NeuroMolecular Medicine (2019) 21:192–203
https://doi.org/10.1007/s12017-019-08536-8

ORIGINAL PAPER

Hydrogen Sulfide Inhibits Formaldehyde-Induced Senescence in HT-22 Cells via Upregulation of Leptin Signaling

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Received: 19 November 2018 / Accepted: 8 April 2019 / Published online: 12 April 2019

Abstract

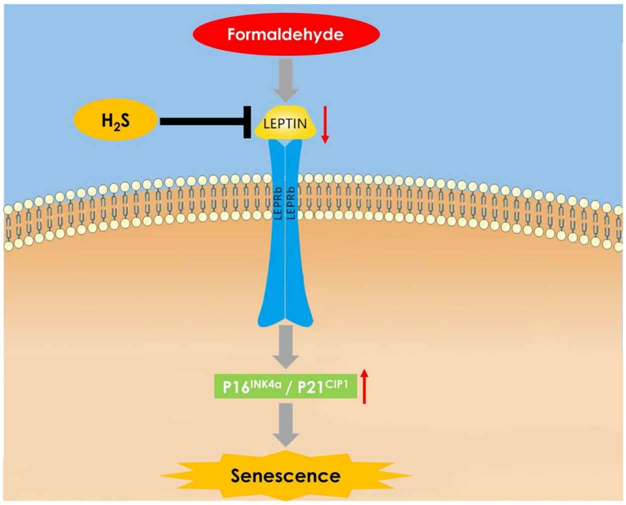
It has been previously demonstrated that hydrogen sulfide (H_2S) prevents formaldehyde (FA)-induced neurotoxicity. However, the exact mechanisms underlying this protection remain to be fully elucidated. Neuronal senescence is involved in FA-induced neurotoxicity. Leptin signaling has anti-aging function. The present work was to investigate the protection of H_2S against FA-induced neuronal senescence and the mediatory role of leptin signaling. FA-exposed HT-22 cells were used as the vitro model of FA-induced neuronal senescence. The senescence-associated β-galactosidase (SA-β-Gal) positive cell was detected by β-galactosidase staining. The expressions of $P16^{INK4a}$, $P21^{CIP1}$, leptin, and lepRb (leptin receptor) were measured by western blot. The proliferation, viability, and apoptosis of cells were evaluated by Trypan blue exclusion assay, Cell Counting Kit-8 (CCK-8) assay, and Flow cytometry analysis, respectively. We found that H_2S suppressed FA-induced senescence, as evidenced by the decrease in SA-β-Gal positive cells, the downregulations of $P16^{INK4a}$ and $P21^{CIP1}$, as well as decrease in cell growth arrest, in HT-22 cells. Also, H_2S upregulated the expressions of leptin and lepRb in FA-exposed HT-22 cells. Furthermore, leptin tA (a specific inhibitor of the leptin) abolished the protective effects of H_2S on FA-induced senescence and neurotoxicity (as evidenced by the increase in cell viability and the decrease in cell apoptosis) in HT-22 cells. These results indicated that H_2S prevents FA-induced neuronal senescence via upregulation of leptin signaling. Our findings offer a novel insight into the mechanisms underlying the protection of H_2S against FA-induced neurotoxicity.

Wei-Wen Zhu, Min Ning, and Yi-Zhu Peng have contributed equally to this work.

Extended author information available on the last page of the article

Graphical Abstract

FA upregulates the expressions of P16^{INK4a} and P21^{CIP1} via inhibiting leptin signaling, which in turn induces senescence in HT-22 cells; H_2S downregulates the expressions of P16^{INK4a} and P21^{CIP1} via reversing FA-downregulated leptin signaling, which in turn prevents FA-induced senescence in HT-22 cells.



Keywords Formaldehyde · Senescence · Hydrogen sulfide · Leptin signaling

Introduction

Recently, the neurotoxic effects of formaldehyde (FA), a common environmental pollutant, in the human has attracted more and more attention (Tulpule and Dringen 2013; Songur et al. 2010; Yu et al. 2014). It has been reported that the accumulation of endogenous FA induced by aging contributes to the pathology of neurodegenerative disease (Tong et al. 2015; Mei et al. 2015). Therefore, the suppression of FA-induced neurotoxicity may provide a promising approach for the FA-dependent neurodegenerative disease. Hydrogen sulfide (H_2S) is a novel endogenous gaseous neuromodulator and neuroprotectant (Kimura 2002; Zhou and Tang 2011; Zhang and Bian 2014). We have previously demonstrated that FA induces neurotoxicity by inhibiting the endogenous generation of H_2S (Tang et al. 2013a, b) and exogenous H_2S ameliorates FA-induced neurotoxicity (Tang et al. 2012; Li et al. 2014), suggesting the therapeutic potential of H_2S for the neurotoxicity of FA. However, the underlying mechanisms that H_2S inhibits FA-induced neurotoxicity have not yet been clarified.

Cellular senescence, a permanent state of cell cycle arrest in response to various stressors, has emerged as a potentially important aging mechanism, and it is an attractive target for therapeutic exploitation (Baker and Petersen 2018; Childs et al. 2017, 2015). The DNA damage and oxidative stress are currently known the critical risk factors for cellular senescence (Kammeyer and Luiten 2015; Pinto and Moraes 2015). It has been reported that FA leads to DNA damage (Yu et al. 2015; Costa et al. 2015; Spencer 2018) and oxidative stress (Chen et al. 2017; Wang et al. 2018; Duan et al. 2018). Furthermore, we have previously confirmed that FA accelerates cellular senescence by upregulation of P16^{INK4a} and P21^{CIP1} in HT-22 cells (K. Zhan et al. 2016). Therefore, it is established that cellular senescence is one of the most critical effects during FA-induced neurotoxicity and that therapeutically targeting FA-induced senescent cells is a novel strategy for treating neurotoxicity of FA. Thus, the present work aimed to expand current understanding of the protective effect of H_2S in FA-triggered neurotoxicity by exploring whether H_2S has a potential protection against FA-elicited neuronal senescence.

Leptin, a hormone with various functions, exerts its physiological functions by binding to leptin receptor (lepRb) (Y. Zhang and Chua 2017). In view of lepRb is expressed mainly in the brain, the neuroprotective effect of leptin signal is attracting more and more attention (Signore et al. 2008; Davis et al. 2014). Previous studies have provided important evidence that leptin signaling pathway has a potential anti-aging effect in brain (Folch et al. 2012; Filippi and Lam 2014). It is noteworthy that leptin exhibit its biological effects in different cell models by reducing the expressions of P16^{INK4a} (Al-Khalaf et al. 2017) and P21^{CIP1} (Feng et al. 2017; Noda et al. 2015; Wen et al. 2015; Ptak et al. 2013), two senescence-associated cyclin-dependent kinase inhibitors. Furthermore, our previous work demonstrated that downregulation of leptin signaling is involved in FA-induced upregulation of $P16^{INK4a}$ and $P21^{CIP1}$ as well as senescence in HT-22 cells (Zhan et al. 2016). Therefore, we further investigated whether leptin signaling mediates the protection of H_2S against FA-induced senescence.

In the present work, we demonstrated that H_2S significantly not only inhibited FA-induced senescence in HT-22 cells, but also upregulated leptin signaling in FA-exposed HT-22 cells. Leptin tA, a specific inhibitor of the leptin signaling, abolished the protection of H_2S against FA-induced senescence and neurotoxicity in HT-22 cells. These data indicated the protection of H_2S against FA-induced neuronal senescence via upregulating leptin signaling.

Materials and Methods

Materials

Formaldehyde (FA), NaSH, and Trypan Blue Solution were supplied by Sigma Chemical CO (St Louis, MO, USA). Leptin Antogonist Triple Mutant Pegylated Rat Recombinant (Leptin tA Rat, PEG) was purchased from ProSpect (Israel). Cell Counting Kit-8 (CCK-8) and Bicinchoninic Acid (BCA) Protein Assay Kit were obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Senescence-associated β-galactosidase (SA-β-gal) activity staining kit was purchased from Cell Signaling Technology (Danvers, MA, USA). Specific monoclonal antibody to $P16^{INK4a}$ and $P21^{CIP1}$ were purchased from OriGene (Rockville, MD, USA). Specific monoclonal antibody to leptin and leptin Rb were supplied by Santa Cruz Biotechnology. RPMI-1640 medium and fetal bovine serums (FBS) were supplied by Gibico, BRL (Ground Island, NY, USA).

Cell Culture

HT-22 cells, a mouse hippocampal neuronal cell line, were provided by China Center for Type Culture Collection (Wuhan, China), are widely used in studies of neurodegenerative diseases (Liu et al. 2009; Choi et al. 2010). Cells were grown in DMEM medium containing 10% fetal bovine serum and incubated at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every other day.

Cell Viability Assay

The viability of HT-22 cells was assessed by Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocol. Briefly, When HT-22 cells were grown to about 70% confluent and then exposed to specific experimental treatment. After incubation, the medium was removed and then rinsed with phosphate-buffered saline (PBS) twice. Then, CCK-8 reagent (5 µl) was added to each well and incubating for another 1-4 h. Finally, the optical density of each well was detected using a microplate reader (ELX800, BioTek Instruments Inc, Winooski, USA). Cell viability was expressed as the percentage of the normal control group value, assuming that the optical density of control cells was 100%. The experiment was repeated 3 times.

Senescence-Associated β-Galactosidase Staining

The SA-β-gal activity of HT-22 cells was determined by SA-β-gal staining according to the protocol described previously (Debacq-Chainiaux et al. 2009). After HT-22 cells were treated with specific drugs, the cells of each well were rinsed once with preheat PBS (2 ml, 37 °C) and then fixed in 1 ml 4% paraformaldehyde fixed solution for 10–15 min. After rinsed thrice with 2 ml PBS, the cells of each well were incubated with 1 ml freshly prepared SA-β-gal staining solution (930 µl staining solution, 10 µl staining supplement A, 10 µl staining supplement B, 50 µl 20 mg/ml X-gal in DMF) at 37 °C overnight. Stained HT-22 cells were observed and imaged under a Nikon XSZ-D2 optical microscope (NIKON, Nikon Corporation, Chiyoda-ku, Tokyo, Japan) and the number of SA-β-gal positive cells was calculated. Results are expressed as the percentage of SA-β-gal positive cells.

Cell Proliferation Assay

Cell proliferation was evaluated using Trypan blue exclusion method during the period of 7 days. Cells were plated at a density of 0.5×10^4 per well and incubated in the presence of specific drugs. After that, cells were harvested by digestion with 0.25% trypsin-EDTA and centrifuged at 8000×*g* for 10 min. Next, the cells were resuspended in 1 ml of medium and then incubated with a mixture of 10 µl of cell suspensions plus 10 µl of 0.4% trypan blue solution for 5 min. Finally, the number of viable cells (colorless) and dead cells (blue) were counted by using hemocytometer under a Nikon XSZ-D2 optical microscope (NIKON, Nikon Corporation, Chiyoda-ku, Tokyo, Japan), and then the living cell density calculated was calculated as follows: living cell density = (number of viable cells/4×10^4)/ml.

Western Blot Analysis

The expressions of $P16^{INK4a}$, $P21^{CIPL}$, leptin, and lepRb were measured by Western Blot. After receiving different experimental treatments, the scraped HT-22 cells were lysed in an ice-cold lysis buffer (20 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 1% Triton X-100, 1 mmol/l phenylmethylsulphonyl-fluoride (PMSF), 1 mmol/l Na_3VO_4, leupeptin, and EDTA). Next, the samples were transferred to a microcentrifuge tube and supernatants were collected following centrifugation for 30 min at 8000×*g* at 4 °C. Protein concentration was analyzed by BCA protein assay kit. Equivalent amounts of protein for each sample were separated by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvivylidene difluoride (PVDF) membranes by electro blotting. Non-specific protein binding was blocked with TBS-T (50 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 0.05% Tween-20) containing 5% non-fat milk for 2 h at room temperature and the membranes were incubated overnight at 4 °C with primary antibodies (1:1000). After three times washing with TBS-T buffer, the membranes were incubated with anti-rabbit secondary antibody (1:5000) conjugated to horseradish peroxidase for 2 h. Finally, the membranes were washed with TBS-T buffer (5 × 5 min) and electrogenerated chemiluminescence reaction solutions were added for 30 s. The signal of the immunoblots was visualized using Tanon-5600 Imaging System (Tanon, Shanghai, China). The quantitative analysis of each bolt was performed by Sigma Scan Pro5 software (San Jose, CA, USA) and normalized to that of β-actin.

Flow Cytometry Analysis of Apoptosis

HT-22 cells (1×10^6) in logarithmic phase growth were seeded in 6-well plate and received different treatment. After washing twice with cold PBS, the cells were digested with trypsin (2.5 g/l) and then harvest in an Eppendorf tube. The sample was centrifuged for 30 min at 8000×*g* at 4 °C, supernatants were discarded and then the collected HT-22 cells were resuspended in 400 µl binding buffer at a concentration of 10×10^5 cells/ml. Next, 500 ml PBS, 5 ml Annexin-V-FITC and 5 ml PI were added to each tube, and the mixture was placed in the dark at room temperature for 15 min, followed by flow cytometric testing (FCM, Beckman-Coulter, Miami, FL, USA). Data acquisition and analysis were performed by Quest software (Becton–Dickinson) and the data are expressed as percentage of cells.

Statistical Analysis

All experiments were repeated at least three times and statistical analyses were performed by SPSS 20.0 software (Chicago, IL, USA). Data are presented as mean ± SEM and the significance of intergroup discrepancy was assessed by oneway analysis of variance (ANOVA, least-significant difference's test for post hoc comparisons). Differences were considered significant at two tailed $p < 0.05$.

Results

NaHS Inhibits FA-Induced Senescence in HT-22 Cells

To elucidate whether H_2S prevents FA-induced senescence in HT-22 cells, we explored the effects of H_2S on SA-β-Gal positive cells, the expression levels of $P16^{INK4a}$ and $P21^{CIP1}$ as well as cell growth state in FA-exposed HT-22 cells. We found that pretreatment with NaHS (200, 400 µM) significantly decreased the percentage of SA-β-Gal positive cells (Fig. 1a, b), downregulated the expressions of $P16^{INK4a}$ (Fig. 1c) and $P21^{CIP1}$ (Fig. 1d), as well as increased the number of cell proliferation (Fig. 1e) in HT-22 cells treated by FA (100 µM, for 48 h). These data indicated that H_2S prevents FA-induced senescence in HT-22 cells.

NaHS Upregulates Leptin Signaling in HT-22 Cells

To explore whether leptin signaling mediates the protective effects of H_2S against FA-induced senescence, we first determined whether H_2S regulates the leptin signaling in HT-22 cells. After treatment with NaHS (100, 200, and 400 µM) for 48 h, the expressions of leptin (Fig. 2a) and lepRb (Fig. 2b) were significantly increased in HT-22 cells. Next, we further investigated the effect of H_2S on the leptin signaling pathway in FA-treated HT-22 cells. Pretreatment with NaHS (100, 200, and 400 µM, for 30 min) significantly prevented FA-induced downregulation of the expressions of leptin (Fig. 2c) and lepRb (Fig. 2d). These data indicated the upregulatory effect of H_2S on leptin signaling in HT-22 cells.

Inhibition of Leptin Signaling Reverses the Protection of NaHS Against FA-Induced Senescence in HT-22 Cells

We explored whether inhibition of leptin signaling by leptin tA reverses the protection of H_2S against FA-induced senescence in HT-22 cells to confirm the mediatory role of leptin signaling in the protection of H_2S against FA-induced senescence. Pretreatment with leptin tA (50 nM) for 30 min not only obviously abolished the inhibitory role of NaHS (400 µM) on FA (100 µM)-induced increased percentage of SA-β-Gal positive cells (Fig. 3a, b) but also reversed NaHS-provided suppression on the FA-induced upregulations of $P16^{INK4a}$ (Fig. 3c) and $P21^{CIP1}$ (Fig. 3d). In addition, from the cell growth curve we found that leptin tA abolished the reversal effect of NaHS on the FA-triggered reduced cell proliferation (Fig. 3e). These data indicated that upregulation of leptin signaling mediates the inhibitory role of H_2S in FA-induced senescence.

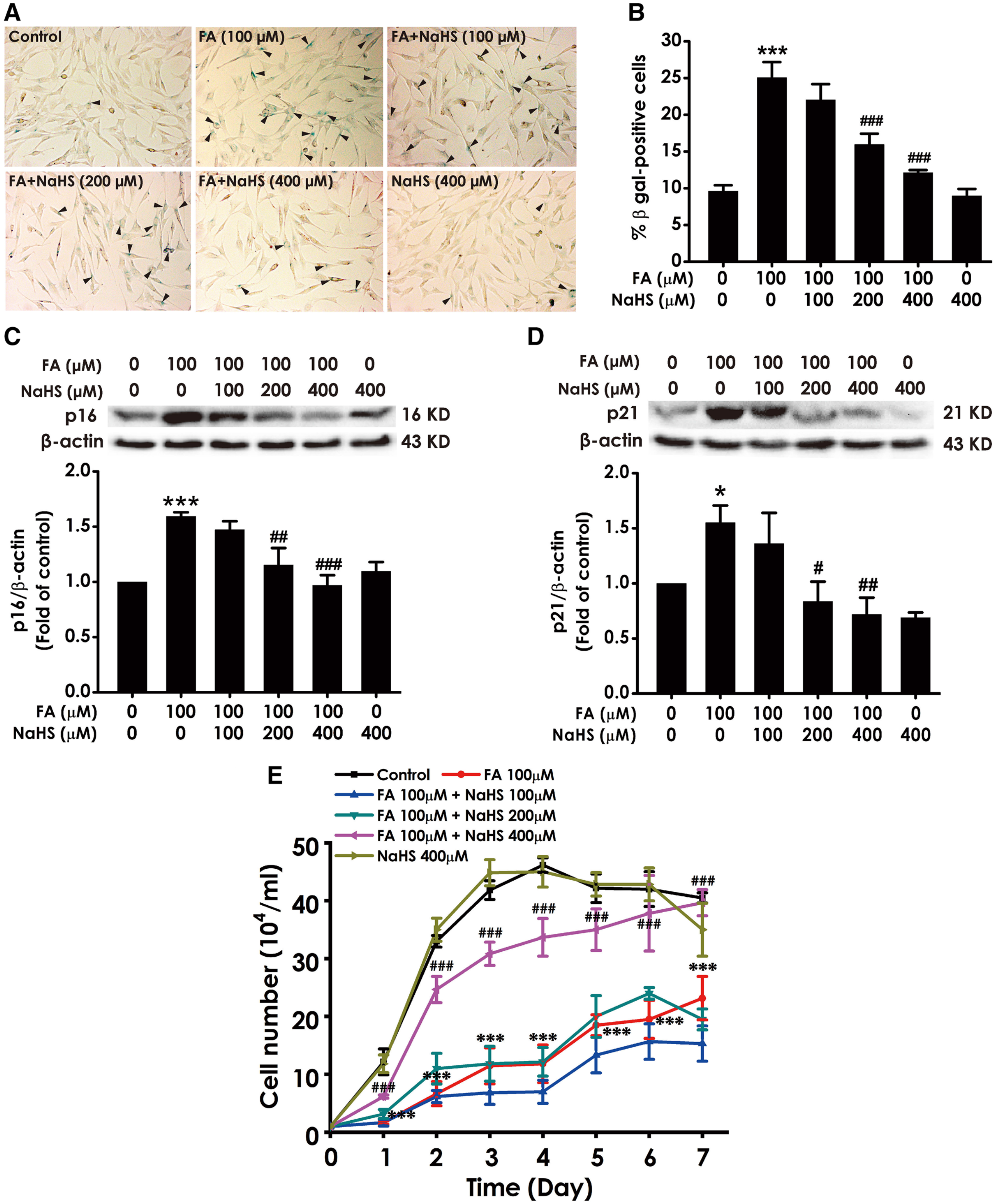


Fig. 1 Effect of NaHS on FA-induced senescence in HT-22 cells. HT-22 cells were pre-treated with NaHS (100, 200, 400 μM) for 30 min and then cotreated with FA (100 μM) for 48 h. **a** Representative images of SA-β-Gal positive cells. **b** Quantification of the percentage of SA-β-Gal positive cells. **c** and **d** The expressions of $\mathrm{P16^{INK4a}}$ (**c**) and $\mathrm{P21^{CIP1}}$ (**d**) were detected by western blot. **e** The state of cell growth was evaluated by Trypan blue exclusion assay. Data are the mean ± SEM of 3 independent experiments. $^{*}P<0.05$, $^{***}P<0.001$, compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the FA-treated alone group

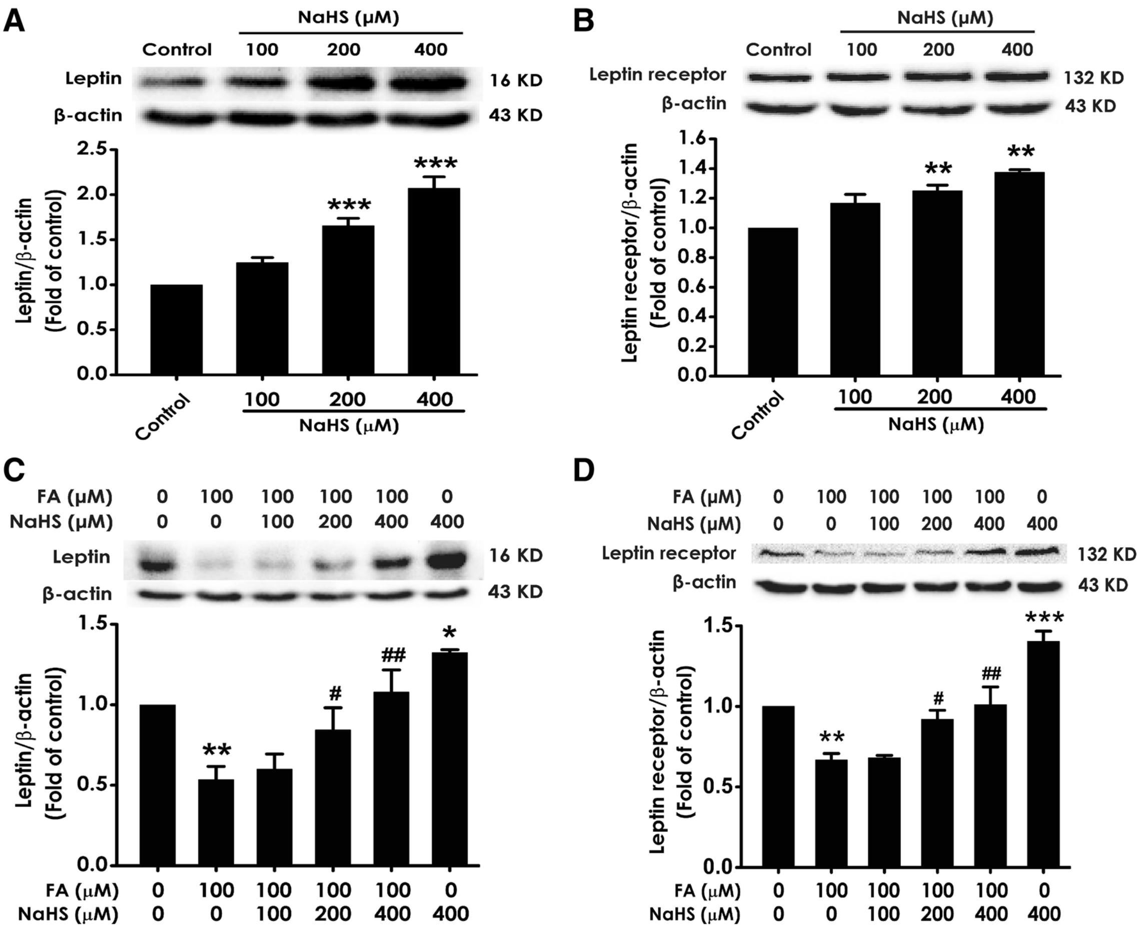


Fig. 2 Effect of NaHS on leptin signaling in HT-22 cells. **a** and **b** HT-22 cells were treated with NaHS (100, 200, 400 μM) for 48 h. **c** and **d** After pretreatment with NaHS (100, 200, 400 μM) for 30 min, HT-22 cells were co-exposed to formaldehyde (FA, 100 μM) for 48 h. The expression levels of leptin and lepRb in HT-22 cells were detected by Western Blot. Data are the mean ± SEM of 3 independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with the FA-treated alone group

Inhibition of Leptin Signaling Reverses the Protection of NaHS Against FA-Induced Neurotoxicity in HT-22 Cells

To further confirm the mediatory role of leptin signaling in the protective effect of H_2S on FA-induced neurotoxicity, we explored whether leptin tA reverses the protection of H_2S against FA-induced the loss of cell viability and the increase in apoptosis in HT-22 cells. Pretreatment with leptin tA (50 nM) for 30 min obviously decreased the cell viability (Fig. 4a) and increased the cell apoptosis (Fig. 4b, c) in HT-22 cells cotreated with FA (100 μM) and NaHS (400 μM), indicating that leptin tA reverses the protection of H_2S against FA-induced neurotoxicity in HT-22 cells.

Discussion

Our previous studies have demonstrated that H_2S has a protective effect against FA-evoked neurotoxicity (Tang et al. 2012; Li et al. 2014). Given the cellular senescence is prominent in the neurotoxicity of FA (Yu et al. 2015; Costa et al. 2015; Spencer 2018; Wang et al. 2018; Chen et al. 2017; Duan et al. 2018; Zhan et al. 2016), the present work was designed to explore whether the protection of H_2S in the neurotoxicity of FA is associated with regulating neuronal senescence. The main findings of the present work are the following: (i) H_2S inhibited FA-induced senescence in HT-22 cells; (ii) H_2S upregulated the leptin signaling in HT-22 cells; and (iii) Inhibited leptin signaling by Leptin tA abolished H_2S-provided protective effects on FA-induced

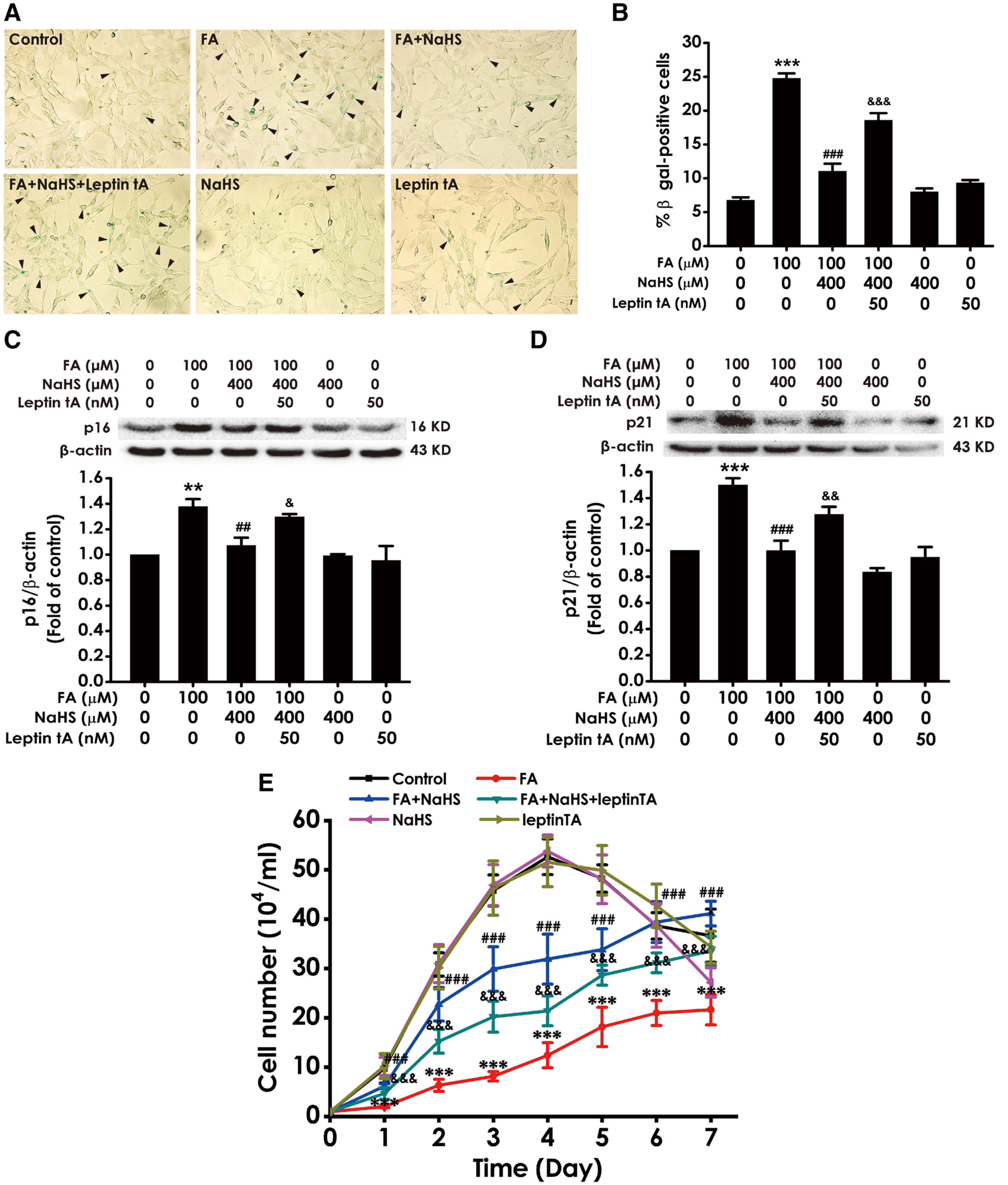


Fig. 3 Effect of leptin tA on NaHS-exerted protection against formaldehyde-induced senescence in HT-22 cells. HT-22 cells were preincubated with leptin tA (50 nM) for 30 min before pretreatment with NaHS (400 μM) for 30 min prior to 48 h co-exposure of formaldehyde (FA, 100 μM). **a** Representative images of SA-β-Gal positive cells. **b** Quantification of the percentage of SA-β-Gal positive cells. **c** and **d** The expressions of P16^{INK4a} (**c**) and P21^{CIP1} (**d**) were detected by western blot. **e** The state of cell growth was evaluated by Trypan blue exclusion assay. Data are the mean ± SEM of 3 independent experiments. $^{*}P<0.05$, $^{***}P<0.001$, compared with the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, compared with the FA-treated alone group alone; $^{\&}P<0.05$, $^{\&\&}P<0.01$, $^{\&\&\&}P<0.001$, compared with cotreated with NaHS and FA group

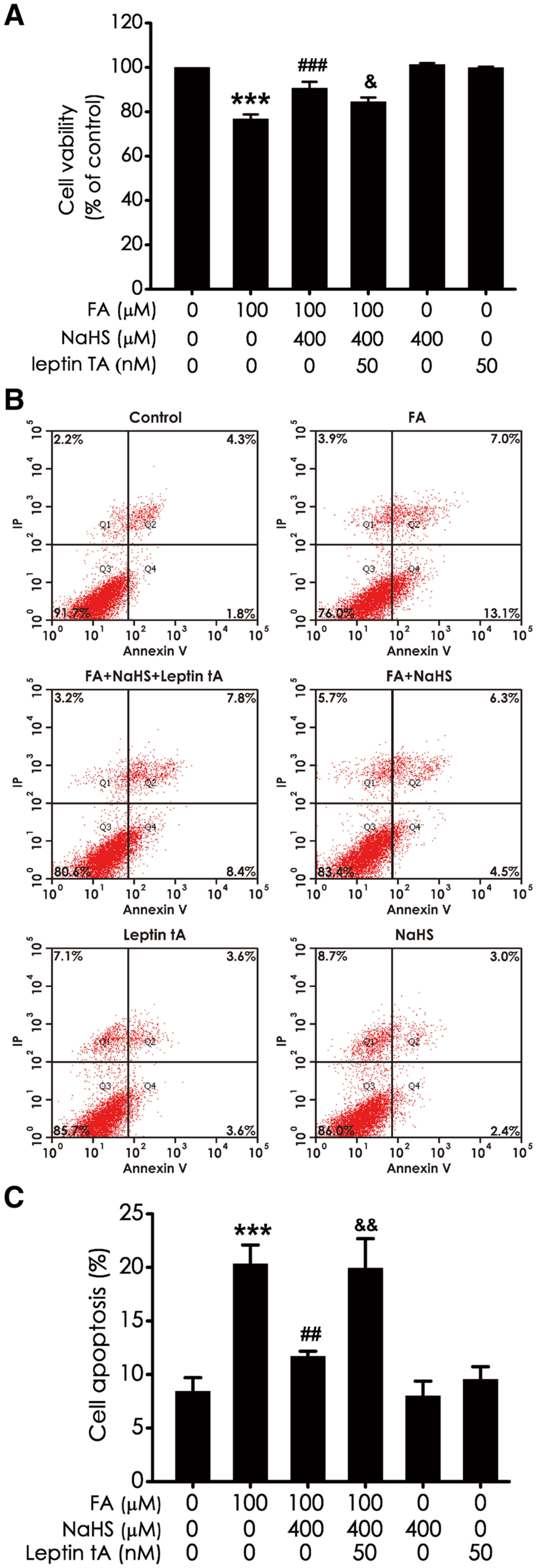


Fig. 4 Effect of leptin tA on NaHS-exerted protection against FA-induced neurotoxicity in HT-22 cells. HT-22 cells were preincubated with leptin tA (50 nM) for 30 min before pretreatment with NaHS (400 μM) for 30 min prior to 48 h co-exposure of formaldehyde (FA, 100 μM). **a** The cell viability was determined by CCK-8 assay. **b** and **c** The apoptosis of HT-22 cells was assessed by flow cytometry after PI and Annexin-V double staining (the annexin-V2/PI2 population is made up of normal healthy cells, while annexin-V+/PI2 cells exist in early apoptotic stage, and annexin-V+/PI+ cells exist in late apoptotic stage). Data are the mean ± SEM of three independent experiments. $^{***}P < 0.001$, compared with control group; $^{\#\#\#}P < 0.001$, compared with FA (100 μM)-treated alone group; $^{\&}P < 0.05$, compared with cotreated with NaHS and FA group

senescence and neurotoxicity. Therefore, we suggest that the protection of H_2S against FA-induced neurotoxicity is involved in inhibition of neuronal senescence through upregulating leptin signaling.

Cellular senescence, a process that imposes permanent proliferative arrest on cells in response to various stressors, has emerged as a potentially important contributor to aging and age-related disease, and it is an attractive target for therapeutic exploitation (Childs et al. 2015). Interestingly, it has been reported that accumulated FA in brain contributes to the age-related neurodegenerative symptoms (Tong et al. 2015; Qiang et al. 2014; Mei et al. 2015). Thus, understanding whether FA induces neuronal senescence is necessary for a greater understanding of the neurotoxicity of FA. It is known that the high SA-β-Gal activity is a main hallmark of senescent cell (Dimri et al. 1995; Debacq-Chainiaux et al. 2009). We investigated the impact of FA on SA-β-Gal activity in HT-22 cells and found that FA-treated HT-22 cells display obviously increased the percentage of SA-β-Gal positive cells. In addition, cellular senescence is associated with increased expressions of senescence biomarker $P16^{INK4a}$ and $P21^{CIP1}$ (Serrano et al. 1997; Baker et al. 2011; Carreira et al. 2005; Mowla et al. 2014). We also demonstrated that FA upregulated the expression levels of $P16^{INK4a}$ and $P21^{CIP1}$ in HT-22 cells. Furthermore, we found that FA led to cell growth arrest. These data clearly indicated that FA is able to induce cellular senescence to HT-22 cells. Therefore, modulation of neuronal senescence might represent a novel therapeutic strategy to overcome FA-induced neurotoxicity.

H_2S is a protective gaseous signaling molecules (Kimura 2002; Zhou and Tang 2011; Zhang and Bian 2014). Interestingly, our previous study confirmed that H_2S prevents FA-induced neurotoxicity in PC12 cells (Tang et al. 2012; Li et al. 2014). However, the exact mechanisms underlying this protection of H_2S need to be further studied. Given the mediatory role of neuronal senescence in FA-induced neurotoxicity (Zhan et al. 2016; Yu et al. 2015; Wang et al. 2018), to develop the current understanding of the protective role of H_2S in the neurotoxicity of FA, we explored whether H_2S inhibits FA-induced neuronal senescence. The present work showed that H_2S reduced the percentage of

SA-β-Gal positive cells, downregulated the expression levels of P16^{INK4a} and P21^{CIP1}, increased the number of cell proliferation, in FA-treated HT-22 cells. These data indicated that H_2S has the ability to suppress FA-induced senescence in HT-22 cells. It has been confirmed that H_2S attenuate the process of senescence in the endothelium (Latorre et al. 2018), kidney (Hou et al. 2016; Lee et al. 2018), vascular (Das et al. 2018), heart (Ma et al. 2018), and brain (Zhan et al. 2018) of mice. Furthermore, increasing the content of endogenous H_2S by proper diet extends the life span of the aged mice (Yoshida et al. 2018; Hine et al. 2018). These previous findings offered a reasonable explanation for the results obtained in the present study. Therefore, the regulation of neuronal senescence offers insights into the protection of H_2S against the neurotoxicity of FA.

The present study also investigated the possible underlying mechanism for the protective role of H_2S against FA-induced senescence. Leptin signaling is considered to have important neuroprotective effects in a series of neuropathy (Signore et al. 2008; Davis et al. 2014). Increasing evidence demonstrates that Alzheimer's disease (AD) patients have leptin resistance and impaired leptin signaling in the brain (Maioli et al. 2015; Bonda et al. 2014) and that aging-related processes such as oxidative stress and inflammation also result in leptin resistance and decreased leptin signaling (Purkayastha and Cai 2013). Furthermore, it has been confirmed that leptin reduces pathology and improves memory in a transgenic mouse model of AD (Greco et al. 2008, 2009, 2010), which suggest that leptin signaling may delay the senescence process in brain. Our previous study has found that FA-induced HT-22 cellular senescence may be related to the downregulation of leptin signaling (Zhan et al. 2016). Therefore, we speculated that leptin signaling is a possible mediator in the protection of H_2S against FA-induced senescence. The present work focuses first the effect of H_2S on the leptin signaling in HT-22 cells. We found that H_2S not only increased the expressions of leptin and lepRb in HT-22 cells, but also reversed FA-reduced the expressions of leptin and lepRb in HT-22 cells. These results implied that the upregulation of leptin signaling contributed to the protective effect of H_2S on FA-induced senescence. To further confirm whether the leptin signaling mediates the protection of H_2S against FA-induced senescence, we explored whether the blockage of leptin signaling abolishes this protection of H_2S. Our results showed that inhibited leptin by Leptin tA abolished the protection of H_2S against FA-induced increase in the percentage of SA-β-Gal positive cells, upregulations of P16^{INK4a} and P21^{CIP1}, and the arrest of cell growth. Taken together, these results indicated that the regulation of leptin signaling mediates the H_2S-exerted protection against FA-induced senescence in HT-22 cells. More importantly, recent research found that leptin upregulated CBS/H_2S system by activation FOXO3a in neurons of PVN (Zheng et al. 2018). Taken together, it seems reasonable to make a hypothesis that there is a positive interaction regulation between H_2S and leptin signaling in neuron biology. However, it remains to be explored that how does H_2S and leptin interact in neurons. It has been confirmed H_2S activates FOXO3a in H9c2 cardiac cells as well (M. H. Liu et al. 2016), suggested that the mutual regulation between H_2S and leptin signals may be achieved through the FOXO3a pathway. In the future study, we will in depth explore the underlying mechanisms of this interaction between H_2S and leptin signaling in the neurons. Simultaneously, the blockage of leptin signaling also eliminated the protective effect of H_2S against FA-induced neurotoxicity, which further demonstrated that the involvement of reduced cellular senescence in the H_2S-exerted protection against the neurotoxicity of FA. Finally, we get a model of H_2S regulates the levels of P16^{INK4a} and P21^{CIP1} through the leptin signaling to prevent FA-induced senescence in HT-22 cells.

In summary, the present study demonstrated that H_2S has the ability to inhibit FA-induced neuronal senescence and upregulates leptin signaling in FA-treated HT-22 cells. We also found that inhibition of leptin signaling reversed the protection of H_2S against FA-induced senescence and neurotoxicity in HT-22 cells. These results clarified that H_2S antagonizes FA-induced neuronal senescence by upregulation of leptin signaling. Our findings provide a novel mechanistic explanation for the protection of H_2S against FA-induced neurotoxicity and suggest that leptin signaling may be a promising therapeutic target for preventing FA-dependent neurodegenerative disease.

Acknowledgements This study is supported by Natural Science Foundation of China (81671057), the Major Research Topics of the Health and Family Planning Commission of Hunan province (A2017011), Hunan Provincial Innovation Foundation for Postgraduate (CX2017B570, CX2018B619) and Postgraduate Research Innovation Foundation of University of South China (2018KYZ048).

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