Replicative Senescence in Human Fibroblasts Is Delayed by Hydrogen Sulfide in a NAMPT/SIRT1 Dependent Manner

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Abstract

Recent evidence suggests that hydrogen sulfide (H₂S) has cytoprotective and anti-aging effects. However, the mechanisms for such properties are not fully understood. Here, we show that the expression of the main H₂S producing enzyme, CBS, and production of H₂S are coordinately diminished in replicative senescent adult human dermal fibroblasts. The reduced production of H₂S falls within the same time-frame that the hallmarks of replicative senescence appear including accumulation of SA–β-Gal, enhanced expression of p16, p21, and RRM2B while the expression of RRM2, hTERT, SIRT1, NAMPT, and NAD/NADH ratio all fall. Exogenous H₂S increases the expression of hTERT, NAMPT, SIRT1 and NAD/NADH ratio in treated cells. Moreover, H₂S safeguards the expression of hTERT in a NAMPT and SIRT1 dependent manner and delays the onset of replicative senescence as evidenced by reduced accumulation of age associated SA–β-Gal and cessation of proliferation. Postponement of loss of cell proliferative capacity without risk of mutagenesis shows implications for use of H₂S in delaying the adverse effects of senescence in organisms.

Introduction

There are several lines of evidence that the gasotransmitter, H₂S has cytoprotective and life extension properties. Recently, it was shown that the generation of reactive oxygen species is increased in knockouts of mpst-1, a major enzyme that drives the production of hydrogen sulfide in C. elegans and this deficit is overcome by the administration of GY4137 that exposes the short-lived mutants to hydrogen sulfide [1]. This treatment also extends the lifespan of normal animals and delays the onset of detrimental impact of senescence as assessed by pharyngeal contraction and defecation [1]. The extension of lifespan by hydrogen sulfide, which requires SIR-2.1 activity, affords the animals other health-promoting effects including stress resistance and improved thermotolerance [2].

It is known that calorie restriction promotes longevity by increasing SIRT1 expression [3]. In yeast and Drosophila, calorie restriction extends life-span by increasing Sir2 activity and by activating Sir2 deacetylase. Senescence is thought to be due to a progressive loss of cell function and/or cell loss over time. SIRT1 reduces stress induced apoptotic cell loss by deacetylation of
the DNA repair factor, Ku70. Deacetylated Ku70, in turn, reduces apoptosis by sequestering the proapoptotic factor, Bax, away from the mitochondria. Thus, by inducing SIRT1 expression, calorie restriction promotes long-term survival of cells which are irreplaceable [3]. It was recently shown that the effect of calorie restriction on life extension is associated with an increase in production of hydrogen sulfide with a cysteine and methionine deficient diet being required for such an enhanced production [4].

In light of such evidence, here, we tested the hypothesis that replicative senescence is associated with a progressive loss in ability of cells to produce hydrogen sulfide and that supporting fibroblasts with an exogenous source of hydrogen sulfide delays replicative senescence that ultimately leads to cessation of proliferation. Data shown here support the view that the life extension properties of hydrogen sulfide, at least in part, is due to its impact in safeguarding against senescence in a NAMPT and SIRT1 dependent manner.

Materials and Methods
Reagents and cell culture
Cell viability was confirmed by Trypan Blue staining (Sigma-Aldrich, St Louis, MO). Chemicals were from Sigma-Aldrich, TRIZOL® and reverse transcriptase (RevertAid® Reverse Transcriptase) were from Thermo Scientific (Carlsbad, CA). NAMPT siRNA was purchased from Santa Cruz Biotechnology (Dallas, TX). Oligonucleotides were generated by IDT (Coralville, IA). Transfection reagent was purchased from Santa Cruz Biotechnology. Adult human dermal fibroblasts (aHDF) cells were obtained from ATCC (Manassas, VA) or Lonza (Walkersville, MD). Cells were maintained in Fibroblast Growth Medium (FGM, Lonza) with 2% fetal bovine serum and growth factors in a 37°C incubator with 5% CO₂.

Determination of Population Doublings (PD)
Culture dishes were seeded in triplicates with 3 x 10⁵ aHDF cells. We calculated PD based on the following formula: log ((number of cells harvested)/(number of cells seeded))/log2 + previous PD. Since the PD of cells received from manufacturer was not known, we defined the first PD after initial culture as 0 [5].

Staining for Senescence-Associated β-Galactosidase (SA-β-Gal)
SA-β-Gal staining was performed as described previously [6].

Measurement of H₂S production
H₂S production was measured by WPI instrument as described previously (Sarasota, FL) [7,8].

Real-time PCR
Real-time PCR was performed using iTaq® Universal SYBR® Green Supermix (Bio-Rad; Hercules, CA) and LightCycler® 96 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instruction. Primers were purchased from IDT (S1 Table).

Immunoblotting
Immunoblotting is described previously [8]. 10 µg of total protein lysates for Nampt and β Actin and 30 µg of total protein lysates for Sirt1 were used. For hTERT blotting, nuclear extraction was performed [9] and 100 µg of nuclear extracted lysates were used. Used antibodies are; anti-hTERT mouse monoclonal (clone 2C4; EMD Millipore), anti-Nampt mouse monoclonal
(Sigma), anti-Sirt1 rabbit polyclonal (Sigma), and anti-β Actin–HRP (sc1616-HRP; Santa Cruz). The substrate used in this study was ECL® Prime Western Blotting Detection Reagent (GE Healthcare). The membranes were scanned using C-Digit® (LI-COR) and analyzed by Image Studio® (LI-COR).

Telomerase activity assay

Cells were harvested and lysed in CHAPS buffer (0.5% CHAPS, 10 mM Tris-HCl, pH = 7.5., with 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol). The telomeric repeat amplification protocol (TRAP) assay was performed as described by Kim et al. [10,11]. Briefly, PCR was performed using primers listed in S1 Table as follows: first incubation at 30°C for 30 min, second incubation at 95°C for 3 min, followed by a 30 cycle amplification (95°C for 30 s, 59°C for 30 s, and 72°C for 1 min). The products were run on a 15% polyacrylamide gel (Bio-Rad) in 0.5x TBS and the bands were stained with SYBR Gold Nucleic Acid Gel Stain (Life Technologies). Relative activity of telomerase was calculated by dividing the density of the all ladders to the density of the bands in internal control (The TRAP internal control “TSNT” was synthesized as reported previously (S1 Table) [11]. Densitometric analysis was performed using ImageJ (NIH, Bethesda, MD).

NAD assay

NAD assay was performed using EnzyChrom® NAD⁺/NADH⁺ Assay kit (BioAssay Systems, Hayward, CA), according to the manufacturer’s instruction.

siRNA transfection

NAMPT siRNA and Scrambled siRNAs (control siRNAs) were purchased from Santa Cruz Biotechnology. SIRT1 siRNA and oligonucleotides were generated by IDT. The sequence of SIRT1 siRNA was described previously [12]. Each oligonucleotide was dissolved in 100 μM Duplex Buffer (100 mM Potassium Acetate, 30 mM HEPES, pH 7.5) and mixed in equal molar amounts, with a final concentration of 10 μM per oligonucleotide. Oligonucleotides were annealed at 94°C for 2 minutes and then cooled to room temperature for 2 hours. Transfection of siRNA into aHDF cells was carried out at 37°C and with 5% CO₂ for 2 days using transfection reagent (Santa Cruz Biotechnology) in DMEM medium without serum and antibiotics. The culture medium was replaced with fibroblast medium (FGM2, Lonza, Walkersville, MD) without or with 1 μM NaHS and cells were cultured for an additional 3 days.

SIRT1 activity assay

SIRT1 activity assay was performed using Universal SIRT Activity Assay Kit (Abcam, Cambridge, MA) according to the manufacturer’s instruction. Briefly, 5 x 10⁵ of aHDF cells were transfected with NAMPT siRNA or Scrambled-siRNA. After 2 days, fresh medium was added without or with 1 μM NaHS and cells were cultured for an additional 3 days. Sample cells were collected and nuclear fractions were prepared, as described previously [13]. SIRT1 activity was normalized to total protein of each sample.

Statistics

All assays were done in 3–6 replicate cultures, in at least three independent experiments. Data are shown as means ± SEM. p values were determined by comparing the data from treated cells against control cells. Data were subjected to the two tailed t-test for determination of means.
and $p$ values. $p$ values less than 0.05 were considered significant. $p$ values are shown as $<$ 0.05 (*), $<$ 0.005 (**) or $<$ 0.0005 (***)

**Results**

Replicative senescence leads to reduced production of hydrogen sulfide

We assessed the production of H$_2$S in young (population doubling; PD: 5.9) and replicative senescent (PD: 18.8) aHDF cells. Consistent with previous reports, senescent cells show accumulation of senescence-associated $\beta$-galactosidase (SA-$\beta$-Gal) (68%, Fig 1A), increase in expression of $p16$, $p21$, $RRM2B$ and decreased expression of $RRM2$ [14] (S1 Fig). The

![Fig 1. Production of H$_2$S is downregulated in replicatively senescent cells.](image)
expression hTERT was also diminished by about 16 fold in senescent aHDF cells (Fig 1B). As compared to young cells, the NAD/NADH ratio which is a measure of metabolic activity was also decreased in senescent cells (Fig 1C).

Then, we assessed whether the expression of three H₂S-producing enzymes, CBS, MST and CSE [15–17] is altered by senescence. The expression of CBS was decreased in senescent cells while the expression of MST and CSE remained the same in young and replicatively senescent cells (Fig 1D–1F). The production level of H₂S in replicative senescent cells was diminished by 63% in senescent cells (Fig 1G).

**Exogenous H₂S upregulates the expression of hTERT and increases PD in aHDF cells**

We tested whether the lower expression of hTERT in senescent aHDF cells is due to down-regulation of H₂S production in senescent cells. Treatment of young aHDF cells with NaHS within the reported physiological level of H₂S [18] (0.01 to 100 μM) significantly upregulated the expression of hTERT with 1 μM being the optimal concentration for maximum hTERT expression (Fig 2A). This finding was confirmed by using other cell types (S2A–S2C Fig), and as evidenced by immunoblotting (NaHS enhanced hTERT in treated cells; Fig 2B). However, treatment with NaHS failed to reduce SA-β-Gal or to upregulate the expression of hTERT in senescent aHDF cells (~65% SA-β-Gal⁺) (S3A and S3B Fig), suggesting that H₂S induced hTERT expression is suppressed in senescent cells.

Up-regulation of expression of hTERT in young aHDF cells treated with NaHS was associated with an increase in the activity of telomerase (Fig 2C). We, then, investigated whether the

![Fig 2. Exogenous H₂S increases the expression of hTERT as well as the activity of telomerase.](image)

**(A)** Real-time PCR analysis of the expression of hTERT in young (PD: 5.9) aHDF cells, treated with NaHS for 3 days. The expression of hTERT was normalized to the level of expression of β-ACTIN. Expression of untreated control was regarded as 1.0. **(B)** Immunoblotting of hTERT in aHDF cells without or with 1 μM NaHS for 7 days. 100 μg of the indicated nuclear extracts were subjected for immunoblotting. β-Actin was used as a loading control. **(C)** Telomerase activity in young (PD: 3.2) aHDF cells without or with treated with 1 μM NaHS for 7 days. Positive control was MDA-MB-231 cell lysate, and negative control was buffer alone. Bottom panel shows quantified means ± error bars from three independent assays. Relative activity of telomerase was calculated by dividing the density of all ladders to the density of the bands in internal control, indicated as internal control (I.C.).

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increased expression of hTERT and telomerase activity increases PD, young aHDF cells were
treated weekly with 0, 1, and 100 μM of NaHS for 84 days, and PD was calculated. As shown in
Fig 3A, as compared to PD of the untreated control groups, treatment of aHDF cells with 1 μM
NaHS caused a significant increase in PD. This increase was lost in cells that were treated with
greater (100 μM) concentration of NaHS (S4 Fig). Consistent with these data, the percentage of
SA-β-Gal positive cells was reduced in aHDF cells that were treated with 1 μM of NaHS (Fig 3B).

**H$_2$S mediated hTERT expression is NAMPT and SIRT1 dependent**

The life extension afforded by hydrogen sulfide [2,19,20] might be mediated, at least in part, by
increasing the expression of NAMPT (nicotinamide phosphoribosyl transferase) that regulates
metabolism together with SIRT1, a factor involved in the maintenance of integrity of telomeres
[21]. Among the seven Sir2 homologues in mammalian cells (SIRT1 to -7), SIRT1 is most
closely related to Sir2 which is known to be a major life-span regulator in C. elegans [2]. Nicotinamide
adenine dinucleotide (NAD$^+$) is a coenzyme that mediates many redox reactions and
regulates NAD$^+$-consuming enzymes such as Sirtuin family of NAD—dependent protein dea-
cetylases. The biosynthesis of NAD$^+$ is mediated by NAMPT. For these reasons, we examined
whether the effect of H$_2$S on hTERT is NAMPT and SIRT1 dependent. In senescent aHDF
cells, the expression of NAMPT and SIRT1 diminished with age (Fig 4A–4C). Treatment of young aHDF cells (PD: 5.9) with NaHS increased the expression of NAMPT and SIRT1, in a dose dependent manner with 1 μM inducing maximum expression (Fig 4D and 4E). These data were further verified by immunoblotting (Fig 4F). The treatment also caused a coordinate increase in the ratio of NAD to NADH (Fig 4G). Treatment of aHDF cells with SIRT1 siRNA suppressed the NaHS induced expression of SIRT1 (Fig 5A and S5 Fig) and concomitantly prevented the expression of hTERT (Fig 5B). Whereas the siRNA to NAMPT reduced expression of NAMPT, it did not reduce the expression of SIRT1 (S5 Fig). However, suppression of

Fig 4. NaHS-treatment increases expression of NAMPT and SIRT1. (A and B) The expression of NAMPT and SIRT1 in young (PD: 5.9) and senescent (PD: 18.8) was assessed by real-time PCR and normalized to the expression level of β-ACTIN. (C) Immunoblotting of Nampt and Sirt1 in young (PD: 5.9) and senescent (PD: 18.8) aHDF cells. β-Actin was used as a loading control. (D and E) Young (PD: 5.9) aHDF cells were treated without and with NaHS for 3 days, and RNA samples were then subjected to real-time PCR for assessment of NAMPT and SIRT1. The expression levels of NAMPT and SIRT1 were normalized to the levels of expression of β-ACTIN. (F) Immunoblotting of Nampt and Sirt1 in NaHS-treated young (PD: 5.9) aHDF cells. β-Actin was used as a loading control. (G) NAD/NADH ratio in young (PD: 5.9) aHDF cells treated without and with NaHS for 7 days. Data were normalized to the total amount of protein.

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NAMPT decreased the activity of SIRT1 (Fig 5C) and led to a decrease in the expression of hTERT (S5 Fig).

**Discussion**

We demonstrated that the production of H₂S as well as the expression of CBS both decrease upon aging. We further show that the downregulation of hTERT, SIRT1, NAMPT, and NAD/NADH ratio can be delayed by H₂S and that long-term effect of H₂S is to maintain telomerase expression, and to postpone replicative senescence as evidenced by increasing population doublings in aHDF cells treated with exogenous H₂S. Thus, H₂S maintains a threshold level of telomerase activity which contributes to its life-span extension properties.

H₂S plays a bioenergetics role in Krebs cycle in mitochondria [22]. Modis et al showed that low concentrations of H₂S elicited an increase of mitochondrial function, including an increase cellular pool of ATP and improved cell viability, whereas higher concentrations of H₂S were inhibitory [22,23]. Our data show 1 μM of NaHS is optimal for promoting hTERT expression (Fig 2A) and in increasing PD (Fig 3A and S4 Fig). Regardless of site of action, H₂S leads to an increase in cellular pool of ATP energy yield which results in suppressing cellular senescence in aHDF cells.

Previously, we have shown that H₂S upregulates NAMPT and increases mitochondrial bioenergetics [7,8]. Although it is still not clear how H₂S controls NAMPT, Huang et al reported that H₂S suppresses the expression of microRNA34a by activating Nrf2 after hepatic ischemia/reperfusion injury [24]. Choi et al demonstrated that microRNA34a reduced NAMPT/NAD+ level [25]. Based on such finding H₂S might negatively regulate NAMPT by suppression of...
microRNA34a. However, further studies are required to address the molecular mechanisms of NAMPT by H$_2$S.

Others have shown the relation of NAMPT, NAD and SIRT1 [26,27]. It has been shown that SIRT1 by deacetylation of c-MYC [28] transcriptionally increases the activation of c-MYC and correspondingly increases the amount of acetylated H4 histone at the hTERT promoter [21]. In addition, FOXO3a, a downstream target of SIRT1, potentiated hTERT gene transcription by binding to c-MYC promoter. This upregulated c-MYC which was recruited to the hTERT promoter, leads to the of hTERT gene activation [29]. Intriguingly, NaHS-treatment increases the expression of FOXO3a in aHDF cells (data not shown); thus, it seems that SIRT1 upregulates hTERT through FOXO3a/c-MYC and increases the lifespan of human fibroblasts.

Mammalian senescence is dependent on the mammalian NAD-dependent deacetylase, Sirt1, and Nampt-mediated systemic NAD biosynthesis [30]. Based on our findings, H$_2$S regulates the expression of NAMPT and SIRT1 in a dose dependent manner and coordinately sets the NAD/NADH ratio. H$_2$S also regulates hTERT expression, and this function is dependent on both NAMPT mRNA expression and SIRT1 activity. Sir2 and its orthologues play an important role in controlling longevity in model organisms as diverse as yeast to worms and flies [31]. Among sirtuins, it has been shown that SIRT1 delays senescence and extends life-span in both male and female mice [32]. In light of such findings, the life extension afforded by H$_2$S might be mediated, at least in part, through activation of SIRT1 (Fig 5D). Consistent with these results, the treatment of human umbilical vascular endothelial cells with H$_2$S, delayed the H$_2$O$_2$ and nicotinamide induced pre-mature senescence by SIRT1 activation [33,34]. The impact of H$_2$S on NAMPT/Sirt1, likely, has global effects since it has been shown that RNA-mediated knockdown of NAMPT or NMNAT-1 in MCF-7 breast cancer cells reduced total cellular NAD$^+$ levels and globally altered pattern of gene expression [35]. Together, the postponement of loss of cell proliferative capacity by H$_2$S without the risk of mutagenesis suggests that H$_2$S can be used in delaying the adverse effects of senescence in organisms.

Supporting Information
S1 Fig. Verification of senescence.
(DOC)

S2 Fig. Effect of NaHS-treatment on other human fibroblasts.
(DOC)

S3 Fig. Treatment of senescence cells with NaHS is not effective to suppress cellular senescence.
(DOC)

S4 Fig. Treatment of aHDF cells with 100 μM NaHS does not increase PD.
(DOC)

S5 Fig. Down regulation of NAMPT suppresses the expression of hTERT, but not SIRT1.
(DOC)

S1 Table. Primer sequences.
(DOC)

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Author Contributions

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Data curation: RSA.
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Investigation: RSA.
Methodology: RSA SA.
Project administration: ST.
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Visualization: RSA SA.
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