high dose (100μM) of Hcy for 24 hour as per the published report [29], and determined the expression of β-MHC, ANP and c-fos. We found that HHcy induces both the early hypertrophy maker c-fos (Figure 1A), and late hypertrophy markers β-MHC (Figure 1B), and ANP (Figure 1C) in the cardiomyocytes. To assess the cardioprotective effect of H₂S in HHcy mediated hypertrophy, the cardiomyocytes were treated with both Hcy and H₂S (30μM). Interestingly, the levels of both early and late hypertrophy markers were significantly down regulated in Hcy and H₂S (Hcy+ H₂S) treated group as compared to the Hcy group (Figure 1A–C) suggesting that H₂S mitigates the initiation and progression of HHcy mediated hypertrophy in cardiomyocytes. Furthermore, there was no significant difference between CT and H₂S groups (Figure 1A–C) suggesting that H₂S maintains the normal size of the cardiomyocytes. The one-way ANOVA showed significant difference among the mean value of c-fos, ANP and BNP among the groups. These molecular studies clearly show that H₂S retains the size of healthy cardiomyocytes but has an anti-hypertrophic effect in HHcy cardiomyocytes. To reinforce our results, we observed the changes in cell morphology in the four groups (CT, Hcy, Hcy+ H₂S, and H₂S) by H-E staining. Our results show that cells were enlarged and morphology of the cells were more round –shape in Hcy as compared to the CT, where the shape of the cells were triangular with tapering ends. Notably, in Hcy+ H₂S, the cell size and morphology were more like CT (Figure 1D). These result are consistent with the molecular data that H₂S alleviates Hcy mediated hypertrophy in cardiomyocytes (Figure 1A–C). In cells, which were treated with H₂S only, the cell size and morphology were similar to that of the CT cells suggesting that the treated dose of H₂S did not have any significant effect on the size and morphology of the cardiomyocytes.

3.2. HHcy attenuates and H₂S induces miR-133a in cardiomyocytes

Since miR-133a is recognized as an anti-hypertrophy miRNA [24], and H₂S is reported to induce miR-133a [30], we were interested to evaluate the effect of HHcy on miR-133a in cardiomyocytes. For that, we measured the levels of miR-133a in the above-mentioned four groups using individual miR-133a assay as described elsewhere [31]. The miR-133a assay revealed that HHcy down regulates miR-133a, whereas concomitant treatment with H₂S induces miR-133a in the cardiomyocytes (Figure 2A). However, there was no significant difference between CT and H₂S groups. One-way ANOVA showed significant difference in miR-133a among the four groups. These findings elucidate that HHcy inhibits miR-133a, which is ameliorated by H₂S in cardiomyocytes. Since miR-133a inhibits hypertrophy [24], we were interested to understand whether the mitigation of HHcy mediated hypertrophy by H₂S is due to up regulation of miR-133a. For that, we treated Hcy+ H₂S with anti-miR-133a (AMO). If H₂S inhibits HHcy mediated hypertrophy through induction of miR-133a, then treatment with AMO in Hcy+ H₂S should blunt the anti-hypertrophic effect of H₂S. We evaluated the levels of β-MHC and ANP in the five (four above and Hcy+ H₂S+AMO) groups, which revealed that AMO treatment nullified the anti-hypertrophy effect of H₂S in HHcy cardiomyocytes (Figure 2B). It suggests that miR-133a plays crucial role in H₂S

mediated mitigation of hypertrophy in HHcy cardiomyocytes. These results provide evidence that H₂S inhibits Hcy mediated hypertrophy by inducing miR-133a in the cardiomyocytes.

3.3 H₂S augments miR-133a by activating MEF2C in HHcy cardiomyocytes

After showing that H₂S induces miR-133a, we were interested to understand how H₂S up regulated miR-133a in HHcy cardiomyocytes. To investigate the underlying mechanism, we first measured the protein and cellular levels of MEF2C- a transcriptional inducer of miR-133a [25] in the four groups. Our results show that HHcy down regulates whereas H₂S induces MEF2C in HHcy cardiomyocytes at both protein and cellular levels (Figure 3A–B). However, there was no significant difference between CT and H₂S group (Figure 3A–B). The significant increase in MEF2C in Hcy+H₂S as compared to Hcy group suggests that H₂S induces miR-133a by up regulating MEF2C in HHcy cardiomyocytes. Next, we determined whether the Hcy has any effect on activation of MEF2C. Since MEF2C remains inactivate if it binds to HDAC [26], we performed co-IP of MEF2C with HDAC1. The bound form of MEF2C with HDAC1 was evaluated by immunoprecipitation of HDAC1 (input) and probing it with MEF2C. The co-IP analyses revealed that the bound form of MEF2C (MEF2C-HDAC1) was robust in HHcy cardiomyocytes but dramatically decreased in Hcy+H₂S (Figure 3C). To rule out unequal loading of proteins for co-IP, we separately performed Western blot with the same amount of protein and measured the levels of β -actin, which showed equal loading of proteins in the four groups (Figure 3C). To reinforce the co-IP results, we determined the cellular levels of MEF2C and HDAC1 in the four groups. The immunofluorescence results show that HDAC1 was up regulated in Hcy as compared to CT, and mitigated in Hcy+ H₂S (Figure 3D(i)). The cellular expression of MEF2C was less in Hcy but elevated in Hcy+H₂S. Interestingly, co-localization of MEF2C with HDAC1 revealed that the number of cells expressing both HDAC1 and MEF2C was comparatively higher in Hcy group (Figure 3D(ii)). To evaluate the co-expression of MEF2C with HDAC1, we determined the ratio of MEF2C with HDAC1 in the four groups. If the HDAC1 level is higher than MEF2C, there will be more probability of bound (MEF2C-HDAC1) or inactive form of MEF2C. The fluorescence analyses showed that in Hcy group MEF2C/HDAC1 was decreased than that of CT indicating that HHcy induces the formation of MEF2C-HDAC1 complex. On contrary, treatment with H₂S increased MEF2C and decreased HDAC1, thereby increasing the ratio of MEF2C/HDAC1 and decreasing the bound form of MEF2C in HHcy cardiomyocytes (Figure 3D(i)–(ii)). These results demonstrate that HHcy attenuates miR-133a by inhibiting MEF2C, whereas H₂S up regulates miR-133a by inducing MEF2C in HHcy cardiomyocytes.

Overall, our results show that HHcy down regulates and inactivates MEF2C causing attenuation of miR-133a, which induces hypertrophy in cardiomyocytes. On contrary, H₂S up regulates and activates MEF2C, and increases the level of miR-133a that mitigates HHcy mediated hypertrophy in cardiomyocytes (Figure 4).

4. Discussion

We demonstrate a novel mechanism of Hcy mediated inhibition of miR-133a and induction of hypertrophy, and H₂S mediated mitigation of hypertrophy in HHcy cardiomyocytes

(Figure 4). Hcy is reported to play crucial role in pathological cardiac remodeling [2, 3, 5, 18, 32–34]. However, there is debate on whether Hcy is a cause or effect of cardiovascular diseases [27]. The negative results of clinical trials, where lowering Hcy level by folic acid supplements did not improve the conditions in heart failure patients poses a major setback on HHcy being a cause for heart failure [35–37]. Additionally, the underlying molecular mechanisms that elicit the causative role of Hcy in the heart disease is poorly understood [38]. Our results demonstrate that HHcy induces cardiomyocyte hypertrophy by suppressing miR-133a, which show a causative role of Hcy in pathological remodeling of the heart. Further, we provided a detailed mechanism by which Hcy down regulates miR-133a. HHcy inactivates and down regulates MEF2C in the cardiomyocytes (Figure 3A–C). Since miR-133a is attenuated in the failing human hearts [24] and in diabetic hearts [22, 28], and its role is established as anti-hypertrophy [24, 39] and anti-fibrosis [22, 23], our results suggest a new role of Hcy in miR-133a mediated pathological remodeling in the heart, which is relevant to the human heart failure.

H₂S is a metabolic product of Hcy [7]. Mutation of cystathionine beta-synthase or cystathionine gamma- lyase genes or enzymatic inactivation of these proteins impairs the endogenous production of H₂S [7]. H₂S is emerged as a novel cardioprotective gas with therapeutic potential for heart diseases [8, 10, 11, 40]. It has multifaceted role such as vasorelaxant [41], inducer of anti-stress AKT [42, 43], preserver of mitochondrial function [12], and inhibitor of hypertrophy and fibrosis [44]. H₂S also mitigates Hcy mediated induction of oxidative and endoplasmic reticulum stress [45]. Since HHcy can be caused due to inhibition of metabolic enzymes that produce H₂S from Hcy [7], one of the fundamental question could be whether the inhibition of H₂S production is the major cause of HHcy mediated cardiovascular diseases. To answer this question, we treated cardiomyocytes with both Hcy and H₂S concomitantly. Our results show that treatment of H₂S mitigates the hypertrophic effect of Hcy (Figure 1A–C) supporting the notion that inhibition of H₂S production in HHcy cardiomyocytes leads to hypertrophy. Since H₂S is reported to mitigate phenylephrine mediated hypertrophy in cardiomyocytes [30], we also measured the levels of miR-133a in HHcy cardiomyocytes treated with and without H₂S. Our results show that H₂S mitigates Hcy mediated attenuation of miR-133a in cardiomyocytes (Figure 2) suggesting that effect of HHcy is alleviated by H₂S in cardiomyocytes. Our results also support the anti-hypertrophic effect of H₂S in cardiomyocytes [30]. To get insight into how Hcy suppresses and H₂S induces miR-133a, we measured the levels of MEF2C, a known inducer of miR-133a [25] in HHcy cardiomyocytes treated with and without H₂S. Interestingly, we observed that Hcy inactivates MEF2C by promoting the binding of MEF2C with HDAC1 in cardiomyocytes. On contrary, H₂S treatment mitigates binding of MEF2C with HDAC1 releasing more MEF2C that in turn increases transcription of miR-133a in HHcy cardiomyocytes. To our knowledge this is first study showing the mechanism of Hcy and H₂S mediated regulation of miR-133a transcription in cardiomyocytes. These findings also suggest that inhibition of H₂S could be the major cause of HHcy mediated pathological remodeling, and supplement of H₂S could be a major therapeutic strategy to ameliorate Hcy mediated cardiovascular pathology.

Hypertrophy is marker for cardiac pathogenesis [46]. Our results show that c-fos, an early marker of hypertrophy [47] is up regulated in HHcy cardiomyocytes, and mitigated by H₂S

in HHcy cardiomyocytes (Figure 1A). Our results are consistent with the previous report showing that HHcy induces c-fos in hepatocytes [48]. HHcy is also reported to induce left ventricular (LV) to body weight ratio, which is an indicator of cardiac hypertrophy [49]. Our results show up regulation of β -MHC and ANP- late markers of cardiac hypertrophy in HHcy cardiomyocytes (Figure 1B–D) supporting HHcy mediated induction of LV hypertrophy. It is documented that H₂S mitigates cardiac hypertrophy in pathological hearts [44, 50], which is consistent with our results, where H₂S mitigates Hcy mediated hypertrophy in cardiomyocytes (Figure 1A–D). The anti-hypertrophic effect of miR-133a is due to its regulatory effect on RhoA and CDC42, which are involved in cytoskeletal and myofibrillar arrangements during cardiac hypertrophy [24]. One of the key molecules involved in regulation of cardiac remodeling is connective tissue growth factor (CTGF). CTGF regulates collagens synthesis by inducing fibroblast and is a direct target of miR-133a [51]. In cardiomyocytes, knockdown of miR-133a robustly increased the mRNA and protein levels of CTGF, which is blunted by overexpression of miR-133a [51]. Our results show that Hcy induces hypertrophy by suppressing miR-133a, whereas H₂S mitigates hypertrophy by inducing miR-133a in HHcy cardiomyocytes. The cardioprotective effect of H₂S is also mediated through miR-21, which attenuates myocardial ischemic and inflammatory injury in mice [52].

In conclusion, our findings provide new insight on Hcy mediated down regulation and inactivation of MEF2C, attenuation of miR-133a, and induction of hypertrophy in cardiomyocytes. It also suggests that the pathophysiology of HHcy is attributed to the decreased levels of H₂S, and H₂S has therapeutic potential for HHcy pathogenesis. Our findings also provide a novel mechanism of H₂S mediated induction of miR-133a in cardiomyocytes.

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Abbreviations

HHcy	Hyperhomocysteinemia
H₂S	Hydrogen sulfide
Hcy	Homocysteine
ANP	atrial natriuretic peptide
β-MHC	beta-myosin heavy chain
MEF2C	myosin specific enhancer factor 2c

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Figure 1A

Figure 1B

Figure 1C

Figure 1.

H₂S mitigates Hcy mediated induction of hypertrophy in the cardiomyocytes.

Immunofluorescence (IF) of c-fos, β -MHC, and ANP in the four groups of HL1 cardiomyocytes: control (CT), homocysteine treated (Hcy), Hcy and H₂S treated (Hcy+H₂S), and H₂S treated (H₂S). A (i) Representative IF of c-fos (green) in the four groups. Dapi (blue) represents nucleus. A (ii) Bar graph shows quantitative analyses of green color and represented as mean $\pm$ S. E. B (i). Representative IF of β -MHC (red) in the four groups. B (ii) Bar graph shows quantitative analyses of red color and values are mean $\pm$ S. E. C (i). Representative IF of ANP (green) in the four groups C (ii). Bar graph shows quantitative analyses of green color and values are represented as mean $\pm$ S. E. Scale bar: 100 μ m. D (i) – (iv). Hematoxylin and Eosin staining of HL1 cardiomyocytes in the four groups. The changes in the size and morphology of cardiomyocytes are shown in high magnification. Scale bar: 100 μ m. N=3. *, p<0.05 compared to CT; †, p<0.05 compared Hcy; ††, p<0.05 compared Hcy+H₂S.

Figure 2A

Figure 2B

Figure 2.

H₂S mitigates Hcy mediated attenuation of miR-133a in the cardiomyocytes. Individual miR-133a assay in the four groups of HL1 cardiomyocytes: control (CT), homocysteine treated (Hcy), Hcy and H₂S treated (Hcy+ H₂S), and H₂S treated (H₂S). U6 is used as an endogenous control for miR-133a. Data represents expression in fold change (mean± S. E.) of miR-133a in treated samples compared to control (CT). Experiments repeated at least three times, N=7. *, p<0.05 as compared to CT; †, p<0.05 as compared Hcy. B. MiR-133a in H₂S mediated recovery in HL1 cells treated with homocysteine (Hcy). B (i). Representative Western blot of β-MHC and ANP in different groups. β-actin is a loading control. HL1 cells treated with Hcy and Hcy+H₂S for 24 hour and expression of ANP was analyzed. AMO-133a was transfected before Hcy+H₂S treatment. B (ii). Bar graph shows densitometric analyses of β-MHC bands and represented as fold change of control. B (iii). Bar graph represents densitometric analyses of ANP bands and presented as fold change of

control. N=3. *, p<0.05 as compared to CT; †, p<0.05 as compared Hcy; ††, p<0.05 as compared Hcy+H₂S.

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Figure 3 A-B

Figure 3C

Figure 3D

Figure 3.

H₂S induces expression and activity of MEF2C expression in HHcy HL1 cardiomyocytes. A (i). Representative Western blot of MEF2C in the four groups. GAPDH is a loading control. A (ii). Bar graph shows densitometric analyses of MEF2C bands and represented as fold change of control. B (i). Immunofluorescence of MEF2C in HL1 cells treated with homocysteine (Hcy), H₂S donor (Na₂S) and Hcy+H₂S. Red color represents MEF2C and blue color shows DAPI. Scale bar: 100μm. (ii) Bar graph shows quantitative analyses of color and represented as mean ± S. E. C. Co-Immunoprecipitation of MEF2C with HDAC in cell lysate of HL1 cells treated with homocysteine (Hcy), H₂S donor (Na₂S) and Hcy+H₂S. Immunoprecipitation was performed with anti-HDAC1 antibody and Western blotting was performed for MEF2C. Input was electrophoresed in a separate gel and developed for β-actin. D (i). Immunofluorescence showing co-localization of MEF2C with HDAC1 in the four groups of HL1 cardiomyocytes. Red color represents MEF2C, green color HDAC1, and blue color DAPI (nucleus). D (ii). Bar graph shows quantitative analysis of MEF2C/HDAC1 immunofluorescence intensity in the above groups and represented as mean± S. E. Scale bar: 100μm. N=3. *, p<0.05 compared to CT; †, p<0.05 compared Hcy; ††, p<0.05 compared Hcy+H₂S.

Figure 4.

A schematic representation showing cardioprotective mechanism of H₂S in Hcy induced hypertrophy in cardiomyocytes. Elevated level of Hcy causes hypertrophy by increasing the level of c-fos, ANP, and β-MHC; and decreasing the level of miR-133a in cardiomyocytes. H₂S treatment induces MEF2C that in turn up regulates miR-133a, which alleviates c-fos, ANP, and β-MHC; and ultimately mitigates hypertrophy.