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ORIGINAL ARTICLE

CO ameliorates cellular senescence and aging by modulating the miR-34a/Sirt1 pathway

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ABSTRACT

Oxidative stress is recognised as a key factor that can lead to cellular senescence and aging. Carbon monoxide (CO) is produced by haemoxygenase-1 (HO-1), which exerts cytoprotective effects in aging-related diseases, whereas the effect of CO on cellular senescence and aging has not been elucidated. In the current study, we clearly demonstrated that CO delays the process of cellular senescence and aging through regulation of miR-34a and Sirt1 expression. CO reduced H_2O_2-induced premature senescence in human diploid fibroblast WI-38 cells measured with SA-β-Gal-staining. Furthermore, CO significantly decreased the expression of senescence-associated secretory phenotype (SASP), including TNF-α IL-6, and PAI-1 and increased the transcriptional levels of antioxidant genes, such as HO-1 and NQO1. Moreover, CO apparently enhanced the expression of Sirt1 through down-regulation of miR-34a. Next, to determine whether Sirt1 mediates the inhibitory effect of CO on cellular senescence, we pre-treated WI-38 cells with the Sirt1 inhibitor Ex527 and a miR-34a mimic followed by the administration of H_2O_2 and evaluated the expression of SASP and antioxidant genes as well as ROS production. According to our results, Sirt1 is crucial for the antiaging and antioxidant effects of CO. Finally, CO prolonged the lifespan of Caenorhabditis *elegans* and delayed high-fat diet-induced liver aging. Taken together, these findings demonstrate that CO reduces cellular senescence and liver aging through the regulation of miR-34a and Sirt1.

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Caenorhabditis *elegans*; carbon monoxide; haemoxygenase; miR-34a; oxidative stress; senescence-associated secretory phenotype; Sirt1

Introduction

Cellular senescence is a durable cell cycle arrest generated through various stressors [1–3]. Oxidative stress induced by hydrogen peroxide (H_2O_2) contributes to cellular senescence [2,4,5]. Senescent cells have several distinguishing characteristics, including senescence-associated secretory phenotype (SASP), including various cytokines, chemokines, and growth factors, increased activity of senescence-associated β-galactosidase (SA-β-Gal), abnormal activation of p53, and upregulation of p21, which can inhibit cyclin-dependent kinase 2 (CDK2) and result in cell cycle arrest [6–8]. Furthermore, cellular senescence affects embryonic development, wound healing, and tissue repairment, whereas the persistent accumulation of senescent cells and SASP causes aging and pathogenesis of aging-associated diseases [9–13]. Therefore, reduced severe cellular senescence is important for protection from aging-related disorders.

Endogenous carbon monoxide (CO) is produced by haemoxygenase-1 (HO-1), inducible stress–response protein and regulator of oxidative stress [14–16]. CO exhibits potent antioxidant, anti-inflammatory, and anti-apoptotic effects [17–19]. CO-releasing molecules (CORMs), safe pharmaceutical agents, can be used as deliverers of CO rather than the inhalation of gaseous CO [20,21]. Furthermore, exogenous CO gas and CORMs exert cytoprotective effects in aging-related diseases [22–24]. Moreover, chronic treatment with haemin, an HO-1 inducer, attenuates fibroblast senescence [25,26]. However, whether CO can attenuate and delay the process of cellular senescence and aging remains unclear.

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Supplemental data for this article can be accessed here.

Sirtuin 1 (Sirt1) is an NAD^+-dependent deacetylase, which regulates various biological processes through deacetylation of transcription factors, histones and other proteins [27]. Sirt1 plays an important role in the attenuation of aging-related diseases and extension of the lifespan in mice and *Caenorhabditis elegans* [28–33]. We previously reported that CO increases Sirt1 expression and activity through inhibition of microRNA-34a (miR-34a) [34]. Moreover, Sirt1 induces an antioxidant response through the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidant genes, and p53 deacetylation [35–37]. However, whether CO exerts a protective effect against oxidative stress-induced cellular senescence through the miR-34a-Sirt1 pathway is still unclear.

In the present study, we demonstrated that CO ameliorated H_2O_2-induced cellular senescence in human diploid fibroblasts (HDFs) and high-fat diet (HFD)-induced liver aging through the regulation of the miR-34a-Sirt1 pathway. Furthermore, CO decreased SASP and prolonged the lifespan of *C. elegans*. Thus, we confirmed that CO attenuated oxidative stress-induced premature senescence and delayed the process of aging via regulation of the miR-34a-Sirt1 pathway.

Materials and methods

Reagents and chemicals

Sodium boranocarbonate (CORM-A1), hydrogen peroxide solution (H_2O_2), Ex527, and pifithrin (PFT) were purchased from Sigma Aldrich (St. Louis, MO, USA). To detect the effects of CO on senescence-associated phenotypes was used with a water-soluble CO-releasing molecule (CORM-A1). Inactive CORM-A1 (iCORM-A1) was applied as a negative control by dissolving CORM-A1 in 0.1 M HCl and bubbling pure N_2 [21].

Animals

The mice in this study were with pure C57BL/6 background. Animal experiments were approved by the University of Ulsan Animal Care and Use Committee. All mice were maintained under specific pathogen-free conditions at 18–24 °C and 40–70% humidity with a 12 h light-dark cycle. To identify the protective effect of CO on HFD-induced aging liver, 6-week-old C57BL/6 male mice were fed with HFD (Research Diet, New Brunswick, NJ) or normal chow diet (NCD) for 16 weeks. After 8 weeks HFD administration, mice in the HFD + CO and CO groups were inhaled with CO gas (250 ppm, Core Gas, Ulsan, Korea) for 2 h daily for the remaining 8 weeks. After 16 weeks of treatment, mice were sacrificed, and liver tissues were extracted for various assays.

Cell culture

Human diploid lung fibroblast WI-38 cells (KCLB, Seoul, Korea) were cultured in minimal essential medium (MEM) (Gibco, Grand Island) supplemented with 10% FBS (Gibco, Melbourne, Australia) and 1% penicillin-streptomycin (Gibco, Grand Island). Cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Caenorhabditis elegans *lifespan analysis*

The wild-type N2 strain used in this study was kindly provided by Seung-Jae V. Lee (Korea Advanced Institute of Science and Technology, Daejeon, Korea). The *C. elegans* were grown and maintained on NGM (Duchefa Biochemie, Haarlem, Netherlands) plates seeded with *Escherichia coli* OP50 at 20 °C [38]. To assess the beneficial effect of CO on the *C. elegans* lifespan, gravid adult worms were first placed on fresh plates and allowed to lay eggs for synchronisation. After 48 h, 120 young adult worms were transferred to new plates that contained 50 μM 2′-fluoro-5′-deoxyuridine (FUDR; Sigma Aldrich, MO). After further 24 h, adult worms were transferred again to new plates that contained 125 and 250 μM CORM-A1. Dead worms were counted; however, worms that died from vulva bursting and internal hatching or crawled off the plate were censored. The survival data were analysed by using the online application for the survival analysis of lifespan assays (Oasis) (http://sbi.postech.ac.kr/oasis/surv/).

Western blot

Total proteins were extracted from tissues and cells using mammalian buffer (Thermo Fisher Scientific, Waltham, MA) containing phosphatase inhibitor cocktail 2 and 3, as well as a protease inhibitor (Sigma Aldrich, St. Louis, Mo, USA). The proteins were resolved by SDS-PAGE and then transferred to PVDF membranes (Millipore, Schwalbach, Germany). After blocking with 5% skim milk in PBS-Tween 20, membranes were probed with anti-Sirt1 (Millipore, Schwalbach, Germany) and anti-β-actin (Invitrogen, CA, USA) primary antibodies by incubation at 4 °C overnight. Next, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. The chemiluminescence signal was detected with an Azure Biosystems C300 analyser (Azure Biosystems, Dublin, CA, USA) by using an ECL detection system (GE

Healthcare Bio-Sciences, Little Chalfont, UK). The band density was analysed with ImageJ2x software (US National Institutes of Health, Bethesda, MD, USA).

Transfection

For transfection of p53 wild type (WT) plasmid DNA, WI-38 cells were seeded in 12-well plates and transfected with pCMV-p53WT (Clontech, CA) using Lipofectamine 2000 reagent (Invitrogen, CA, USA). After 48 h, cells were harvested and analysed. For transfection of miR-34a, WI-38 cells were transfected with microRNA human has-miR-34a-5p-mimic (Thermo Pierce, IL) using HiPerFect transfection reagent (Qiagen, CA). After 48 h, cells were harvested and analysed.

Reverse transcriptase PCR

Total RNA was isolated from tissues or cells using Trizol reagent (Invitrogen, CA), according to the manufacturer's instructions. Briefly, cDNA was synthesised from mRNA (2 μg) by an oligo(dT) primer (Bioneer, Daejeon, Korea) and M-MLV reverse transcriptase (Promega, WI, USA). The synthesised cDNA was subjected to PCR-based amplification. The expression of GAPDH was determined as an internal control. The following primers were used: human GAPDH forward, 5′-CCACCC ATGGCAAATTCCATGGCA-3′, reverse, 5′-TCTAGACGGCA GGTCAGGTCCACC-3′; human Sirt1 forward, 5′-GCTTAT TTGTCAGAGTTCCCACCC-3′, reverse, 5′-CAGCATTTTCTC ACTGTTCCAGCC-3′; human TNF-α forward, 5′-GAGCA CTGAAAGCATGATCCG-3′, reverse, 5′-AAAGTAGACC T G CCCAGACTCGG-3′; and human IL-6 forward, 5′-CTCTAT GGAGAACTAAAAGT-3′, reverse, 5′-ACTGCATAGCCACT TTCCAT-3′.

Real-time quantitative RT-PCR

The synthesised cDNA was analysed by real-time quantitative PCR using SYBR Green qpcr MASTER MIX (USB, CA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, CA). The target gene expression was normalised against the GAPDH gene. The following primers were used: human GAPDH forward, 5′- CAATGACCCCA TTGACCTC-3′, reverse, 5′-AGCATCGCCCCACTTGATT-3′; human p21 forward, 5′-CGATGGAACTTCGACTTTGTC A, reverse: 5′-GCA CAA GGG TAC AAG ACA GTG-3′; human PAI-1 forward, 5′- TGATGGCTCAGACCAACAAG-3′, reverse, 5′-CAGCAATGAACATGCTGAGG; human HO-1 forward, 5′-C AGGAGCTGCTGACCCAT GA-3′, reverse, 5′-AGCAACTG TCGCCACCAGAA-3′; and human Sirt1 forward, 5′-TG CTGGCCTAATAGAGTGGCAAAG-3′, reverse, 5′-TCTGGCAT GTCCCACTATCACTGT-3′

SA-β-galactosidase staining

To observe the senescent cells, WI-38 cells were treated with 150 μM H_2O_2 for 4 days to induce oxidative stress-induced premature cellular senescence. Then, senescence-associated (SA)-β-galactosidase (gal) staining was performed by utilising a cellular senescence cell histochemical stain kit (Sigma, CS0030, St. Louis, MO, USA) according to the manufacturer's protocol.

ROS measurement

For measurement of ROS by confocal microscopy, WI-38 cells were treated with 10 μM Ex527 for 1 h and then with 20 μM CORM-A1 for 2 h followed by exposure to 150 μM H_2O_2 for 12 h. Next, the cells were stained with 10 μM CM-H_2DCFDA (Invitrogen, CA, SUA) for 1 h followed by PBS washing. Images were captured using an Olympus FV1200 confocal microscope (Olympus, Tokyo, Japan). The fluorescent intensity per group was analysed with the ImageJ2x software (US National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

For statistical comparisons, all values were expressed as mean ± SD. Statistical differences between all experimental groups were determined by one-way ANOVA with Tukey's post hoc tests, and all statistical analyses were performed with GraphPad Prism software version 5.03 (San Diego, CA). Probability values of $p \leq .05$ were considered statistically significant differences among the groups.

Results

CO delays aging in C. elegans and oxidative stress-induced cellular senescence in WI-38 cells

Although CO has considerable potential to attenuate aging-related diseases in vivo [22–24], the effects and molecular mechanisms, by which CO confers benefits on aging and cellular senescence, have not been experimentally dissected. To determine the effect of CO on aging, we first assessed the longevity impact of CORM-A1 on *C. elegans*. *C*aenorhabditis *elegans* raised on 125 and 250 μM CORM-A1 exhibited a prolonged median lifespan (Supplementary material Table S1) and a significantly right-shifted survival curve ($p < .05$ by the Log-rank test) (Figure 1(A)). However, there were no obvious differences in the lifespan between *C. elegans* raised on 125 and 250 μM CORM-A1.

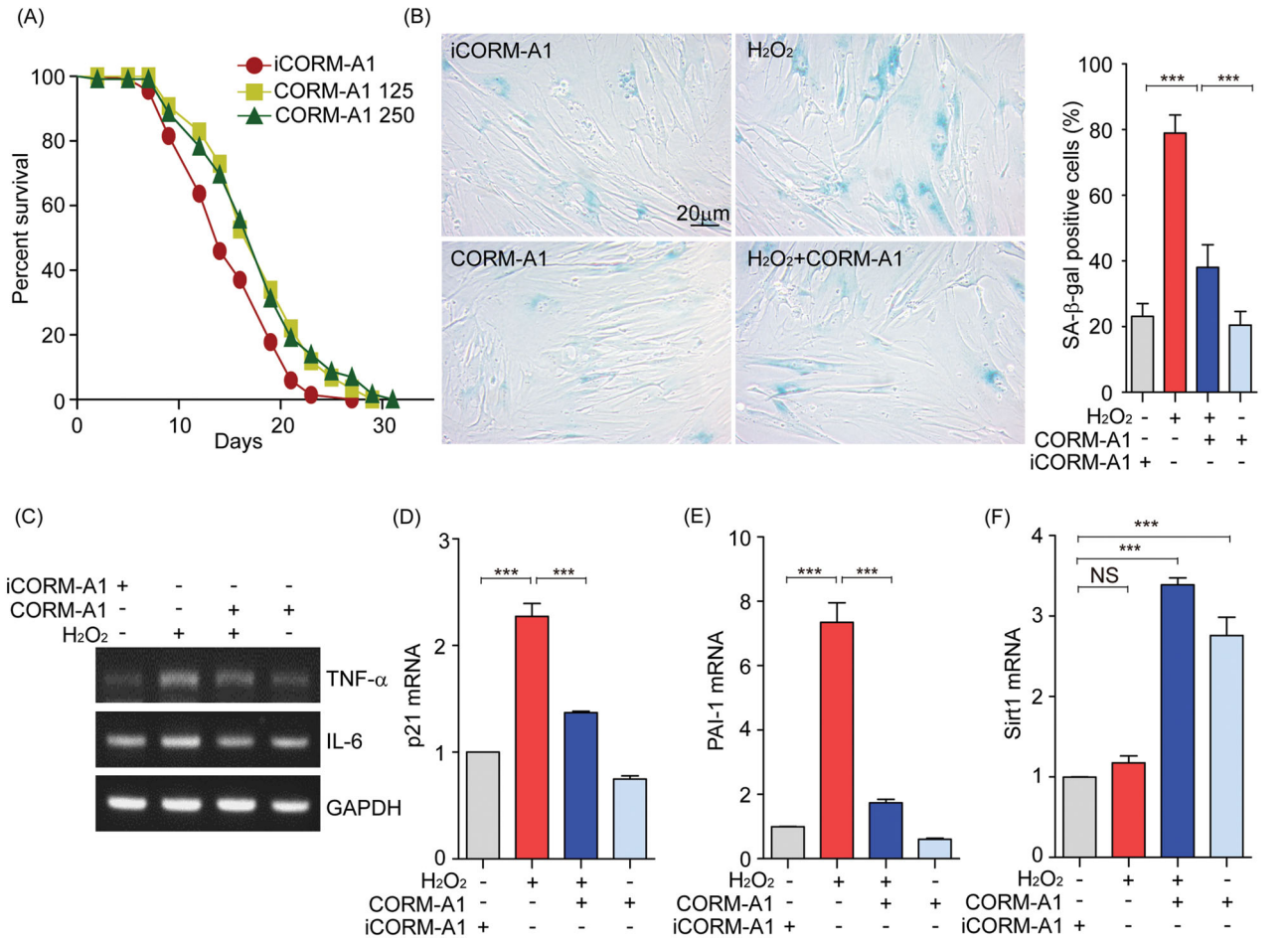

Figure 1. CO delays aging in *C. elegans* and oxidative stress-induced cellular senescence in WI-38 cells. (A) Survival curves of *C. elegans* raised on 0, 125, and 250 μM CORM-A1 plates at 20 °C. Survival curves are significantly shifted to the right in *C. elegans* raised on 125 and 250 μM CORM-A1 (p=.0179 and p=.0115, respectively). (B) To evaluate the beneficial effect of CO on oxidative stress-induced senescence, WI-38 cells were pre-treated with CORM-A1 (20 μM) for 2 h followed by H_2O_2 stimulation (150 μM) for another 24 h. After 24 h, cells were changed to fresh medium and then incubated for 4 days. The senescent cells were measured by SA-β-Gal staining (left panel). Quantitative analysis of the senescent cell number from three biological replicates. For each replicate, at least 10 random microscopic fields were counted (right panel). Scale bar = 20 μm. (C) The mRNA levels of TNF-α and IL-6 were analysed by RT-PCR. GAPDH was used as an internal control. (D–F) The expression of p21, PAI-1, and Sirt1 was assessed by RT-qPCR. Data were expressed as mean ± SD; ***p<.001.

Cellular senescence affects integrative aging hallmarks [39]. Furthermore, some senescent cells persist in tissues and can induce aging and pathogenesis of aging-associated diseases [11–13]. Even though HO-1 reportedly attenuates cellular senescence in fibroblasts and cardiomyocytes, it is unclear whether CO contributes to this process. To investigate the effect of CO on oxidative stress-induced senescent cells, we evaluated SA-β-Gal activity, which is a marker of senescent cells. In line with previous studies [4,5], exposure of WI-38 cells to H_2O_2 for 4 days significantly increased the number of SA-β-Gal-positive cells compared to the control group (Figure 1(B)). However, treatment with CORM-A1 markedly reversed the number of H_2O_2-induced SA-β-Gal-positive cells (Figure 1(B)). Senescent cells secrete various SASP, including proinflammatory cytokines, chemokines, and growth factors. Proinflammatory SASP reportedly promotes the pathogenesis of aging-related diseases [6,13,38]. To determine the suppressive effects of CO on SASP expression in oxidative stress-induced senescence, we analysed the levels of senescence-related molecules such as TNF-α IL-6, p21, and PAI-1 in oxidative stress-induced senescent cells. CORM-A1 significantly reduced the expression of TNF-α IL-6 (Figure 1(C)), p21 (Figure 1(D)), and PAI-1 (Figure 1(E)) in H_2O_2-induced senescent cells. According to previous reports [19,40], CO might involve in a positive feed forward loop in which CO induces HO-1 expression, which produces more CO, which induces further HO-1 expression. Thus, to investigate whether the endogenously produced CO implicate in attenuation of cellular senescence, we treated with zinc protoporphyrin (ZnPP), an HO-1 inhibitor in H_2O_2-induced senescent cells. As shown in Supplementary

material Figure S1, ZnPP treatment reversed CO-reduced p21, PAI-1, and SA-β-Gal-positive cells in H_2O_2-induced senescent cells. Therefore, we suggest that the endogenous CO induced by HO-1 leads to a synergistic effect on the CO-induced attenuation of cellular senescence.

Sirt1 attenuates the aging of *C. elegans* and mice, cellular senescence and its related diseases [25–32]. We previously demonstrated that CO increases Sirt1 expression [33]. To investigate whether CO upregulates Sirt1 in cellular senescence, we analysed the levels of Sirt1 in CORM-A1-treated senescent cells. Sirt1 expression in CORM-A1-treated cells was dramatically increased compared to nontreated cells (Figure 1(F)). Taken together, these data suggest that the antiaging and antisenescence effects of CO are potentially associated with Sirt1 induction.

CO attenuates cellular senescence through the miR-34a-Sirt1 pathway

The expression of the miR-34 family, an upstream regulator of Sirt1, was transcriptionally induced by p53 activation [41,42]. Up-regulation of miR-34a leads to p53-mediated apoptosis or senescence [43]. In our previous study, CO inhibited miR-34a, resulting in the activation of Sirt1. To determine whether CO can reverse oxidative stress-induced senescence by miR-34a inhibition, we first assessed the effect of CORM-A1 on Sirt1 and miR-34a expression in WI-38 cells. CORM-A1 treatment significantly increased both mRNA and protein of Sirt1 levels in a dose-dependent manner (Figure 2(A,B)). Furthermore, CORM-A1 strongly decreased the expression of miR-34a (Figure 2(C)).

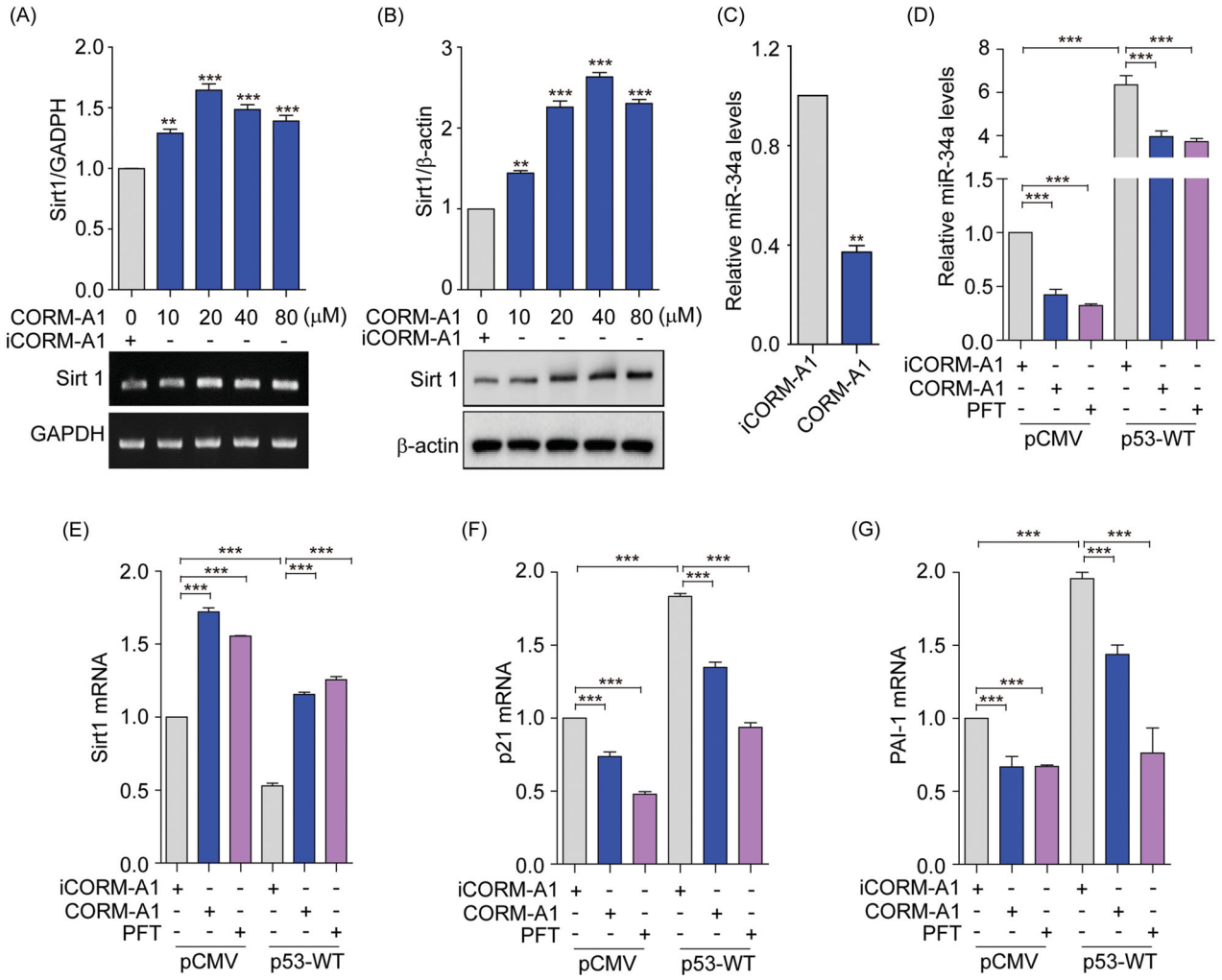

Figure 2. CO attenuates cellular senescence through p53-dependent miR-34a inhibition. (A and B) To determine the effect of CORM-A1 on Sirt1 induction, cells were treated with CORM-A1 (0, 10, 20, 40, and 80 μM) for 6 h. Sirt1 mRNA and protein levels were measured by RT-PCR and western blotting, respectively. (C) To detect the miR-34a reduction by CORM-A1, WI-38 cells were treated with iCORM-A1 or CORM-A1 (20 μM) for 6 h. The miR-34a expression was measured by RT-qPCR. To evaluate the effect of CORM-A1 on miR-34a-related senescent cells, (D–G) WI-38 cells were transfected with p53 WT for 36 h and then treated with iCORM-A1 or CORM-A1 (50 μM) and PFT (10 μM) for another 6 h. The levels of miR-34a (D), Sirt1 (E), p21 (G), and PAI-1 (F) were measured by RT-qPCR. Data were expressed as mean ± SD; $^{**}p<.01$, $^{***}p<.001$.

Next, we determined whether CO could decrease the expression of miR-34a and SASP in cells overexpressing with p53. Consistent with our previous study [34], CORM-A1 or the p53 inhibitor pifithrin-α (PFT) reduced miR-34a levels, contrarily, p53 overexpression significantly induced miR-34a expression (Figure 2(D)). Congruent with the inhibition of miR-34a by CORM-A1, PFT or CORM-A1 increased Sirt1 expression, whereas p53 overexpression decreased Sirt1 levels (Figure 2(E)). Furthermore, p21 and PAI-1 levels were enhanced in p53-overexpressing cells (Figure 2(F,G)).

To confirm that the effect of CO on cellular senescence is mediated through the miR-34a-Sirt1 pathway, we used miR-34a-overexpressing WI-38 cells. CORM-A1 significantly decreased the number of miR-34a-overexpressing SA-β-Gal-positive cells (Figure 3(A)). Cells transfected with a miR-34a mimic significantly exhibited the increase of miR-34a levels compared with cells transfected with a mimic control (Figure 3(B)). As shown in Figure 3(C), CORM-A1 decreased miR-34a levels in a dose-dependent manner, and this effect was mimicked by PFT. Notably, p21 and PAI-1 were upregulated by miR-34a overexpression, whereas CORM-A1 significantly reduced miR-34a-induced p21 and PAI-1 levels (Figure 3(D,E)). Furthermore, the miR-34a-induced Sirt1 downregulation was reversed by CORM-A1 (Figure 3(F)). These data indicated that CO attenuates cellular senescence through the miR-34a-Sirt1 pathway.

CO ameliorates oxidative stress and H_2O_2-induced cellular senescence via Sirt1 activation in WI-38 cells

Sirt1 exhibits an antioxidant effect through Nrf2 and p53 deacetylation [35–37]. Furthermore, antioxidant activity plays an important role in the attenuation of cellular senescence [44,45]. To determine whether the antioxidant and antisenescent effects of CO were mediated by Sirt1, we treated oxidative stress-induced senescent WI-38 cells with the Sirt1 inhibitor Ex527. Administration of CORM-A1 to H_2O_2-induced senescent cells increased the expression of antioxidant genes such as HO-1 and NQO1 (Figure 4(A,B)). Contrarily,

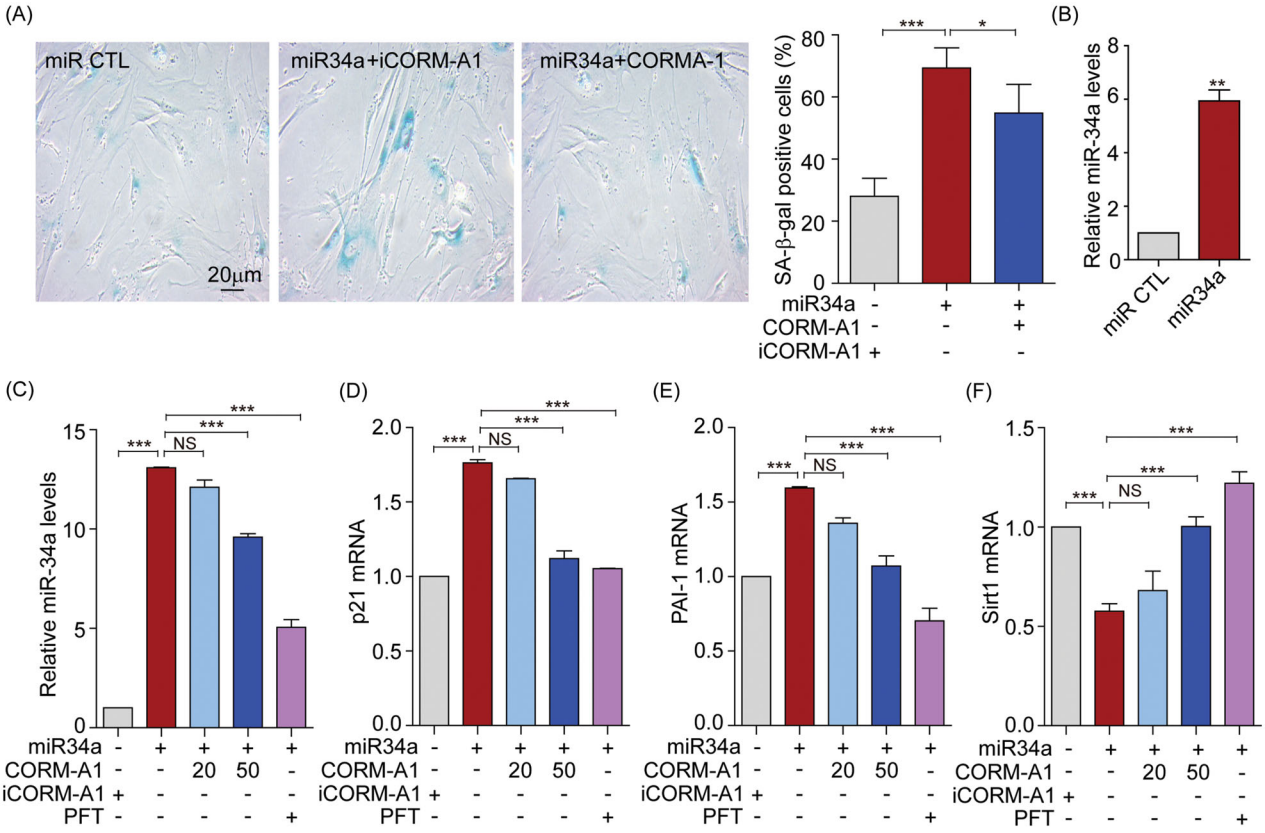

Figure 3. CO ameliorates cellular senescence through the miR-34a-Sirt1 pathway. WI-38 cells were transfected with a miR-34a mimic for 36 h and then treated with iCORM-A1 or CORM-A1 (20 μM) for another 6 h. (A) Senescent cells analysed by SA-β-Gal staining (left panel). Quantitative analysis of the senescent cell number from three biological replicates. For each replicate, at least 10 random microscopic fields were counted (right panel). Scale bar = 20 μm. (B) After transfection of WI-38 cells with control miRNA (miR CTL) or mimic miR-34a (miR-34a) for 36 h, the levels of miR-34a were verified by RT-qPCR. (C–F) WI-38 cells were transfected with a miR-34a mimic for 36 h and then treated with iCORM-A1 or CORM-A1 (20 and 50 μM) and PFT (10 μM). The expression of miR-34a (C), p21 (D), PAI-1 (E), and Sirt1 (F) was analysed by RT-qPCR. Data were expressed as mean ± SD; $^{*}p<.05$, $^{**}p<.01$, $^{***}p<.001$, and NS: not significant.

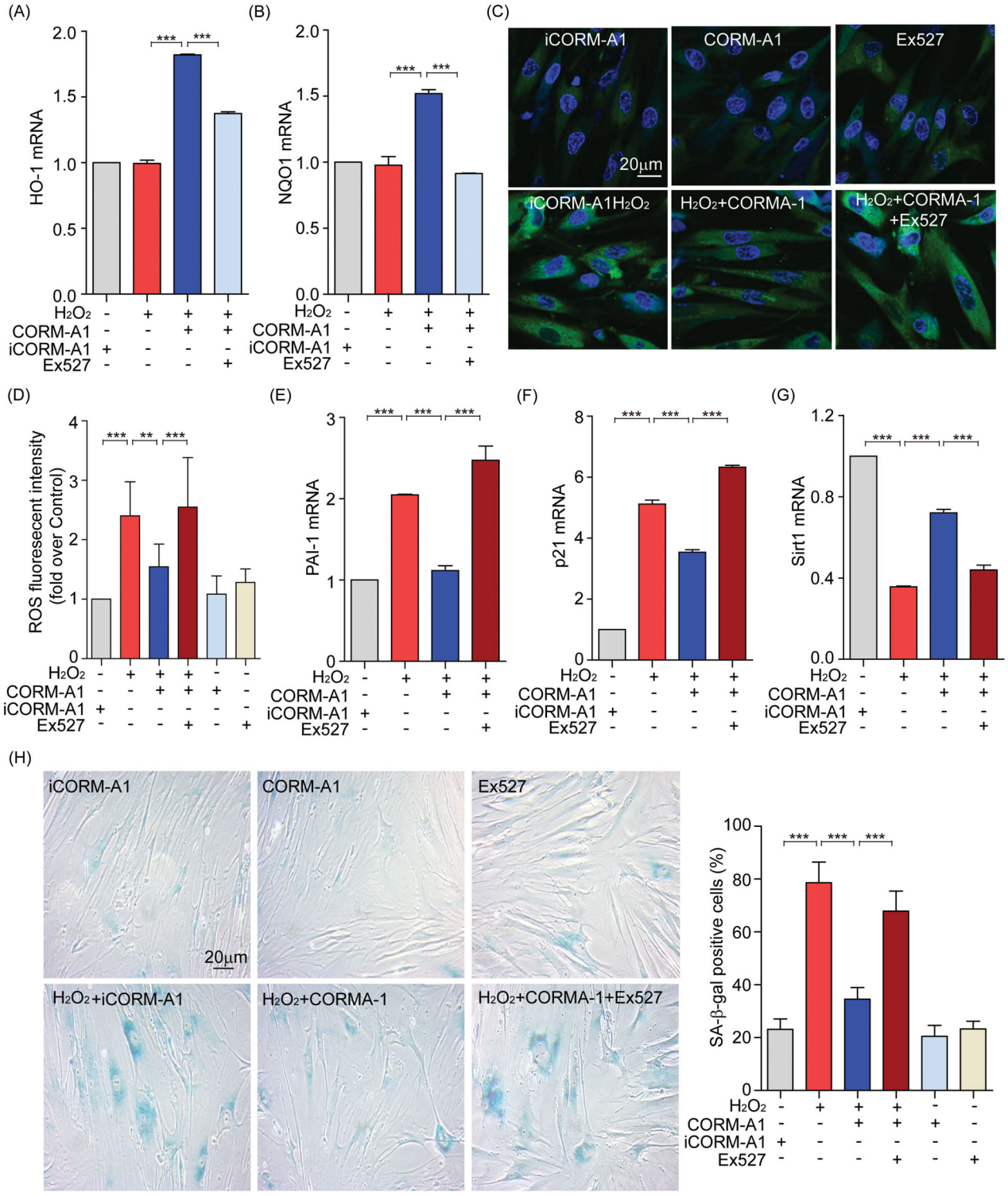

Figure 4. CO inhibits oxidative stress and H_2O_2-induced cellular senescence *via* Sirt1 activation in WI-38 cells. WI-38 cells were pre-treated with Ex527 (10 μM) and iCORM-A1 or CORM-A1 (20 μM) for 1 or 2 h, respectively. Then, WI-38 cells were stimulated with H_2O_2 (150 μM) for 12 h and subsequently were changed to fresh medium. (A and B) The mRNA levels of HO-1 and NQO-1 were measured by RT-qPCR. (C) The production of ROS was analysed by DCF-DA staining using confocal microscopy. (D) The fluorescent intensity was quantified utilising ImageJ software. (E–G) The mRNA levels of PAI-1, p21 and Sirt1 were measured by RT-qPCR. (H) Senescent cells analysed by SA-β-Gal staining (left panel). Quantitative analysis of the senescent cell number from three biological replicates. For each replicate, at least 10 random microscopic fields were counted (right panel). Scale bar = 20 μm. Data were expressed as mean ± SD; ***$p<.01$, ***$p<.001$.

Ex527 treatment inhibited the CORM-A1-induced HO-1 and NQO1 upregulation (Figure 4(A,B)). Furthermore, ROS production in H_2O_2-induced senescent WI-38 cells was reduced by CORM-A1, and the effect was reversed by Ex527 (Figure 4(C,D)). Next, we assessed whether Sirt1 is required for the inhibition of SASP expression by CORM-A1 in H_2O_2-induced senescent WI-38 cells. As expected, Ex527 treatment reversed the decrease of PAI-1 and p21 in H_2O_2-induced senescent WI-38 cells treated with CORM-A1 (Figure 4(E,F)); furthermore, it abolished Sirt1 up-regulation (Figure 4(G)). Moreover, we confirmed that Sirt1 was required for the inhibitory effect of CORM-A1 on H_2O_2-induced cellular senescence using SA-β-Gal staining (Figure 4(H)). These data suggest that Sirt1 plays a critical role in the regulation of antioxidant activity and oxidative stress-induced cellular senescence.

CO attenuates high-fat diet-induced hepatocyte senescence through the increase of antioxidant genes and Sirt1 expression

Since hepatic fat accumulation drives hepatocyte senescence, the elimination of senescent cells may ameliorate liver steatosis [12]. Therefore, the inhibition of hepatocyte senescence has emerged as a new therapeutic target for non-alcoholic fatty liver disease. Here, we used HFD-fed mice to study the regulation of hepatocyte senescence by CO in vivo. HFD significantly increased the expression of p21, TNF-α and IL-6 in the liver (Figure 5(A–C)). Additionally, CO inhalation decreased the levels of p21, TNF-α and IL-6 upregulated by HFD (Figure 5(A–C)). Antioxidant activity also plays an important role against HFD-induced metabolic problems [46,47]. Thus, we assessed the effect of CO on the

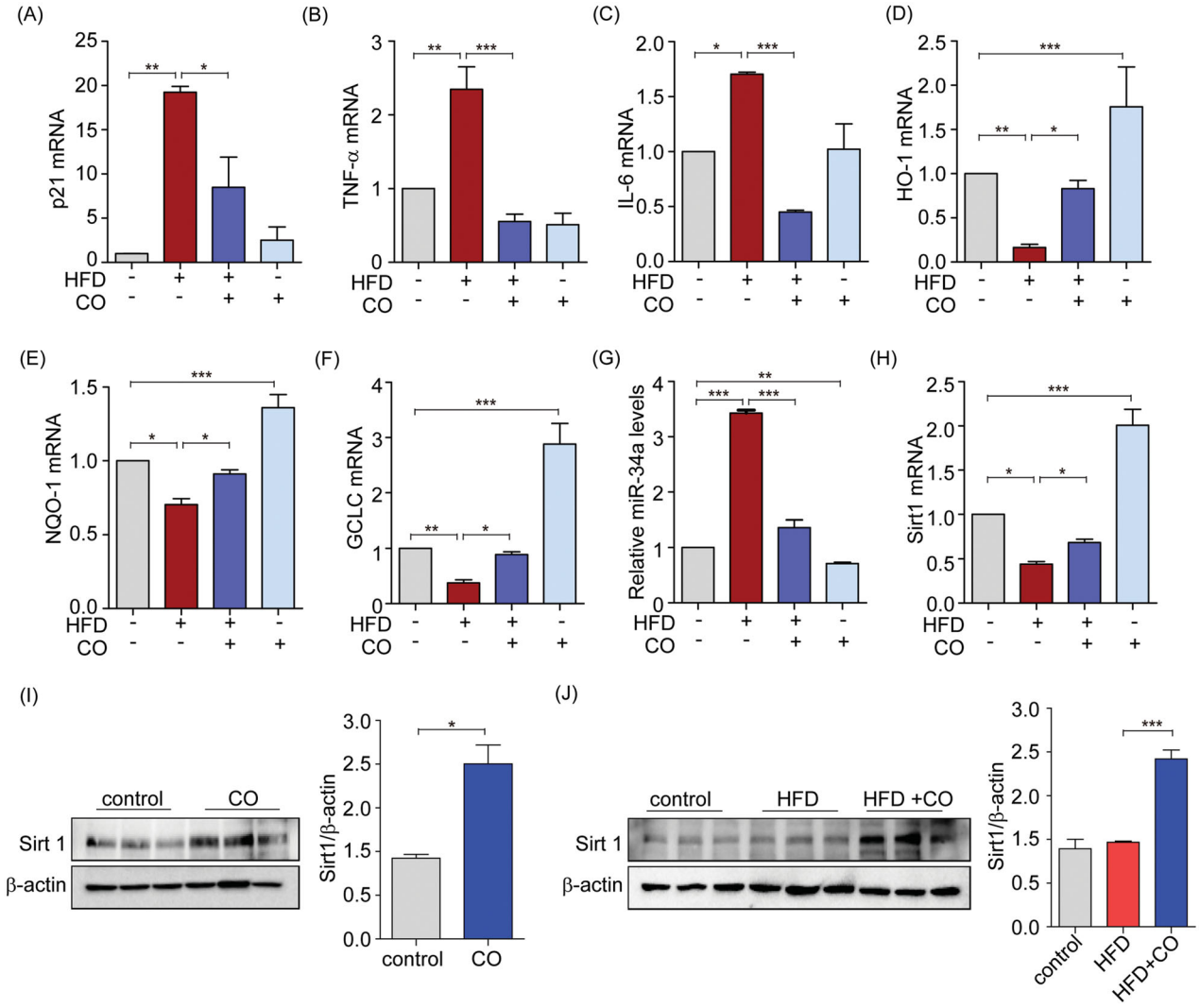

Figure 5. CO attenuates high fat diet-induced hepatocyte senescence through the increase of antioxidant genes and Sirt1. Six-week-old mice were fed an NCD or HFD for 16 weeks. After 8 weeks, mice were inhaled with CO (250 ppm) for 2 h daily for 8 weeks. (A–H) The mRNA levels of SASP (p21 (A), TNF-α (B) and IL-6 (C)); antioxidant genes (HO-1 (D), NQO1 (E), and GCLC (F)); miR-34a (G); and Sirt1 (H) in liver tissues were measured by RT-qPCR. (I and J) The protein levels of Sirt1 in liver tissues were analysed by Western blotting. Data were expressed as mean ± SD; $^{*}p<.05$, $^{**}p<.01$, $^{***}p<.001$.

levels of antioxidant genes such as HO-1, NQO1, and GCLC in liver. As expected, CO-inhaled mice showed a significant increase of HO-1, NQO1, and GCLC (Figure 5(D–F)). In HFD-fed mice, expression of HO-1, NQO1, and GCLC was decreased; however, CO inhalation led to a marked reversal in the expression pattern of these genes (Figure 5(D–F)). In line with a previous investigation [48], HFD feeding upregulated miR-34a in the liver, and CO suppressed HFD-induced miR-34a (Figure 5(G)). Consistent with our previous study [34], CO inhalation alone markedly increased both mRNA and protein of Sirt1 levels in liver tissues (Figure 5(H–J)), and CO reversed the expression of Sirt1 after HFD administration (Figure 5(H,I)). These data strongly indicated that CO attenuated HFD-induced hepatocyte senescence, which was associated with the upregulation of Sirt1 and antioxidant genes.

Discussion

CO is a byproduct by HO-1, an inducible stress response protein involved in haem degradation [16]. CO and CORMs exert protective effects on antioxidation, anti-inflammation, and antiapoptosis [17–19]. Furthermore, CO provides a therapeutic benefit in aging-related diseases, such as cardiovascular disease, hypertension, hepatic steatosis and type 2 diabetes [22–24]. Moreover, the HO-1 inducer haemin attenuates chronic obstructive pulmonary disease (COPD)-induced lung fibroblast senescence and myocardial infarction-induced cardiomyocyte senescence [25,26]. However, the protective effects of CO on cellular senescence and aging remain undefined. Our study demonstrated the protective activity of CO in cellular senescence and aging and the involvement of Sirt1 in its mechanism of action.

Aging is a universal feature of biological organisms, which undergo a gradual functional decline [39]. Cell cycle arrest is a cell senescence characteristic, which is caused by p53-induced p21 expression, CDK2 inhibition, and increases in SASP secretion, cell size, and number of SA-β-Gal-positive cells [6–8]. Notably, oxidative stress contributes to the induction of cellular senescence in mesenchymal stem cells and reduction of the lifespan in haematopoietic cells [2–5]. Furthermore, cellular senescence plays an important role in the pathogenesis of aging-related diseases [11–13]. Our results showed that CO prolonged the *C. elegans* lifespan and decreased the expression of senescence markers in HFD-induced aging liver and the number of SA-β-Gal-positive cells in H_2O_2 -induced senescent WI-38 cells.

Senescent cells promote immune responses because of their clearance and secretion of various SASP, such as proinflammatory cytokines and growth factors [13]. SASP secreted by persistently present senescent cells induce several diseases including cancer, hepatic steatosis, and gastrointestinal diseases [11–13]. Thus, SASP inhibition is critical for protection from aging-related diseases. In our present study, CO decreased SASP such as TNF-α IL-6, and PAI-1 in oxidative stress-induced cellular senescence and HFD-induced liver aging. Therefore, we suggest that CO attenuated aging-related diseases.

Sirt1 is increased by miR-34a repression, which can be induced by p53 activation [41,42]. Both Sirt1 inhibition and miR-34a induction reportedly affect cellular senescence [30–33,43]. Also, Sirt1 extends the lifespan, delays aging, and protects against aging-related diseases. We showed that the inhibitory effects of CO on oxidative stress-induced cellular senescence and HFD-induced aging liver are mediated by the restoration of Sirt1 expression and reduction of miR-34a levels. Moreover, both overexpression of p53 and miR-34a

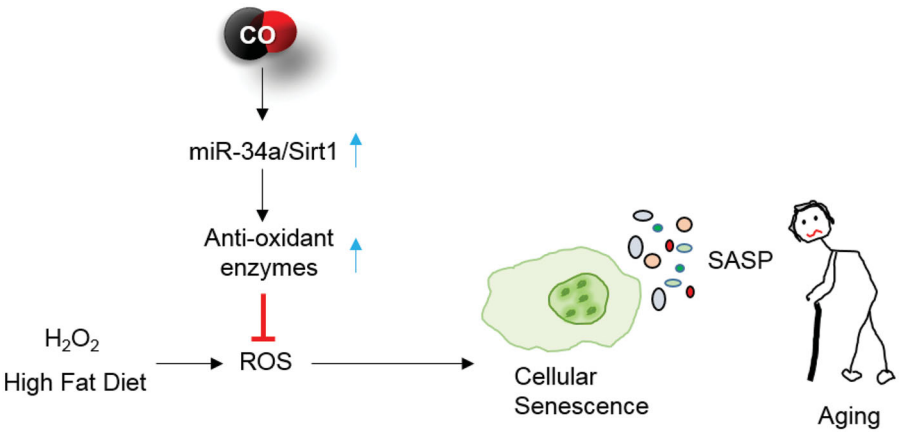

Figure 6. The scheme for the inhibitory effects of CO on H_2O_2-induced cellular senescence and HFD-induced aging, respectively via miR34a/Sirt1 pathway.

induced cellular senescence, which was attenuated by CO. Furthermore, since treatment with a Sirt1 inhibitor reversed the CO-induced attenuation of cellular senescence, we propose that Sirt1 plays a critical role in the antioxidant effect of CO on oxidative stress-induced senescence.

Antioxidant chemicals exert protective activities against oxidative stress-induced endothelial and mesenchymal stem cell senescence [44,45]. Moreover, Sirt1 contributes to the antioxidant activity through Nrf2 and p53 deacetylation [35–37]. Our present study showed that CO increased the expression of antioxidant genes and decreased the production of ROS in oxidative stress-induced senescence, and this effect was reversed by a Sirt1 inhibitor (see schema in Figure 6).

Finally, our results indicated that CO-induced miR-34a inhibition enhanced Sirt1 expression, which contributed to antioxidant and antisenescent activity.

Disclosure statement

All authors have disclosed that they have no conflicts of interest.

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Data availability

All relevant data are presented within the paper and its supplementary Information files.

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