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H_2S protects from oxidative stress-driven ACE2 expression and cardiac aging

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Abstract

Cystathionine gamma-lyase (CSE)-derived hydrogen sulfide (H_2S) plays an essential role in preserving cardiac functions. Angiotensin-converting enzyme 2 (ACE2) acts as the negative regulator of the renin-angiotensin system, exerting anti-oxidative stress and anti-inflammatory properties within the body. The interplays of CSE/H_2S signaling and ACE2 in cardiac aging are unclear. In this study, the regulatory roles of H_2S on ACE2 expression in mouse heart tissue and rat cardiomyocytes under different stress conditions were investigated. It was found that ACE2 protein level was lower in heart tissues from old mice (56-week-old) than young mice (8-week-old), and the knockout of CSE (CSE KO) induced moderate oxidative stress and further inhibited ACE2 protein level in mouse hearts at both young and old age. Incubation of rat cardiac cells (H9C2) with a low dose of H_2O_2 (50 μM) suppressed ACE2 protein level and induced cellular senescence, which was completely reversed by co-incubation with 30 μM NaHS (a H_2S donor). Prolonged nutrient excess is an increased risk of heart disorders by causing metabolic dysfunction and cardiac remodeling. We further found high-fat diet feeding stimulated ACE2 expression and induced severe oxidative stress in CSE KO heart in comparison with wild-type heart. Lipid overload in H9C2 cells to mimic a status of nutrient excess also enhanced the expression of ACE2 protein and induced severe oxidative stress and cell senescence, which were significantly attenuated by the supplementation of exogenous H_2S. Furthermore, the manipulation of ACE2 expression partially abolished the protective role of H_2S against cellular senescence. These results demonstrate the dynamic roles of H_2S in the maintenance of ACE2 levels under different levels of oxidative stress, pointing to the potential implications in targeting the CSE/H_2S system for the interruption of aging and diabetes-related heart disorders.

Keywords H_2S · ACE2 · Heart · Aging · Diabetes

Abbreviations

ACE2	Angiotensin-converting enzyme 2
Ang (1–7)	Angiotensin 1–7
Ang II	Angiotensin II
CSE	Cystathionine gamma-lyase
CVD	Cardiovascular disease
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2S	Hydrogen sulfide
HFD	High-fat diet
KO	Knockout
MDA	Malondialdehyde
RAS	Renin–angiotensin system
ROS	Reactive oxygen species
SIRT 6	Sirtuin 6
SREBP1	Sterol regulatory element-binding protein 1
WT	Wild-type

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Introduction

Angiotensin-converting enzyme 2 (ACE2) acts as the negative regulator of the renin–angiotensin system (RAS), exerting anti-inflammatory, anti-oxidative stress, anti-fibrotic, and anti-hypertrophic properties within the body [1]. ACE2 has a high affinity for Angiotensin II (Ang II) and can hydrolyze the octapeptide Ang II into the heptapeptide Angiotensin 1–7 (Ang 1–7) by the removal of a single phenylalanine on the C terminus [2]. Ang 1–7 then activates the Mas Receptor for a series of downstream signaling [2, 3]. The downregulation of ACE2 and the subsequent increase in Ang II levels may be present in many different pathologies, including

cardiovascular disease (CVD) associated with aging and diabetes [3, 4]. Ang II activation is associated with vasoconstriction, inflammation, hypertrophy, and fibrosis [4]. In the case of CVD associated with aging, it is believed that the decrease in ACE2 allows for an increase in Ang II levels in the hearts of these patients [3, 5]. In the hearts of patients with diabetes, the loss of ACE2 expression is related to a condition known as diabetic cardiomyopathy, in which the heart takes on an inflamed phenotype and loses its ability to function correctly, potentially leading to heart failure [3, 5, 6]. However, accumulating evidence showed an enhanced ACE2 expression in lung, kidney, heart, and pancreas in diabetic rodents and human [7–9]. Wysocki et al. found there were no significant differences in ACE2 activity between diabetic and control mice in cardiac tissue [8]. Interestingly, it has been noted that during the COVID-19 pandemic, the elderly and those with diabetes were more likely to suffer adverse outcomes [10]. The virus responsible for COVID-19, SARS-CoV-2, is believed to downregulate ACE2 as its spike protein binds to ACE2, allowing for its entry into human cells [11–13]. Thus, the worse outcomes of the elderly and those with diabetes may be due to the upregulation of ACE2 expression, which might facilitate the infection with SARS-CoV-2 [14]. On the other hand, it could also be due to the deficiency of ACE2 expression and the increased sensitivity to ACE2 downregulation in older people and patients with diabetes, which might develop and induce the progression of myocardial dysfunction [11].

Hydrogen sulfide (H_2S) is the third most recently identified gasotransmitter [15]. Three enzymes primarily produce H_2S, including 3-mercaptopyruvate sulfurtransferase, cystathionine β-synthase, and cystathionine gamma-lyase (CSE) [15]. CSE is predominantly responsible for generating H_2S within the cardiovascular system [16, 17]. H_2S plays a crucial role in helping heart health via improved oxidative stress, and the loss of H_2S production within the cardiovascular system is associated with increased risks of heart disease and complications [18, 19]. Recent research has found that H_2S signaling is impaired in patients with COVID-19, potentially helping to explain the increase in cardiac complications in these patients [20]. Another mechanism by which H_2S affects heart health includes its regulation of the RAS system [19]. Activation of the RAS results in an increase in blood pressure due to the production of Ang II by ACE. H_2S can protect the heart against overactivation of the RAS by directly inhibiting the activity of ACE [19]. However, the regulatory roles of H_2S on ACE2 remain unknown.

Based on the fact that ACE2 dysregulation and the impairment of H_2S signaling are often present in the elderly and those with metabolic syndromes, such as diabetes, we undertook this study with the hypothesis that H_2S would attenuate oxidative stress-driven cell senescence via regulation of ACE2. We found that CSE was vital in maintaining ACE2 expression in mouse heart tissues during the aging process and high-fat diet (HFD) feeding. We also found that H_2S played a crucial role in maintaining ACE2 expression and mitigating cellular senescence in cardiomyocytes stressed by a lower dose of H_2O_2 or lipid overload.

Materials and methods

Cell culture

Rat cardiomyocytes (H9C2, CRL-1446, ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium containing streptomycin (100 mg/mL), penicillin (100 U/mL) and 10% of heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere supplemented with 5% CO_2. The cells were washed every other day with Dulbecco's phosphate-buffered saline (PBS).

Animal experiments

Mice were bred in-house per proper laboratory animal care. The two groups of mice used were male CSE-knockout (KO) mice and age-matched male wild-type (WT) mice [17]. CSE KO mice were previously generated with lacked CSE expression and reduced H_2S production in various tissues [17, 21]. The young mice used for the experiments were between 8 and 10 weeks old. The older mice used for the experiments were 56 weeks old. These mice were fed a standard rodent chow diet (Zelgler Bros Inc, PA) with constant water and food access. A subgroup of 8-week-old CSE KO and WT mice were selected for feeding either high-fat diet (HFD) containing 60% of kcal/fat (Research Diets, New Brunswick, NJ) or the control diet including 10% of kcal/fat (Research Diets) for 12 weeks. The desired tissues were immediately removed from the mice after being euthanized and flash-frozen in liquid nitrogen.

Ethical statement

All the animal studies were implemented in the light of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and authorized by the Animal Care Committees of Laurentian University, Canada.

Western blotting

H9C2 cells and mouse heart tissues were lysed in 1 mM Tris–EDTA and 0.3 M sucrose buffer (pH 7.2) containing protease inhibitors [21, 22]. The protein content of the lysate from cells and tissues was measured using a Pierce bovine serum albumin assay (Fisher Scientific, Toronto, Ontario).

Equal amount of protein samples (60 µg) were then boiled for 8 min in a loading buffer containing β-mercaptoethanol. Afterward, the protein samples were separated on a 10% polyacrylamide gel for 90 min. The protein in the gels was then transferred to a nitrocellulose membrane, and the membrane was blocked using 3% non-fat skim milk dissolved in PBS containing 0.1% Tween® 20 Detergent (PBST) for 1 h. The membranes were then probed with the primary antibodies diluted in 3% milk for 1 h at room temperature. The primary antibodies were glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology, Santa Cruz, CA, 1:850) and ACE2 (Santa Cruz Biotechnology, 1:200). The membranes were then washed three times for 8 min each with 3% milk, followed by being washed with PBST three times for 8 min each. The membranes were then probed with the secondary antibody at 1:5000 in 3% milk for 1 h at room temperature. Next, the membranes were rewashed, followed by visualization using an enhanced chemiluminescence solution (GE Healthcare, Ottawa, ON) and X-ray films (Kodak Scientific Imaging film, Kodak, Rochester, NY) in dark room.

Real-time PCR

Total RNAs from the cells were isolated using TriReagent (Invitrogen, Carlsbad, CA) and dissolved in nuclease-free water. The concentration of RNA was measured with the spectrophotometer (BMG LABtech, Germany) at 260 nm. First-strand cDNA was prepared by reverse transcription using reverse transcriptase and random hexamer primers according to manufacturer's protocol (New England Biolabs, Pickering, ON). Real-time PCR was performed with an iCycler iQ^5 apparatus (Bio-Rad, Mississauga, ON) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix. The primer sequences for GAPDH were as follows: 5′-CACGGCAAGTTCAACGGC ACAGT-3′ and 5′-AGCGGAAGGGGCGGAGATGAT-3′. The primer sequences for ACE2 were as follows: 5′-GAA CAGTCCAAGATCGCCCAAAAT-3′ and 5′-CCCTCGCCA ATAATCCCCATAGT-3′. Relative mRNA quantification is calculated using the arithmetic formula "$-2^{-\Delta\Delta C_T}$", where ΔC_T is the difference between the threshold cycle of ACE2 cDNA and an endogenous reference of GAPDH gene.

Malondialdehyde (MDA) measurement

The level of MDA was determined in heart tissues and cardiac cells with the lipid peroxidation assay kit from Abcam (Cambridge, MA). Briefly, the tissues or cells were lysed in 20 mM sodium phosphate buffer (pH 3.0) containing 0.5% Triton X-100. A volume of 25 µL lysate was mixed with 10 µL MDA color reagent solution in 96-well plate and incubated at room temperature for 30 min. After that, 40 µL of reaction solution was added for another 30 min. The absorbance was recorded at 600 nm using a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTech). MDA levels were calculated using a standard curve and expressed as nmol/g tissue or nmol/mg protein.

Detection of free thiol contents

The free thiol contents were measured with a thiol detection assay kit (Cayman Chemical, Ann Arbor, MI) as previously described [23]. Briefly, equal amount of protein from tissue or cell lysates were mixed with 50 µL of diluted fluorometric thiol detector in 96-well plate for 5 min at dark. The fluorescence was read at an excitation wavelength of 340 nm and an emission wavelength of 520 nm using a FLUOstar OPTIMA reader. Free thiol content was expressed as µmol/mg proteins.

Analysis of cell senescence

C_{12}FDG probe (Fisher Scientific) was used for detection of cell senescence [24]. Briefly, after different treatments, C_{12}FDG (10 µmol/L) was added to H9C2 cardiomyocyte cells and incubated for 15 min at 37 °C. After wash, the cells were immediately observed with Olympus IX71 microscope. The percentage of cell senescence was calculated based on the ratio of green fluorescence-stained C_{12}FDG-positive cells to the total cell number.

Lipid overload

For the study with lipid overload, H9C2 cells were incubated with 5 µL/mL of a chemically defined lipid mixture (Sigma-Aldrich, Oakville, ON) for 72 h in the presence or absence of NaHS (30 µM) [22]. The lipid mix contains non-animal-derived fatty acids (2 µg/mL arachidonic and 10 µg/mL each linoleic, linolenic, myristic, oleic, palmitic, and stearic), 0.22 mg/mL cholesterol, 2.2 mg/mL Tween-80, 70 µg/mL tocopherol acetate, and 100 mg/mL pluronic F-68.

ACE2 overexpression and knockdown

ACE2 overexpression was obtained by transfecting the cells with the plasmid pcDNA3.1(−)-hACE2 (#145,033, Addgene, Watertown, MA) mixed with lipofectamine 2000 (Invitrogen) in a serum-free medium [25]. The empty plasmid pcDNA3.1(−) acted as a control. For the knockdown of ACE2, the cells were transfected with pre-designed ACE2-targeted siRNA (ACE2-siRNA, sc-41400, Santa Cruz Biotechnology) using the siPORT™ lipid transfection agent from Fisher Scientific.

Statistical analysis

Statistical analysis was performed using SPSS 21.1 software (SPSS Inc., IL). A two-way student's *t* test at 95% confidence or a one-way ANOVA was used. The data were shown as means ± standard error (SEM).

Results

CSE deficiency down-regulates ACE2 expression and increases oxidative stress in heart tissues from aged mice

Compared to age-matched WT mice (8–10-week males), ACE2 protein expression in the heart tissues from CSE KO mice was reduced by almost half (Fig. 1A). Next, we analyzed the effect of age on ACE2 protein expression in mouse heart tissues. ACE2 protein expression from older WT mice (56 weeks) were reduced by 50% compared to that from young WT mice (8–10 weeks). Furthermore, heart ACE2 expression was decreased by 70% in older mice when the CSE gene was completely knocked out. These data suggest that both aging and CSE deficiency have a synergistic effect in suppressing ACE2 expression in heart tissues (Fig. 1A). MDA content in the heart from 8- to 10-week-old CSE KO mice was 1.8 times of that from age-matched WT mice ($p < 0.05$). Age did not affect MDA contents in heart tissues from WT mice; however, CSE deficiency doubled the MDA content in heart tissues from old mice when compared with that from young KO mice (Fig. 1B). In contrast, free thiol content was significantly lower in heart tissues from CSE KO mice, and the age decreased free thiol contents in both WT and CSE KO hearts with more severe in the latter (Fig. 1C). Thus, these data indicate that CSE deficiency exacerbates cardiac oxidative stress in aged mice.

H_2S reverses H_2O_2-suppressed ACE2 expression and induced cellular senescence in cardiac cells

Then, we investigated whether oxidative stress can directly affect ACE2 expression. H9C2 cells were first treated with a low dose of H_2O_2 (50 μM) for 2 h and then continuously cultured with or without H_2S donor NaHS (30 μM) for 70 h. H_2O_2 treatment reduced ACE2 protein expression by 56.7% in comparison to control cells, while the addition

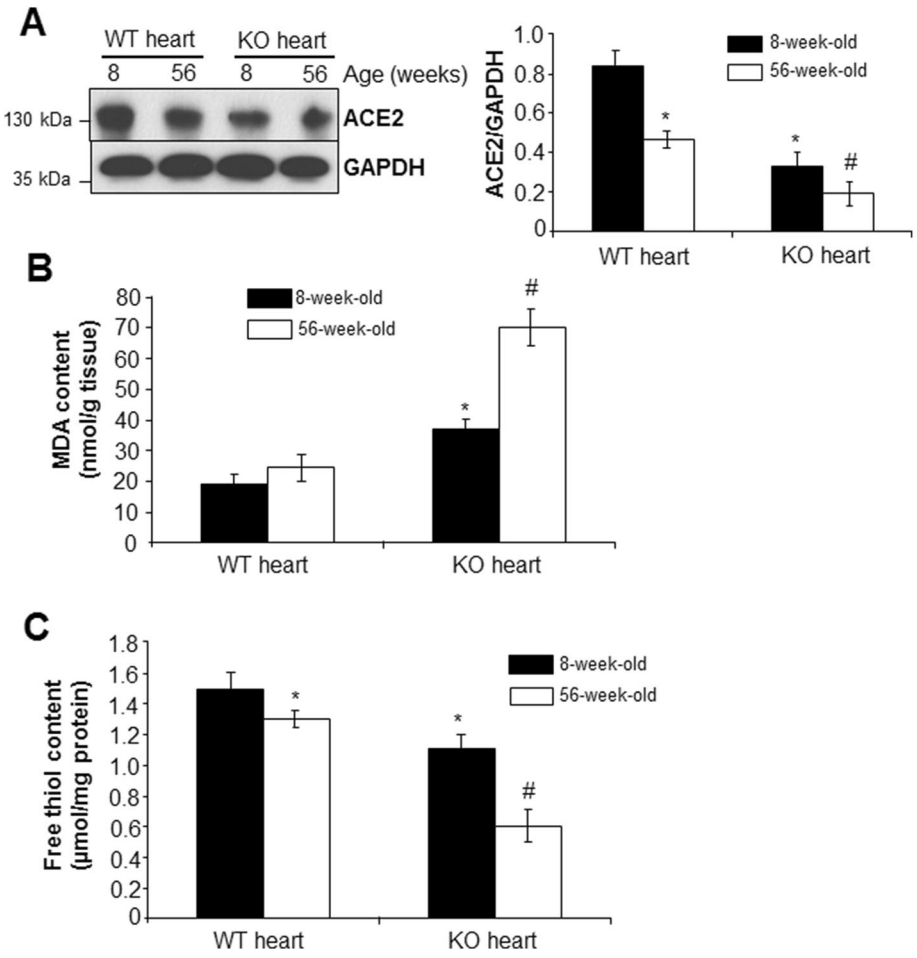

Fig. 1 Lower ACE2 protein level and higher oxidative stress in heart tissues from older CSE KO mice. ACE2 protein expressions (**A**), MDA content (**B**), and free thiol contents (**C**) in heart tissues from both young (8–10 weeks) and old (56 weeks) mice were detected. $^{*}p < 0.05$ vs. WT young mice; $^{\#}p < 0.05$ vs. KO young mice. In each group, 4–6 mice were used

of NaHS almost restored H_2O_2-inhibited ACE2 protein expression (Fig. 2A). The cells treated only with NaHS had no effect on ACE2 protein expression. The change of ACE2 mRNA expression in H9C2 cells was quite similar to ACE2 protein levels as determined using real-time PCR (Fig. 2B). The mRNA expression in the cells treated with H_2O_2 was only 30% of the control cells (Fig. 2). When H9C2 cells were washed out of H_2O_2 and then incubated with NaHS for 70 h, ACE2 mRNA expression was around 80% compared to control (Fig. 2B, $p > 0.05$). NaHS alone did not affect ACE2 mRNA expression. The MA content in H_2O_2-incubated cells (21.6 nmol/mg protein, $p < 0.05$) was significantly higher in comparison with the control cells (9.1 nmol/mg protein) and the cells co-treated with H_2O_2 and H_2S (13.8 nmol/mg protein). In addition, H9C2 cells treated with H_2O_2 showed a significant increase in the number of senescent cells compared to control as determined by staining the cells with C_{12}FDG (Fig. 2D). H9C2 cells treated with H_2O_2 and then NaHS had senescent cell counts similar to those of the control and the cells treated with NaHS alone (Fig. 2D). These results suggest that H_2S would prevent cell senescence induced by oxidative stress.

Next, we tested the direct mediation of ACE2 in oxidative stress-induced cellular senescence by knockdowning ACE2. H9C2 cells transfected with control siRNA or ACE2 siRNA were incubated with 50 μM H_2O_2 for 2 h and then incubated with 30 μM NaHS for another 70 h. As shown in Fig. 2E, ACE2 protein expression was obviously reduced in the cells transfected with ACE2 siRNA. The number of senescent cells in ACE2 siRNA cells was significantly more than that in control siRNA-transfected cells (Fig. 2F). In control siRNA-transfected cells, the senescent cell count by H_2O_2 treatment was 72.6% but only 4.8% by H_2O_2/H_2S co-treatment. However, in ACE2 siRNA-transfected cells, the senescent cell counts by H_2O_2 and H_2O_2/H_2S co-treatments were increased to 87.2% and 60.5%, individually (Fig. 2F). These results indicate that ACE2 deficiency may be directly involved in the oxidative stress-induced cell senescence and H_2S would prevent cellular senescence through regulating ACE2 expression.

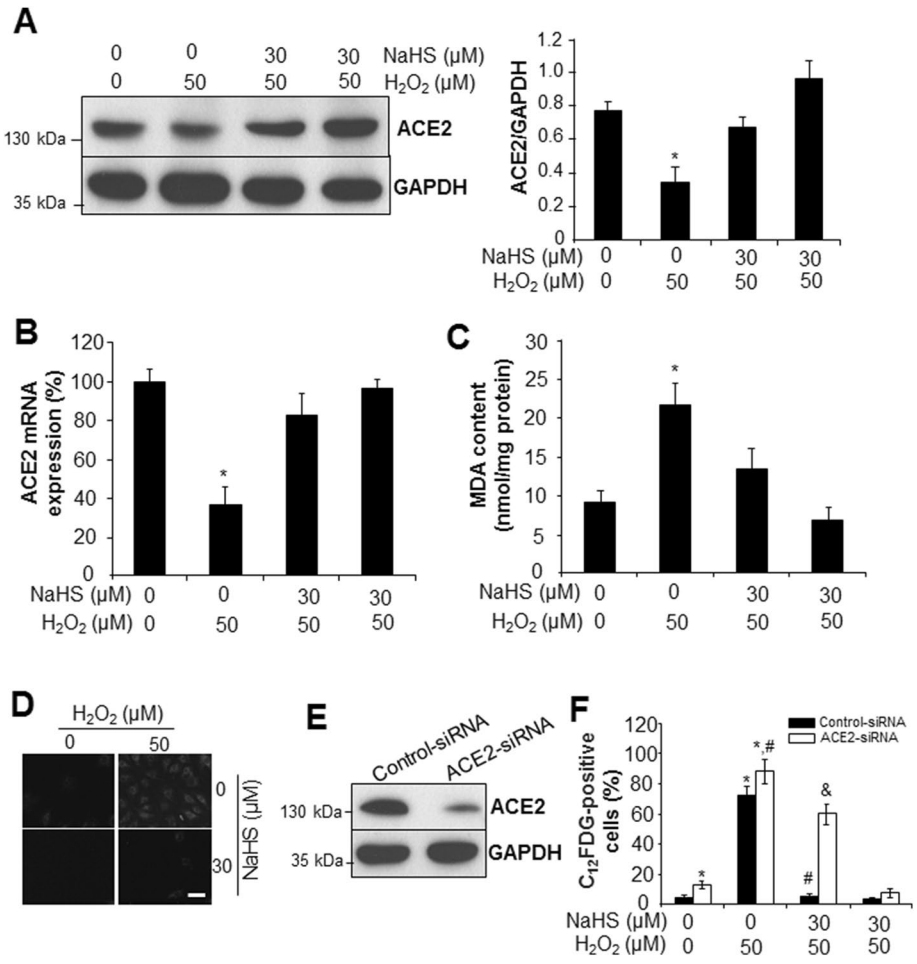

Fig. 2 A lower dose of H_2O_2 suppresses ACE2 expression and induces cellular senescence in cardiac cells. After H9C2 cells were incubated with 50 μM H_2O_2 for 2 h, then incubated with 30 μM NaHS for another 70 h, and the cells were then processed for analysis of ACE2 protein expression (**A**), mRNA expression (**B**), MDA contents (**C**), and cell senescence (**D** and **F**). Scale bar in **D**: 20 μm. $^*p < 0.05$ vs. all other groups. In **E** and **F**, the cells transfected with control siRNA or ACE2 siRNA were incubated with 50 μM H_2O_2 for 2 h, then incubated with 30 μM NaHS for another 70 h, and the cells were then processed for analysis of ACE2 protein expression (**E**) and cellular senescence (**F**). $^*p < 0.05$ vs. control siRNA-transfected cells; $^{\#}p < 0.05$ vs. control siRNA-transfected cells incubated with H_2O_2; $^{\&}p < 0.05$ vs. ACE2 siRNA-transfected cells incubated with H_2O_2. The data were from four independent experiments

H_2S inhibits lipid overload-stimulated ACE2 expression and cellular senescence in cardiac cells

Finally, we investigated whether lipid overload affects ACE2 expression in heart cells. At the animal level, both 8-week-old WT and CSE KO mice were fed with HFD or control diet for 12 weeks. Cardiac ACE2 expression in control diet-fed CSE KO mice was significantly lower than that in age-matched WT mice with the same diet (Fig. 3A), which is consistent with the previous finding as shown in Fig. 1A. When fed with HFD, ACE2 protein expression in heart tissues from both mice were noticeably increased with more significant effect from CSE KO mice (Fig. 3A). HFD induced significantly higher MDA content but decreased more free thiol content in CSE KO hearts when compared with WT hearts (Fig. 3B and C).

At the cellular level, we found in H9C2 cells treated with the lipid overload mixture (5 µL/mL) for 72 h, ACE2 expression was 1.7-fold of that in control cells (Fig. 4A). H9C2 cells treated with the lipid mixture but supplemented with NaHS for 72 h had their ACE2 protein expression returned to levels comparable to control. Consistently, the cells only treated with NaHS for 72 h were with normal ACE2 protein expression as that observed in control cells (Fig. 4A). In contrast, ACE2 mRNA expression was similar across all four groups, with no significant difference observed (Fig. 4B). The MDA content was increased by 390% in lipid overload cells and was almost restored to normal level by NaHS co-incubation (Fig. 4C). We then analyzed the role of ACE2 in lipid overload-stimulated cellular senescence by overexpressing ACE2. H9C2 cells transfected with the control vector or ACE2 cDNA vector were incubated with lipid mix (5 µL/mL) in the presence or absence of 30 µM NaHS for 72 h. Figure 4D demonstrates that ACE2 was overexpressed in ACE2 cDNA vector-transfected cells. Lipid overload mixture induced a similar increase of cell senescence count in the cells transfected with control vector (87.1%) or ACE2 cDNA vector (82.8%) (Fig. 4E). It was further observed that NaHS inhibited lipid overload-induced cell senescence by 68.5% in control vector-transfected cells but only 26.1% in ACE2 cDNA-transfected cells ($p < 0.05$). These data suggest that H_2S may be able to suppress lipid overload-stimulated cardiac senescence through downregulation of ACE2 expression.

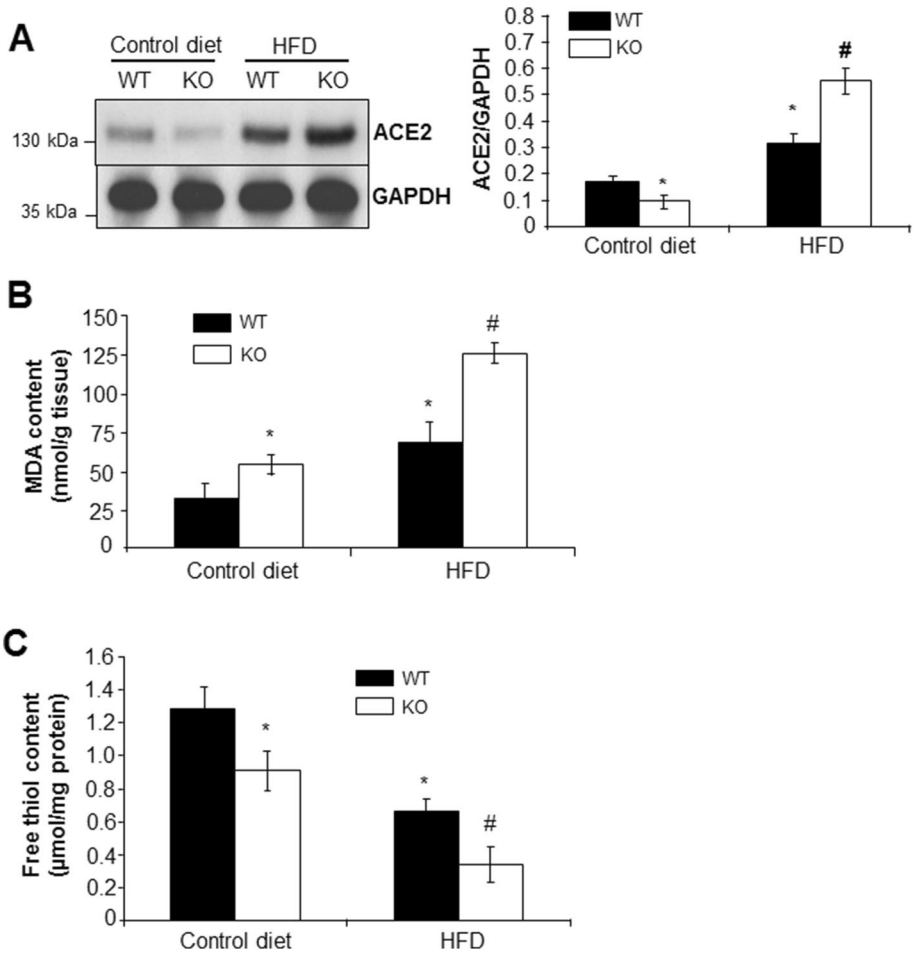

Fig. 3 High-fat diet stimulates ACE2 expression and induces oxidative stress in CSE KO heart. HFD induced more ACE2 protein expression (**A**) and MDA contents (**B**) but inhibited free thiol contents (**C**) in heart tissues from CSE KO mice. Both 8-week-old WT and CSE KO mice were fed with HFD or control diet for 12 weeks. $^{*}p < 0.05$ vs. WT mice fed with control diet; $^{\#}p < 0.05$ vs. WT mice fed with HFD. In each group, 4–5 mice were used

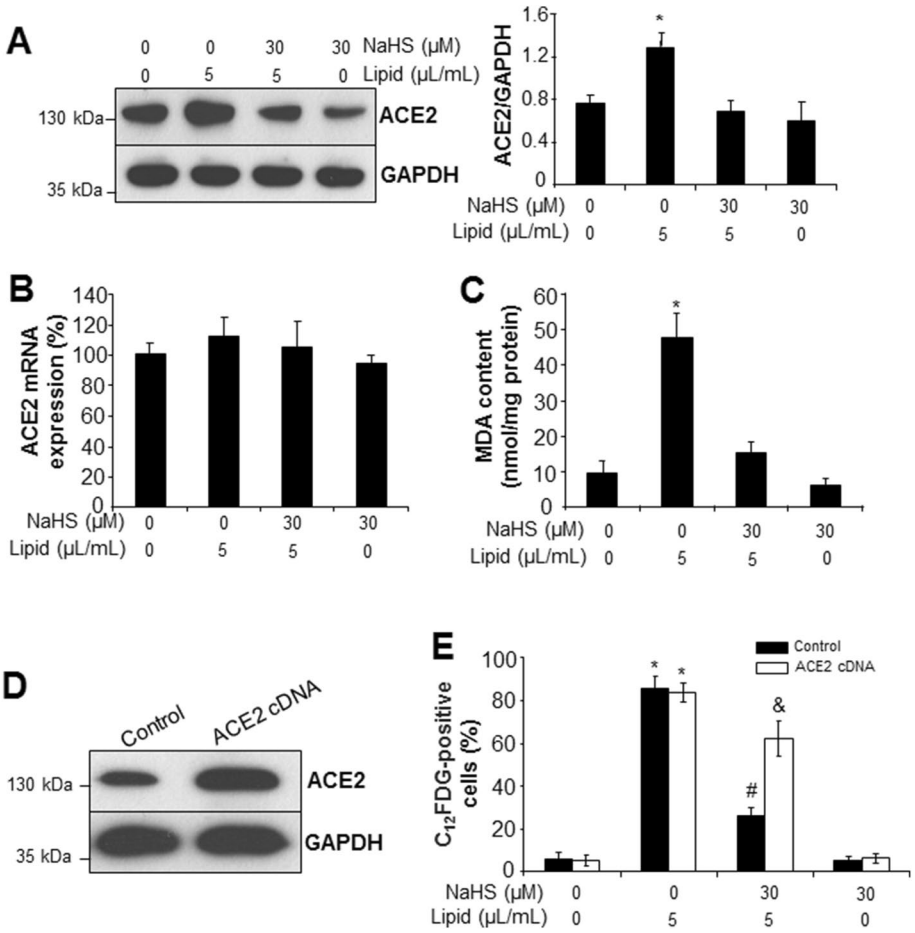

Fig. 4 Lipid overload stimulates ACE2 expression and induces cellular senescence in cardiac cells. H9C2 cells were incubated with lipid mix (5 μL/mL) in the presence or absence of 30 μM NaHS for 72 h; the cells were processed for analysis of ACE2 protein expression (**A**), mRNA expression (**B**), and MDA contents (**C**). $^{*}p < 0.05$ vs. all other groups. In **D** and **E**, the cells transfected with control vector or ACE2 vector were incubated with lipid mix (5 μL/mL) in the presence or absence of 30 μM NaHS for 72 h; the cells were then processed for analysis of ACE2 protein expression (**D**) and cellular senescence (**E**). $^{*}p < 0.05$ vs. control vector-transfected cells; $^{\#}p < 0.05$ vs. control vector-transfected cells incubated with lipid mix; $^{\&}p < 0.05$ vs. ACE2 vector-transfected cells incubated with lipid mix. The data were from four independent experiments

Discussion

The present project indicates that CSE-derived H_2S plays a crucial role in regulating ACE2 in heart tissues. Deficiency of CSE in the mouse heart tissue led to significantly reduced ACE2 protein expression. As found in previous studies, CSE deficiency in the mice caused a more than 80% drop in H_2S levels in heart tissues [17]. The significant loss of H_2S resulted in age-dependent hypertension, one of many possible CVDs that can develop with age. H_2S can mitigate hypertension and relax blood vessels by opening K_{ATP} channels in vascular smooth muscles [17, 26]. While this is the primary mechanism by which H_2S is believed to reduce blood pressure, its regulation of ACE2 could represent a novel mechanism for mitigating hypertension and other CVDs.

Accumulated evidence has demonstrated that as we age, the amount of H_2S decreases significantly [27, 28]. Serum H_2S levels are lower as we age due partially to the increased oxidative stress. The sources of oxidative stress related to aging are numerous and include increased xanthine oxidase activity, nitric oxide synthase uncoupling, and advanced glycation. Since H_2S acts as a direct/indirect scavenger of reactive oxygen species (ROS), an increased ROS level will result in more H_2S being depleted. This eventually decreases the bioavailability of H_2S in the body [27, 28]. ACE2 expression has also been observed to decline during the aging process, indicating a possible link between H_2S and ACE2 [29]. As expected, our experiment confirmed that heart ACE2 protein expression was decreased in older mice (56 weeks) in comparison to the younger (8–10 weeks) mice. It also demonstrated that H_2S produced by CSE was crucial in maintaining ACE2 protein expression as the mice age, as ACE2 was further lowered in CSE KO heart. These results show that a decline in H_2S is partially responsible for the downregulation of ACE2 in the mouse heart. A possible explanation for the regulatory role of H_2S on ACE2 expression is attributed to the involvement of Sirtuin 6 (SIRT6) [30]. Possibly, the supplementation of exogenous H_2S increased the expression and activation of SIRT6, likely by reducing oxidative stress, which further promoted ACE2 expression [31]. Our result also showed that MDA content in CSE KO heart was nearly 1.8-fold of that in WT heart, which indicates that H_2S could significantly alleviate oxidative stress.

After exploring the role of CSE-derived H_2S and aging on ACE2 expression, we then examined how oxidative stress

would affect ACE2 expression and cardiomyocyte senescence. Emerging evidence indicates that increased ROS generation contribute to age-related heart diseases [32]. H_2O_2 has been the most commonly used inducer for stress-induced premature senescence in cardiac cells [33, 34]. We utilized an in vitro cell culture model by incubating H9C2 cells with low dose of H_2O_2 (50 μM) for a prolonged incubation (72 h) to induce cellular senescence. The present study validated that the moderate oxidative stress caused by the addition of a lower dose of H_2O_2 to the cardiomyocytes appreciably increased cellular senescence [35]. In addition to causing an increase in senescent cardiomyocytes, moderate oxidative stress also resulted in the downregulation of ACE2 mRNA and protein expression. The results further demonstrated that the supplementation of H_2S would reverse these phenome. Two mechanisms are proposed here to explain the protective roles of H_2S against cardiomyocyte senescence. Firstly, H_2S would exert a direct or indirect anti-oxidative stress properties. As previously mentioned, H_2S can directly neutralize ROS and/or activate anti-oxidative stress pathways, such as the Nrf2-ROS-AMPK pathway. H_2S modifies Keap1 activity by *S*-sulfhydration, causing nuclear factor erythroid 2-related factor 2 (Nrf2) release from Keap1 and translocation to the nucleus followed by upregulation of antioxidant gene transcription [36]. Secondly, H_2S can reduce cardiomyocyte senescence by upregulating ACE2. ACE2, much like H_2S, exerts anti-oxidative stress properties via its degradation of Ang II and the production of Ang 1–7 [37]. These will help reduce oxidative stress responsible for initiating cardiomyocyte senescence [34]. The mechanism by which H_2S can upregulate ACE2 is likely taking place at the transcriptional level, as evidenced by the increase in ACE2 mRNA expression after H_2S was administered. One possible version of this mechanism could involve the regulation of SIRT6, as previously mentioned [30, 31]. To validate the role of ACE2 in reducing oxidative stress-induced cardiomyocyte senescence, we transfected H9C2 cells with ACE2 siRNA to knockdown ACE2. We found that the effect of H_2S in reducing cellular senescence under oxidative stress was partially abolished when ACE2 was knockdown. Given this evidence, it can be inferred that H_2S can reduce cardiomyocyte senescence via either direct scavenging of ROS and/or upregulation of anti-oxidative stress proteins, such as ACE2.

Lastly, we examined how HFD-fed mice and lipid overload incubation of H9C2 cells would affect ACE2 expression and cardiomyocyte senescence. Obesity-derived nutrient excess is an increased risk of heart disorders leading to metabolic dysfunction and cardiac remodeling [38]. The heart maintains profound metabolic flexibility under stress condition, such as nutrient excess, while sustained metabolic derangements would promote oxidative stress and lipotoxicity, progressively leading to heart failure [39]. H9C2 cells subjected to lipid overload mimic a nutritional excess state in the heart. We found that the lipid overload increased ACE2 protein expression but caused no change in ACE2 mRNA expression. A previous study had also observed a significant increase of ACE2 expression in obese mice [40]. This increase in ACE2 expression was correlated with a decrease in sterol regulatory element-binding protein 1 (SREBP1), which has been shown to have a potential regulatory role on ACE2 expression. SREBP1 is a transcription factor that plays a vital role in reducing lipotoxicity by regulating metabolic pathways related to lipogenesis and adipogenesis. The upregulation of ACE2 and the downregulation of SREBP1 in response to lipid overload are indicators of a dysregulated response to lipotoxicity-induced cell injury [22, 41]. Another explanation underlying the increased cardiac ACE2 expression by lipid overload could be due the severe oxidative stress, because lipid overload cells had about fivefold of MDA content of the control cells. A lower dose of H_2O_2 (50 μM) only induced MDA contents by 1.4-fold but inhibited ACE2 expression in comparison to the control cells. The expression of ACE2 has been found to correlate with oxidative stress in the brains with Alzheimer's disease [42]. This dysregulated response to lipid overload, however, was reversed with the supplementation of H_2S. H_2S restored ACE2 expression to the basal levels and prevented cardiomyocyte senescence induced by lipotoxicity. To investigate what role the ACE2 performs in lipid overload-stimulated cellular senescence, H9C2 cells were transfected with ACE2 vector to overexpress ACE2. We found that the effect of H_2S in reducing cellular senescence under lipid overload was less significant in the ACE2-overexpressed cells than in the control vector cells, which indicate that ACE2 might contribute to lipid overload-induced cellular senescence. In regards to ACE2 mRNA expression being unaffected by the lipid overload, a possible explanation could be due to the target of microRNA by lipid overload. MicroRNAs control the expression of specific genes either by causing mRNA degradation or translational repression. Previous research has established that miR-421 can reduce ACE2 protein expression via translational repression. Translational repression results in a decrease in protein expression while having little effect on mRNA expression [43]. miR-421 level in the serum was found to be decreased in diabetic patients, which may cause upregulation of ACE2 [44]. Our results would seem to indicate that the lipid overload inhibits miR-421 resulting in the loss of translational repression and the subsequent increase in ACE2 protein expression. The addition of H_2S seemed to protect miR-421 from degradation and resulted in translational repression of ACE2 protein expression. This is consistent with previous studies showing that H_2S plays a crucial role in regulating microRNAs responsible for cardiovascular health [45]. However, its role in regulating miR-421 has yet to be established, and more research is required to confirm its regulatory function.

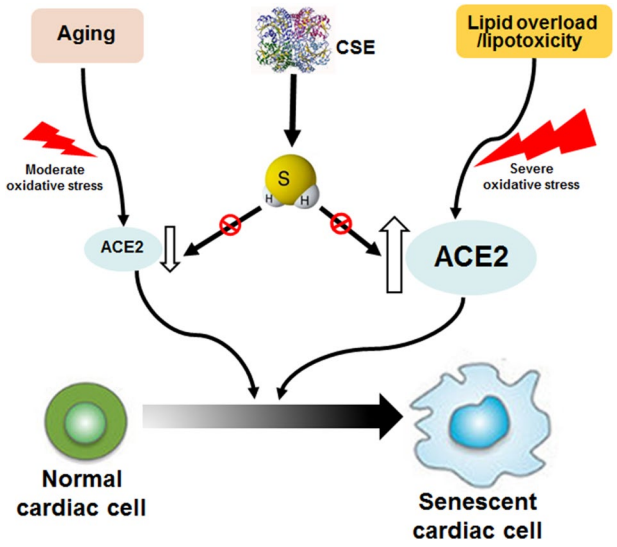

Fig. 5 H_2S protects stressed cardiac cells from senescence via the regulation of ACE2. H_2S has the potential to restore ACE2 expression to control levels and protects the cells from senescence under stress, such as aging-related moderate oxidative stress and lipid overload-related severe oxidative stress

In conclusion, H_2S produced by CSE plays a vital role in regulating cardiac ACE2. In mice deficient in CSE, ACE2 was subsequently downregulated in heart tissues. CSE-derived H_2S was also crucial in maintaining ACE2 expression in the hearts of aged mice. For cardiomyocytes subjected to oxidative stress, H_2S could upregulate ACE2 and protect the cardiomyocytes from senescence. Additionally, for cardiomyocytes subjected to a lipid overload mimicking a status of prolonged nutrient excess, H_2S restored ACE2 expression to control levels and protected the cells from senescence. These results demonstrate the dynamic roles of H_2S in the maintenance of ACE2 levels under different levels of oxidative stress, pointing to the potential implications in targeting the CSE/H_2S system for the interruption of aging and diabetes-related heart disorders (Fig. 5).

Author contributions Conceptualization: KB, YW, and GY; methodology: KB, YW, and RY; formal analysis and investigation: KB, YW, RY, and JZ; writing—original draft preparation: KB and GY; writing—review and editing: JZ and GY, funding acquisition and supervision: GY.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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