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Carbon monoxide protects against hepatic ischemia/reperfusion injury by modulating the miR-34a/SIRT1 pathway

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ABSTRACT

Hepatic ischemia/reperfusion (I/R) injury can arise as a complication of liver surgery and transplantation. Sirtuin 1 (SIRT1), an NAD^+-dependent deacetylase, modulates inflammation and apoptosis in response to oxidative stress. SIRT1, which is regulated by p53 and microRNA-34a (miR-34a), can modulate non-alcoholic fatty liver disease, fibrosis and cirrhosis. Since carbon monoxide (CO) inhalation can protect against hepatic I/R, we hypothesized that CO could ameliorate hepatic I/R injury by regulating the miR-34a/SIRT1 pathway. Livers from mice pretreated with CO, or PFT, a p53 inhibitor, displayed reduced production of pro-inflammatory mediators, including TNF-α, iNOS, interleukin (IL)-6, and IL-1β after hepatic I/R injury. SIRT1 expression was increased by CO or PFT in the liver after I/R, whereas acetylated p65, p53 levels, and miR-34a expression were decreased. CO increased SIRT1 expression by inhibiting miR-34a. Both CO and PFT diminished pro-inflammatory cytokines production *in vitro*. Knockdown of SIRT1 in LPS-stimulated macrophages increased NF-κB acetylation, and increased pro-inflammatory cytokines. CO treatment reduced miR-34a expression and increased SIRT1 expression in oxidant-challenged hepatocytes; and rescued SIRT1 expression in p53-expressing or miR-34a transfected cells. In response to CO, enhanced SIRT1 expression mediated by miR-34a inhibition protects against liver damage through p65/p53 deacetylation, which may mediate inflammatory responses and hepatocellular apoptosis. The miR-34a/SIRT1 pathway may represent a therapeutic target for hepatic injury.

1. Introduction

Hepatic ischemia–reperfusion (I/R) injury is a major cause of liver dysfunction that occurs as the result of resuscitation from hemorrhagic shock or surgical procedures such as liver transplantation and liver resection. The initial hepatic injury during I/R results from increased reactive oxygen species (ROS) production, which can promote apoptosis and acute inflammatory responses. Inflammation during I/R can lead to endothelial and Kupffer cell activation and cytokine/chemokine release associated with increased hepatocyte cell death [1,2]. During I/R injury, activation of Toll-like receptor 4 (TLR4)-dependent innate immune mechanisms can promote liver damage through the increase of pro-inflammatory cytokines, and can lead to chronic inflammation and disease [3].

Carbon monoxide (CO), a reaction product of heme oxygenase (HO) activity, can exert potent anti-inflammatory, anti-proliferative, and anti-apoptotic effects *in vitro* and *in vivo* and thereby mimic the cytoprotective effect of HO-1 [4–6]. Tissue protective effects have been described in organ transplantation models after perfusion with exogenous CO, resulting in graft preservation [6–9]. Furthermore, exogenously applied CO can ameliorate I/R injury in animal models through anti-inflammatory, vasodilatory, and antiapoptotic effects [10–14]. However, the molecular mechanisms underlying the protective effect of CO in I/R injury have not been well elucidated.

Sirtuin 1 (SIRT1) is an NAD^+-dependent deacetylase implicated in diverse cellular processes, including metabolism, development, stress response, neurogenesis, inflammation and apoptosis [15–17]. SIRT1 can act as a robust metabolic protector in several systems, including the liver, brain and immune system [18]. Recent studies indicate that SIRT1 can downregulate inflammatory responses in various models [19–23]. For example, SIRT1 was linked to protection from diabetes and steatosis through reduction of the inflammatory response,

Abbreviations: ALT, alanine aminotransferase; BAX, BCL_2-associated X protein; CO, carbon monoxide; CORMs, carbon monoxide releasing molecules; I/R, ischemia/reperfusion; miRNA or miR, microRNA; MPO, myeloperoxidase; PFT, pifithrin-α *p*-nitro; SIRT1, sirtuin 1

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associated with downregulation of nuclear factor-κB (NF-κB) [19–22]. SIRT1 can protect the heart and brain from I/R injury, and ameliorate inflammation and apoptosis in these models through NF-κB/p65 and p53 deacetylation [24,25]. SIRT1 expression in the liver is specifically regulated by microRNA-34a (miR-34a) [26,27]. Targeting of the miR-34a/SIRT1/p53 pro-apoptotic pathway increased SIRT1 expression, decreased acetylation of p53, reduced apoptosis in primary hepatocytes, and also reduced steatosis *in vivo* [28,29].

On the basis of these observations, we hypothesized that induction of SIRT1 by CO-mediated miR-34a inhibition could play a role in ameliorating hepatic I/R injury. To test this hypothesis, we used CO inhalation or application of pifithrin-α *p*-nitro, a p53 inhibitor, as therapeutic interventions for experimental hepatic I/R injury in mice. We demonstrate that induction of SIRT1 by CO-dependent miR-34a inhibition decreases I/R-induced inflammatory responses and apoptotic hepatic injury *via* SIRT1 deacetylase activity acting on NF-κB/p65 and p53. Thus, we identify a novel mechanism for CO action, as well as a therapeutic target for hepatic I/R injury.

2. Materials and methods

2.1. Chemicals and reagents

Tricarbonyl dichlororuthenium (II) dimer (CORM-2), pifithrin-α *p*-nitro, hydrogen peroxide (H_2O_2), EX527 as a SIRT1 inhibitor, and lipopolysaccharide (LPS) (055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Male C57BL/6 wild-type (WT) mice were used at 6–8 weeks of age (Orient Bio, Seoul, South Korea). Animals were maintained in a specific pathogen-free facility. Animal studies were approved by the University of Ulsan Animal Care and Use Committee.

2.3. Cell culture

The murine macrophage cell line, RAW 264.7 was maintained in DMEM. AML12 mouse hepatocytes (ATCC; CRL-2254) were maintained in DMEM/F12. All media were supplemented with 10% fetal bovine serum (FBS) and a 100 units/ml penicillin/streptomycin mixture (Gibco, Grand Island, NY). Cell lines were incubated at 37 °C with 100% humidity in 5% CO_2 and passaged using standard cell culture techniques.

2.4. Isolation of primary hepatocytes and Kupffer cells (KCs)

Primary hepatocytes and Kupffer cells were isolated from mice by collagenase digestion and by the two-step Percoll gradient method with slight modifications. Mice were anesthetized, and the peritoneal cavity was opened. Livers were perfused with Ca^{2+} and Mg^{2+}-free Hank's buffered salt solution (HBSS) (Gibco) containing EGTA (2.5 mM) and then digested with a collagenase buffer containing collagenase (0.5 mg/ml, C5138, Sigma), NaCl (66.7 mM), KCl (6.7 mM), HEPES (50 mM), and $CaCl_2$ (4.8 mM). Digested livers were dissected and then gently teased with forceps until they were in solution. The cell suspensions were filtered through a 100-μm nylon cell strainer (BD Falcon). The cells were centrifuged for 5 min at 1900 rpm and resuspended with HBSS. After the cell suspensions were centrifuged for 3 min at 450 rpm, the supernatants (non-parenchymal cells such as Kupffer cells) were transferred to a new tube, and the pellets (parenchymal cells such as hepatocytes) were resuspended with HBSS.

For Kupffer cell isolation, the supernatants were layered onto a 50/25% two-step Percoll gradient (GE Healthcare, Uppsala, Sweden) for 15 min at 3200 rpm with the brake option off. KCs in the middle layer were collected and washed with DMEM supplemented with 10% FBS. One hour following seeding, non-adherent cells (cell debris and blood cells) were removed by replacing the culture medium. The purity of KCs was confirmed by flow cytometry using PE-conjugated anti-F4/80 Antibody (BM8) (eBiosciences, San Diego, CA, #12-4801-82). Flow cytometric analysis was performed using a FACSCanto II (BD) cytometer, and data were analyzed using FACSDiva software (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

For hepatocyte isolation, the pellet suspensions obtained as described above were centrifuged using 25% Percoll for 5 min at 800 rpm with the brake option off. The pellets were washed with DMEM supplemented with 10% FBS, and then cells were seeded into a collagen pre-coated 100 mm tissue culture plates. After 24 h, non-adherent cells were removed by aspiration, and fresh media were added.

2.5. Carbon monoxide treatment

To evaluate the protective effect of inhaled CO, mice inhaled CO at 250 parts per million (ppm) in air (Core Gas, Ulsan, South Korea) for 12 h in a sealed exposure chamber (LB Science, Daejeon, South Korea) prior to the experiment. Mice were placed in an exposure chamber (LB Science) at room temperature for exposure to air (control) or to 250 ppm CO as monitored by a CO probe (Tongoy Control Technology, Beijing, China). During reperfusion, mice were exposed to CO (250 ppm) for 6 h.

2.6. Mouse liver I/R injury model

We used a well-established mouse model of warm hepatic ischemia followed by reperfusion [30]. An atraumatic clip was used to interrupt the arterial/portal venous blood supply to the cephalad liver lobes. After 60 min the clip was removed and mice were sacrificed after reperfusion. Sham wild-type (WT) controls underwent the same procedure, but without vascular occlusion. Mice were exposed to compressed air or CO. CO or room air was given to the mouse overnight prior to the liver ischemia and during reperfusion. In some experiments, mice were treated with a p53 inhibitor, pifithrin-α *p*-nitro (2.2 mg/kg, [29]) or the vehicle DMSO, three times for 1 week prior to ischemia.

2.7. Hepatocellular damage assay

To detect serum alanine aminotransferase (ALT), serum was collected from peripheral blood. ALT activity, an indicator of hepatocellular injury, was measured using the EnzyChrom™ Alanine Transaminase Assay Kit (BioAssay System, Hayward, CA).

2.8. Liver histology

For histopathological observations, portions of liver were fixed in 10% neutral-buffered formalin solution and then dehydrated in graded alcohol. The fixed tissue was embedded in paraffin and sliced into 4-μm-thick sections. Tissue sections were mounted on regular glass slides, deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). Overall pathological changes, including immune cell infiltration, and hepatic cell necrosis were diagnosed according to previously described methods [31].

2.9. Immunohistochemistry

For the detection of SIRT1, F4/80, and LY6G by immunohistochemistry, SuperPicTure™ Polymer Detection Kit Invitrogen Polymer Detection System (Invitrogen, Frederick, MD, #879663) was used according to the manufacturer's protocols. Briefly, after administrating Peroxoblock solution (Invitrogen, #002015), the sections were incubated first with anti-SIRT1 (Santa Cruz Biotechnology, Santa Cruz, CA, #15404, 1:100 dilution), anti-F4/80 (Abcam, Cambridge, MA, #6640, 1:100

dilution, and anti-Ly6G (Abcam, #25377, 1:100 dilution). After overnight incubation, the sections were washed and then incubated with HRP polymer conjugate. Tissue sections were visualized with the substrate DAB chromogen (diaminobenzidine); and then the sections were then counterstained with hematoxylin.

2.10. Myeloperoxidase (MPO) activity assay

Neutrophil sequestration in liver was quantified by measuring tissue MPO activity. Tissue samples for MPO analysis were frozen in liquid nitrogen immediately after removal from the animal and were thawed and homogenized and centrifuged to remove insoluble materials. MPO levels were measured using a mouse myeloperoxidase DuoSet ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The supernatants were analyzed for MPO levels by ELISA. The levels of MPO in organ extracts were expressed as ng/mg of protein.

2.11. Transfection of plasmids, miRNAs and siRNAs

The p53 responsive element reporter plasmid (p53-luc) was purchased from Stratagene (La Jolla, CA). A pRL-SV40 *Renilla* luciferase construct was purchased from Promega (Madison, WI). pCMV-p53WT (containing human wild-type p53) was purchased from Clontech (Mountain View, CA) (631922). WT-SIRT1 3′UTR constructs were from Addgene (Cambridge, MA) (Addgene plasmid 20379). Small interfering RNAs (siRNAs) against SIRT1 (SIRT1-siRNA) (sc-40987) and control siRNA (scRNA) (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MicroRNAs (miRNAs) against miR34a (miRIDIAN microRNA Mouse mmu-miR-34a-5p-mimic, C-310529-07 and meridian microRNA human has-miR-34a-5p-mimic, C-300551-07) and control miRNA (meridian microRNA mimic Negative Control, CN-001000-01) were purchased from Thermo Pierce Biotechnology Scientific. Cells were transfected with plasmids, siRNAs, and miRNAs using Lipofectamine 2000 reagent (Life Technologies, New York, USA) and Hiperfect transfection reagent (QIAGEN, Valencia, CA). After 36 h of transfection, cells were harvested for RT-PCR, immunoblotting, or luciferase assay.

2.12. Luciferase assay

AML12 cells were transiently transfected with the firefly luciferase reporter plasmids and the *Renilla* luciferase-expressing plasmid (pRL-SV40; Promega, Madison, USA), the latter as a transfection control, were transiently transfected into AML12 cells using Lipofectamine 2000 reagent (Life Technologies). After 36 h of transfection, cells were harvested and lysed with 100 μl of cell culture lysis buffer (CLB, Promega). Lysates of the transfected cells were analyzed using the dual luciferase assay kit (Promega) according to the manufacturer's protocol. The luciferase activities were measured on a SpectraMax L (Molecular Devices, Sunnyvale, CA). Firefly luciferase activity of p53-luc and WT-SIRT1 3′UTR were normalized to *Renilla* luciferase in each sample. Each experiment was carried out at least three times with triplicate samples.

2.13. Quantitative RT-PCR analysis

Total RNA was prepared using Trizol reagent (Invitrogen). Three microgram of total RNA was used to synthesize the first-strand cDNA by using oligo-dT primers (QIAGEN) and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. The synthesized cDNA was subjected to the PCR-based amplification. Semi-quantitative RT-PCR was performed using Taq polymerase (Bioneer, Daejeon, South Korea). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Real-time PCR primer pairs were as follows: TNF-α: 5′-AGA CCC TCA CAC TCA GAT CAT CTT C-3′, 5′-TTG CTA CGA CGT GGG CTA CA-3′, IL-6: 5′-CGA TGA TGC ACT TGC AGA AA-3′, 5′-TGG AAA TTG GGG TAG GAA GG-3′; IL-1β: 5′-TCG CTC AGG GTC ACA AGA AA-3′, 5′-ATC AGA GGC AAG GAG GAA ACA C-3′; iNOS: 5′-ACT GTG TGC CTG GAG GTT CT-3′, 5′-TCT CTG CCT ATC CGT CTC GT-3′; CXCL10: 5′-TTT TCT GCC TCA TCC TGC TG-3′, 5′-CCT TTC AGA AGA CCA AGG GC-3′; GAPDH: 5′-GGG AAG CCC ATC ACC ATC T-3′, 5′-CGG CCT CAC CCC ATT TG-3′; SIRT1: 5′-CAG ACCCTC AGG CCA TGT TT-3′, 5′-ACA CAG AGA CGG CTG GAA CT-3′. mRNA expression data were normalized to GAPDH gene expression. The nucleotide sequences of the primers used for semi-quantitative RT-PCR are listed in Table S1.

2.14. MicroRNA analysis

Total RNA was isolated with Trizol reagent (Invitrogen). RNA was reverse transcribed using the miScript Reverse Transcription kit (QIAGEN) according to the manufacturer's instructions. A quantitative PCR was performed using a miScript SYBR-Green PCR Kit (QIAGEN) and miScript Primer assays for miR-34a (MS00001428, QIAGEN), with RNU6B (MS00033740, QIAGEN) used as an internal control, according to the manufacturer's protocol. miRNAs were detected using ABI 7500 Fast Real-Time PCR System (Applied Biosystems) by monitoring in real-time the increase in fluorescence of SYBR Green detection.

2.15. Western blot analysis

Harvested liver tissues and cells were lysed with mammalian lysis buffer containing phosphatase and protease inhibitors. Equal amounts of cell lysates was measured with the BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). Lysates were boiled in sample buffer containing β-mercaptoethanol for 5 min. Proteins were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE healthcare). After blocking with 5% skim milk in PBS, membranes were incubated with appropriate dilutions of antibodies at 4 °C overnight as follows: polyclonal rabbit anti-SIRT1 (Millipore, Temecula, CA, #07-131, 1:3000 dilution), rabbit anti-acetylated p65 (Abcam, #52175, 1:1000 dilution), acetylated p53 (Cell Signaling Technology, Danvers, MA, #2570, 1:1000 dilution, Bcl-2 (Cell Signaling, #2876, 1:1000 dilution), Bcl-X_L (Cell Signaling, #2764, 1:1000 dilution), cleaved caspase-3 (Cell Signaling, #9661, 1:1000 dilution), and Bax (Santa Cruz, #526, 1:1000 dilution) and β-actin (Santa Cruz, #1616, 1:1000 dilution) were used. Membranes were then washed with 0.05% PBS–Tween 20 and incubated with a 1/5000 dilution of HRP-conjugated secondary Abs at room temperature for 1 h. Immunoreactivity was detected using the ECL detection system (GE Healthcare). Films were exposed at multiple time points to ensure that the images were not saturated.

2.16. Statistical analysis

All data were expressed as mean ± SD. Differences between experimental groups were compared using the Student's two-tailed unpaired t-test.

3. Results

3.1. Carbon monoxide protects against liver ischemia/reperfusion injury in mice

CO has been shown to exert protective effects in various tissue models of I/R injury [7,11–14,32]. We therefore analyzed the effect of inhaled CO on hepatocellular function in mice subjected to 60 min of warm ischemia followed by 6 h of reperfusion. As shown in Fig. 1A, serum ALT levels in mice subjected to hepatic I/R were decreased in animals pretreated with CO gas (250 ppm), as compared with room air (2688 ± 1054 vs. 8561 ± 3185 U/L respectively, $P < 0.01$). Likewise,

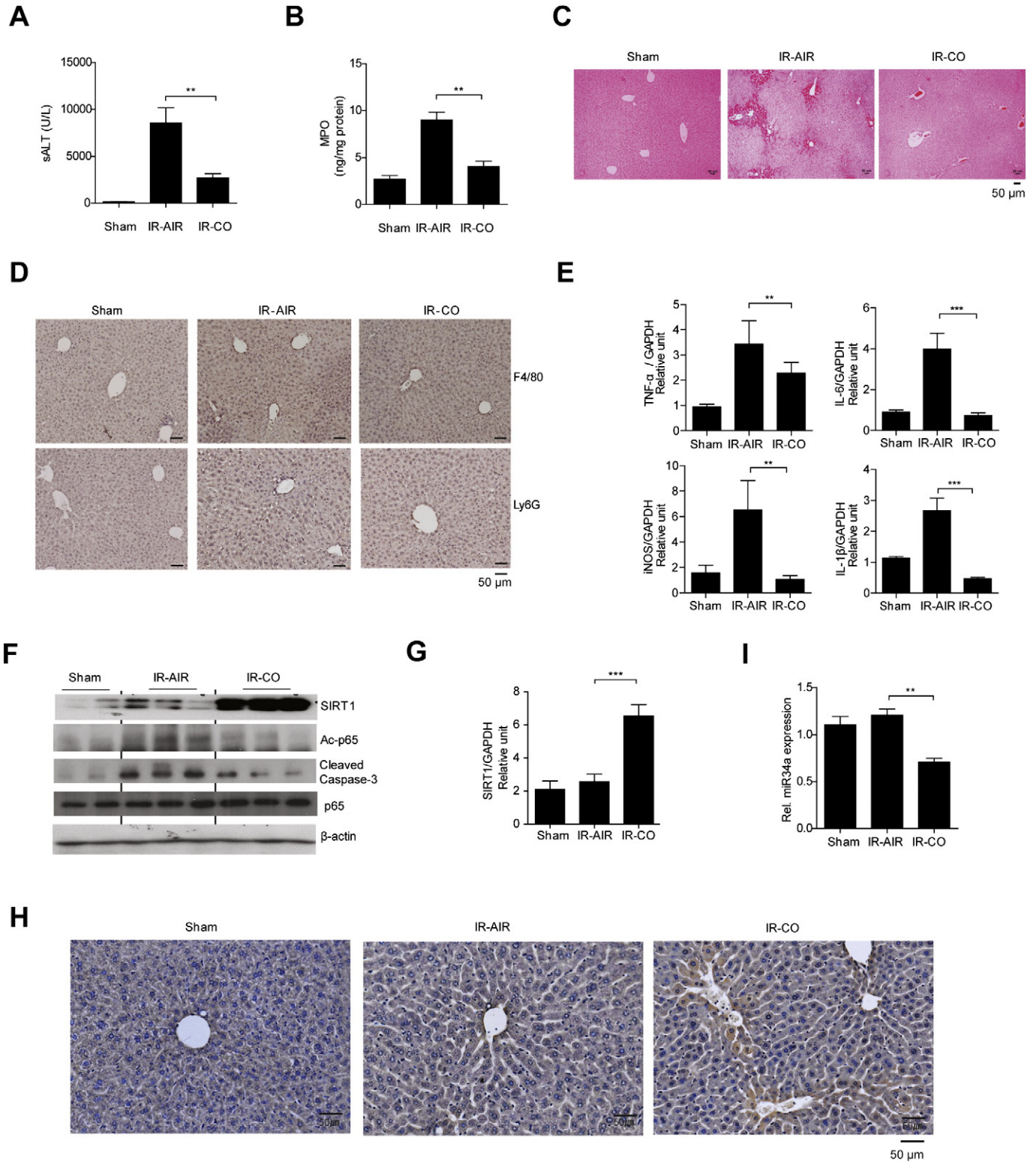

Fig. 1. Carbon monoxide protects against liver ischemia/reperfusion injury through induction of SIRT1 in mice. Mice were sham-operated or subjected to 60 min liver warm ischemia followed by 6 h of reperfusion. Mice were treated with air or CO gas (250 ppm) inhalation. (A) sALT levels (U/L) were measured in blood samples retrieved after I/R. (B) Liver neutrophil accumulation was assessed by MPO level. (C) H&E staining of liver samples from air- and CO-inhaled mice after hepatic I/R or sham treatment. (D) Immunohistochemical staining for F4/80 and Ly6G in the liver tissue was performed. (E) The gene expression of TNF-α, IL-6, iNOS, and IL-1β was evaluated in liver tissue by quantitative RT-PCR. (F) Hepatic expression of SIRT1, acetylated-p65, cleaved-caspase-3 and p65 were evaluated in IR lobes from air- and CO-inhaled mice by immunoblotting. β-Actin was used as an internal control. (G) SIRT1 mRNA expression was as detected by quantitative RT-PCR in liver tissue. (H) Immunohistochemical staining of SIRT1 was performed on liver sections. (I) qRT-PCR analysis of miR-34a expression in liver tissue. miR-34a expression data were normalized to RNU6B. Data shown represent the mean ± SD. (N = 4–6/group), $^{***}P < 0.001$, $^{**}P < 0.01$.

myeloperoxidase (MPO) activity of the liver, an index of neutrophil accumulation, was depressed in mice that inhaled CO, compared with the air-inhalation control group after hepatic I/R (4.026 ± 1.173 vs. 9.034 ± 1.952 ng/mg, respectively, $P < 0.01$) (Fig. 1B). Moreover, histological examination of the liver from CO-pretreated mice revealed reduction of the typical lesions of hepatocellular necrosis and sinusoidal congestion

associated with I/R injury (Fig. 1C). To assess the immunoregulatory function of CO, first we detected inflammatory cell infiltration and then analyzed the expression of hepatic cytokines. In order to investigate inflammatory cell infiltration, we performed immunohistochemical staining for Ly-6G and F4/80 (Fig. 1D). The increase of infiltrated cells after surgery was reduced by CO inhalation. Consistent with the immunohistochemical data, the mRNA levels of iNOS and pro-inflammatory cytokines (i.e., TNF-α, IL-6, and IL-1β) were significantly decreased in the livers of mice subjected to hepatic I/R with CO inhalation, compared to those of the air inhalation group (Fig. 1E). These findings suggest that CO inhalation can ameliorate I/R injury in the liver.

3.2. The protective effect of CO in hepatic I/R injury is associated with SIRT1 induction

SIRT1 can protect against I/R injury in heart and brain tissue [24,25]. Previous studies suggest that p53 acts as a transcription factor to increase the expression of miR-34a which in turn regulates SIRT1 [27,33]. In addition, it was reported that HO-1 can reduce miR-34a expression [34]. To evaluate the potential role of SIRT1 in CO-mediated liver protection, we analyzed the effects of CO on SIRT1 expression *in vivo*. As shown in Fig. 1F, the expression of SIRT1 in mice subjected to hepatic I/R injury and receiving air-inhalation pretreatment was increased when compared with the sham I/R group. Interestingly, mice subjected to CO inhalation pretreatment revealed a markedly increased SIRT1 expression in the liver after hepatic I/R, compared with the sham control, and air inhalation control mice. Induction of SIRT1 in CO-treated mice was also associated with decreased acetylation of the p65 subunit of NF-κB, and the decreased expression of the cleaved form of caspase-3 (Fig. 1F). Consistent with Western blot data, the expression of SIRT1 mRNA was dramatically increased in CO-pretreated mice subjected to hepatic I/R, whereas it was not altered in the other treatment groups (Fig. 1G). Next, to confirm these results, we performed immunohistochemical staining which clearly showed increased SIRT1 expression in CO pretreated mice during hepatic I/R (Fig. 1H). We also examined the effect of CO on the miR-34a signaling pathway *in vivo*, which is known to regulate SIRT1. As shown in Fig. 1I, miR-34a expression was decreased in the CO-inhalation I/R group when compared with I/R mice subjected to air inhalation. These results, taken together suggest that SIRT1 induction following CO inhalation was associated with the reduction of miR-34a expression.

3.3. p53 inhibition confers functional protection against I/R injury in mice

We observed that inhalation of CO reduced miR-34a expression in I/R-treated mice in association with tissue protection. We hypothesized that this may be due to reduction of p53 transcriptional activity. Inhibition of p53 can reduce endotoxemic liver injury and protect against the apoptotic response after renal I/R [35,36]. Therefore, we analyzed the effect of the p53 inhibitor, pifithrin-α (PFT), on hepatocellular function in mouse liver subjected to 60 min of warm ischemia followed by 4 h reperfusion. As shown in Fig. 2A, sALT levels were decreased in mice pretreated with PFT as compared with vehicle (DMSO)-treated mice (1481 ± 53 vs. 2112 ± 165 U/L respectively, $P < 0.01$). Liver MPO activity was decreased in mice pretreated with PFT, compared to vehicle-treated mice (5.696 ± 2.294 vs. 8.884 ± 0.735 ng/mg, respectively, $P < 0.05$) (Fig. 2B). Additionally, we found that PFT reduced hepatocellular necrosis and sinusoidal congestion as well as attenuated the expression of HMGB1, an inflammatory cytokine that can promote liver damage following I/R injury (Fig. 2C–D). Moreover, to assess the immunoregulatory function of p53, we studied hepatic cytokine/chemokine expression patterns. Consistent with the results for liver damage by H&E staining (Fig. 2C), the mRNA levels of pro-inflammatory mediators such as TNF-α, IL-6, IL-1β, iNOS and CXCL10 were significantly decreased in the liver of mice subjected to PFT administration, compared with those of the control group (Fig. 2E). Collectively, these results indicate that inhibition of p53 has a protective effect in hepatic I/R.

3.4. p53 inhibition induces SIRT1 expression after hepatic ischemia/reperfusion

To assess whether attenuation of hepatic I/R injury by p53 inhibition is due to SIRT1 induction, we tested the effect of PFT on SIRT1 expression. Treatment of mice with PFT during hepatic I/R increased SIRT1 protein and mRNA levels when compared with the untreated sham I/R and vehicle control groups (Fig. 2F and G). In contrast, miR-34a expression was decreased in the presence of PFT (Fig. 2H). In addition, induction of SIRT1 was also associated with decreased acetylation of p65 (Fig. 2F). Taken together, these results suggest that SIRT1 induction following PFT injection was associated with decreased expression of miR-34a as the consequence of the inhibition of p53 transcriptional activity. Because SIRT1 can also catalyze the deacetylation of p53, we further performed immunoblot analysis to determine whether the protective effect of SIRT1 induction by PFT during hepatic I/R injury depends on p53 deacetylation. We found that PFT injection diminished the levels of acetylated p53 and cleaved caspase-3; whereas, it increased the expression of antiapoptotic Bcl-2/Bcl-X_L proteins in the liver of mice subjected to hepatic I/R, compared with the DMSO-injected group (Supplementary Fig. 1). These findings demonstrate that inhibition of p53 transcriptional activity by PFT suppresses the apoptotic pathway in association with increased SIRT1 deacetylase activity.

3.5. Carbon monoxide reduces inflammation through SIRT1 induction in macrophages

Previous reports have shown that SIRT1 acts as a regulator of macrophage inflammatory responses [37]. To investigate the cellular mechanisms underlying our *in vivo* findings, we analyzed whether CO-dependent reduction of miR-34a may regulate inflammatory responses through SIRT1 induction *in vitro*. We first examined the effect of CO on SIRT1 expression *in vitro* using Kupffer cells, RAW 264.7 macrophages, and mouse AML12 hepatocytes. After culture of KCs, more than 90% of cells were F4/80-positive macrophages (Supplementary Fig. 2A). When macrophages or hepatocytes were incubated with the CO donor compound CORM-2, SIRT1 expression increased in a dose-dependent manner (Supplementary Fig. 2B). LPS treatment increased miR-34a expression in KCs (Fig. 3A) or RAW 264.7 cells (Supplementary Fig. 3A), which in turn was decreased profoundly in the presence of the CO-releasing molecule (CORM2). We investigated the effect of CORM2 on the acetylation of NF-κB/p65 in response to LPS treatment. In agreement with our *in vivo* data, analysis revealed that treatment with CORM2 significantly increased the protein and mRNA levels of SIRT1 and decreased LPS-induced acetylation of p65 in KCs (Fig. 3B, Supplementary Fig. 4A for semi-RT PCR) or RAW 264.7 cells (Supplementary Fig. 3B, Supplementary Fig. 4D for semi-RT PCR). To further define the role of SIRT1 in the regulation of inflammation, we quantified the expression of several inflammatory genes. The miR-34a reduction elicited by CORM2 was associated with significant reduction of the expression of TNF-α, IL-6, IL-1β, and iNOS, a known NF-κB target gene, in response to LPS in KCs (Fig. 3C, Supplementary Fig. 4B for semi-RT PCR) or RAW 264.7 cells (Supplementary Fig. 3C, Supplementary Fig. 4E for semi-RT PCR). Taken together, these data suggest that CO-mediated SIRT1 induction can negatively regulate the LPS-mediated inflammatory response through deacetylation of NF-κB/p65.

3.6. SIRT1 induction via downregulation of the miR-34a signaling pathway negatively regulates the LPS-mediated inflammatory response in vitro

To examine the functional consequences of SIRT1-dependent inflammatory responses, we analyzed the cellular levels of SIRT1 in macrophages treated with the p53 inhibitor PFT. As the result of PFT

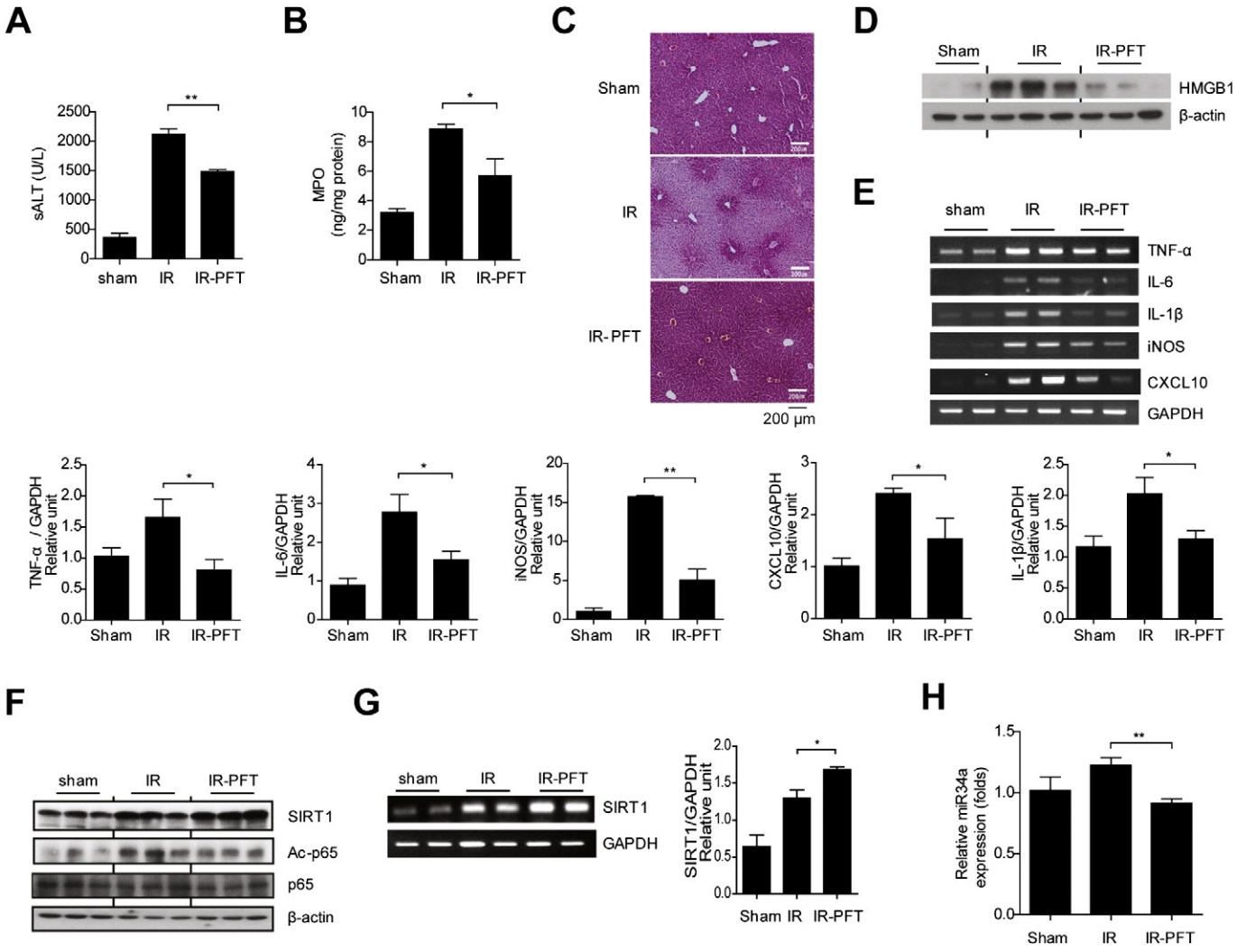

Fig. 2. Administration of the p53 inhibitor, PFT ameliorates liver I/R injury by inducing SIRT1 in mice. Mice were subjected to 60 min liver warm ischemia, followed by 4 h of reperfusion. Liver samples were harvested from unclamped mice (sham) or mice clamped and pretreated with DMSO (IR) or Pifithrim-α (IR-PFT). (A) Hepatocellular function was evaluated by sALT (U/L). (B) MPO level, an index of neutrophil infiltration. (C) H&E staining of liver sections from each group. (D) Hepatic HMGB1 expression in liver tissue was assessed by Western blot analysis. (E) Semi- and quantitative RT-PCR-assisted detection of TNF-α, IL-6, iNOS, CXCL10, and IL-1β gene expression in liver samples. (F) Hepatic expression of SIRT1, Ac-p65 and p65 were evaluated in IR lobes from DMSO (IR) and PFT (PFT-IR) injected mice versus sham mice by immunoblotting. (G) SIRT1 mRNA expression was detected by semi- and quantitative RT-PCR in liver tissue. (H) Expression of miR-34a was evaluated in liver tissue by quantitative RT-PCR. Data shown represent the mean ± SD. (N = 4–6/group), $^{*}P < 0.05$, $^{**}P < 0.01$.

treatment, miR-34a expression was reduced in KCs (Fig. 3D) or RAW 264.7 cells (Supplementary Fig. 3D). LPS-stimulated cells that were pretreated with PFT displayed a marked increase in SIRT1 protein (Fig. 3E, upper panel) and mRNA levels (Fig. 3E, lower panel; Supplementary Fig. 4C for semi-RT PCR) in KCs, or SIRT1 protein (Supplementary Fig. 3E, left) and mRNA levels (Supplementary Fig. 3E, right; Supplementary Fig. 4F for semi-RT PCR) in RAW 264.7 cells, whereas the LPS-induced acetylation of p65 was markedly decreased in KCs (Fig. 3E) or RAW 264.7 cells (Supplementary Fig. 3E). To further define the role of SIRT1 in the regulation of inflammation, we quantified the mRNA levels of several inflammatory genes in response to LPS in the absence or presence of PFT treatment. The inhibition of p53 by PFT significantly reduced the expression of TNF-α, IL-6, and iNOS, a known NF-κB target gene, in response to LPS in KCs (Supplementary Fig. 5A for semi-RT PCR; Supplementary Fig. 5B for quantitative RT-PCR) or RAW 264.7 (Supplementary Fig. 5C for semi-RT PCR; Supplementary Fig. 5D for quantitative RT-PCR). Together, these data show that p53 inhibition by PFT can negatively regulate the LPS-mediated inflammatory response *in vitro*. To confirm the SIRT1-dependent anti-inflammatory mechanism induced by PFT, we silenced SIRT1 by siRNA transfection. SIRT1 knockdown significantly decreased SIRT1 mRNA levels in RAW 264.7 cells (Fig. 3F and G; Supplementary Fig. 4G for semi-RT PCR). SIRT1 knockdown blunted the inhibitory effect of PFT on the acetylation of p65 induced by LPS. These data suggest that the effect of PFT on p65 deacetylation is mediated by SIRT1 induction (Fig. 3H). SIRT1 knockdown also reversed the inhibitory effect of PFT on LPS-induced expression of iNOS and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β (Fig. 3I and Supplementary Fig. 4H for semi-RT PCR). These data indicate that the inhibition of the p53/miR-34a axis resulting in enhanced SIRT1 signaling can negatively regulate inflammatory responses in macrophages in part through p65 deacetylation.

3.7. CO inhibits miR-34a resulting in SIRT1-dependent inhibition of p53 activity in hepatocytes

To characterize the effect of CORM in modulating miR-34a expression, we transfected AML12 hepatocytes with a miR-34a mimic, in the presence or absence of CORM2. The miR-34a expression was markedly increased compared with the miR-control. CORM2 dose-dependently reduced the level of miR-34a in miR-34a transfected cells (Fig. 4A). In addition, miR-34a overexpression led to a 2-fold decrease in SIRT1 expression (Fig. 4B). Notably, the reduction in SIRT1 expression induced

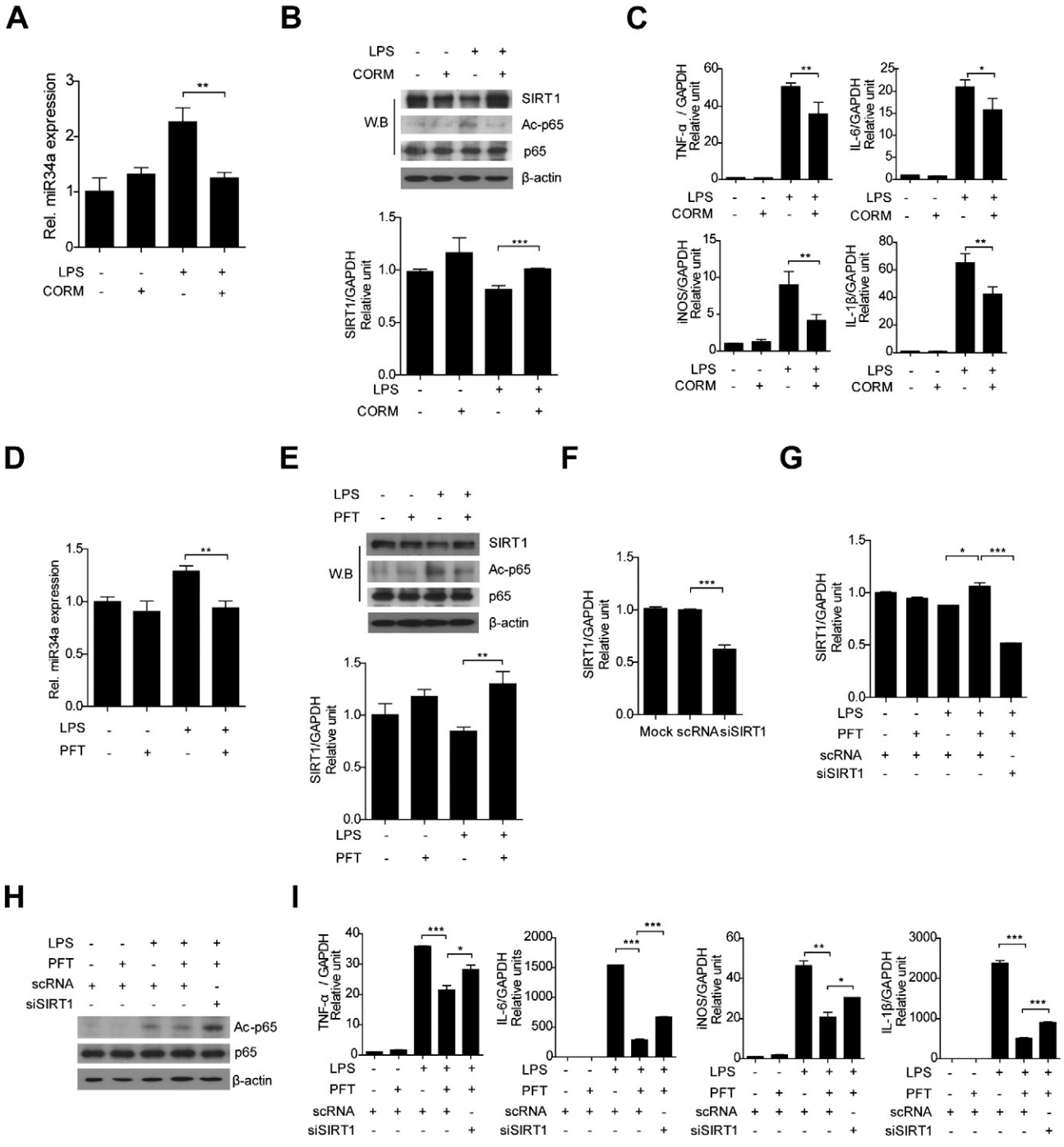

Fig. 3. The protective effect of p53 inhibition by CO or PFT is dependent on the miR-34a/SIRT1 pathway. (A–C) Kupffer cells (KCs) were stimulated with 1 μg/ml of LPS for 3 h in the absence or presence of CORM2 (20 μM). A. The level of miR-34a was evaluated by quantitative RT-PCR. B. Cell lysates were analyzed by Western blotting with antibodies against SIRT1, acetylated-p65, p65, or β-actin (*upper panel*). SIRT1 mRNA expression was also evaluated by quantitative RT-PCR (*lower panel*). C. The gene expression of TNF-α, IL-6, iNOS, and IL-1β was evaluated by quantitative RT-PCR. (D and E) KCs were stimulated with 1 μg/ml of LPS for 3 h in the absence or presence of PFT (10 μM). D. The level of miR-34a was determined by quantitative RT-PCR. E. SIRT1, acetylated-p65, p65, or β-actin was measured by immunoblotting *(upper panel)*. SIRT1 mRNA expression was also measured by quantitative RT-PCR (*lower panel*). (F–I) RAW 264.7 cells were transfected with SIRT1-specific siRNA (siSIRT1) or scrambled siRNA (scRNA). After 48 h, cells were stimulated with 1 μg/ml of LPS for 3 h in the absence or presence of PFT. Total RNA was collected for assessment of transcription levels by (F and G, SIRT1; I, TNF-α, IL-6, iNOS, and IL-1β) quantitative RT-PCR and level of acetylated-p65 and p65 were measured by (H) Western blotting. Data shown represent the mean ± SD. (N = 3), $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$.

by miR-34a overexpression was reversed by CORM2 treatment. These data suggest that CO protects the liver by inducing SIRT1 expression by downregulating the p53/miR-34a pathway. To examine whether the modulation of SIRT1 by CORM2 occurs via miR-34a, we co-transfected cells with a luciferase reporter construct containing the miR-34a binding site within the SIRT1 3′UTR (Luc-SIRT1 3′UTR) together with a miR-34a mimic. While miR-34a overexpression led to a decrease in luciferase activity, CORM2 treatment resulted in increased luciferase activity in both control and miR-34a overexpressing cells (Fig. 4C). To further investigate whether CO can affect p53 transcriptional activity, we assessed p53 transactivation in cells overexpressing p53, in the presence or absence of CORM2 (Fig. 4D). While p53 overexpression increased p53 transcriptional activity approximately 5-fold, treatment with CORM2 significantly inhibited p53 transcriptional activity. These data suggest that p53 is a key molecular target of CORM2 in modulating hepatic I/R injury. To further evaluate the role of CO in

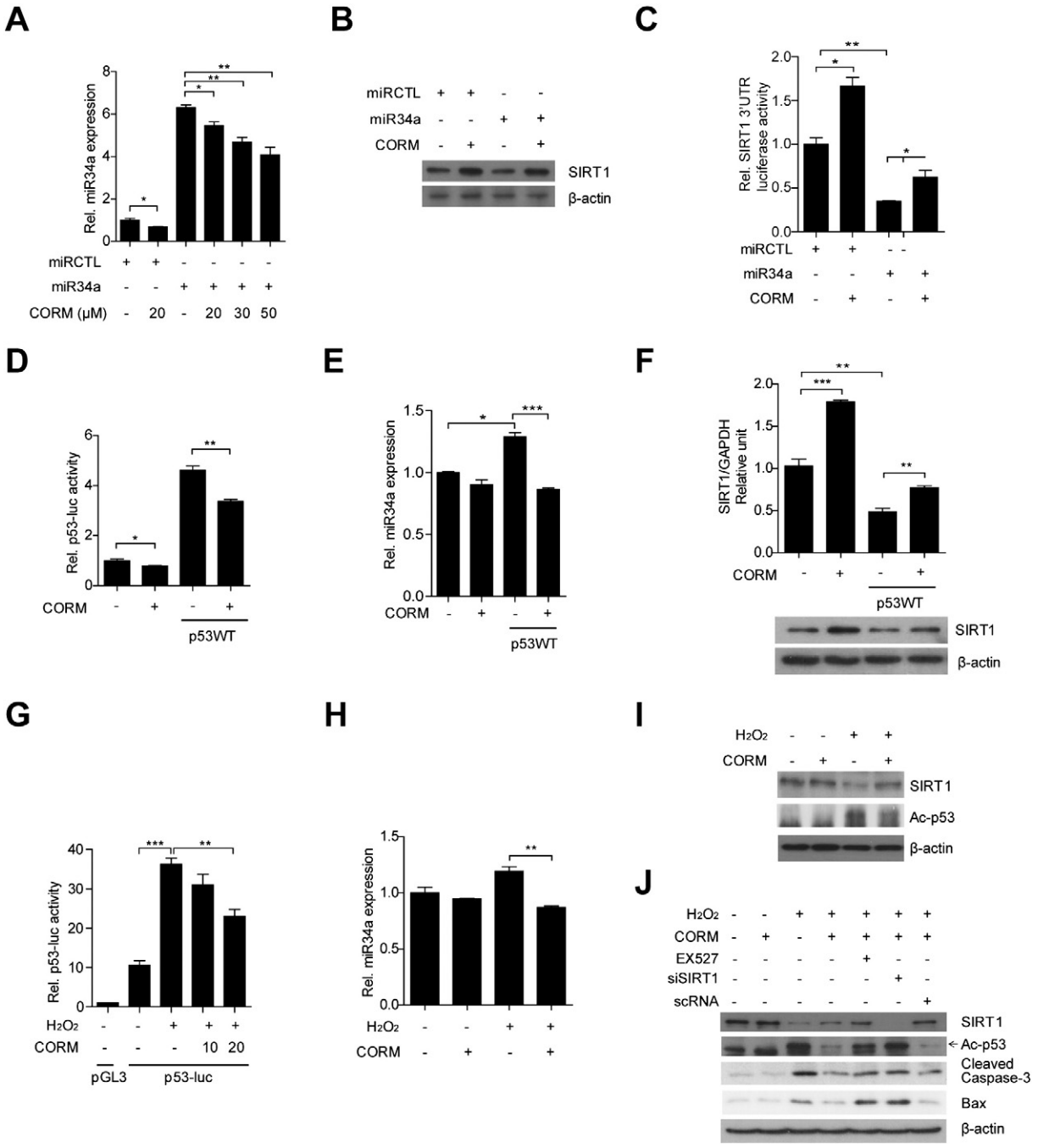

Fig. 4. Carbon monoxide inhibits p53-dependent induction of the miR-34a/SIRT1/p53 pathway in hepatocytes. (A and B) AML12 hepatocytes were transfected with control miRNA (miRCTL) or mimic miR-34a for 48 h and then treated with CORM2. A. the level of miR-34a was evaluated by quantitative RT-PCR. B. The levels of SIRT1 and β-actin were measured by western blotting. (C) AML12 hepatocytes were co-transfected with SIRT1 3'UTR luciferase constructs and control miRNA (miRCTL) or mimic miR-34a for 48 h and then treated with CORM2 (20 μM). Cells were harvested and luciferase activity was determined. (D) AML12 hepatocytes were co-transfected with p53 responsive element reporter constructs (p53-luc) and p53 expressing plasmids for 48 h and then treated with CORM2 (20 μM). Cells were lysed and assayed for luciferase activity. (E and F) AML12 hepatocytes were transfected with p53 expressing plasmid for 48 h and treated with CORM2. E. Expression of miR-34a was evaluated by quantitative RT-PCR. F. The level of SIRT1 was measured by quantitative RT-PCR (*upper panel*) and western blotting (*lower panel*). (G) AML12 hepatocytes were transfected with p53 responsive element reporter constructs (p53-luc) for 36 h. Cells were pretreated with CORM2 and stimulated with 0.2 mM of H_2O_2. After 6 h, luciferase activity was measured. The levels of firefly luciferase activity were normalized to *Renilla* luciferase activity. (H) AML12 hepatocytes were pretreated with CORM2 and stimulated with 1 mM of H_2O_2. Expression of miR-34a was evaluated by quantitative RT-PCR. (I) Primary Hepatocytes were pretreated with CORM2 and incubated with 1 mM of H_2O_2 for 6 h. The levels of SIRT1, ac-p53, and β-actin were measured by western blotting. (J) Primary hepatocytes were transfected with siSIRT1 or scRNA. After 48 h, cells were pretreated with or without the indicated SIRT1 inhibitor (EX527; 10 μM) for 1 h, followed by treatment with CORM2 (20 μM) alone or in combination for 1 h and harvest after incubating with H_2O_2 (1 mM) for 6 h. The levels of SIRT1, acetylated-p53, cleaved caspase-3, Bax, and β-actin were measured by western blotting. Data shown represent the mean ± SD. (N = 3), $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$.

p53-dependent miR-34a regulation, we transfected AML12 cells with a p53 expression vector. Overexpression of p53 led to a 1.3-fold increase in miR-34a expression compared to empty vector-transfected cells. Importantly, CORM2 abrogated the induction of miR-34a by p53 expression (Fig. 4E). Consistently, p53 overexpression also decreased SIRT1 mRNA and protein levels, an effect reversed by CORM2 (Fig. 4F). Because hepatic I/R injury is characterized by the generation of reactive oxygen species (ROS), such as superoxide anion and H_2O_2, we utilized H_2O_2-mediated oxidant injury as an *in vitro* model of reperfusion injury. To determine the functional significance of the p53/miR-34a/SIRT1

pathway and its regulation by CO *in vitro*, we analyzed p53 expression in H_2O_2-challenged AML12 hepatocytes treated with the CO donor compound CORM2. Treatment with H_2O_2 increased p53 gene expression and accumulation. As expected, CORM2 decreased p53 transcriptional activity in a dose-dependent manner (Fig. 4G). To investigate the effect of CORM2 on the apoptotic response of AML12 hepatocytes, we induced oxidative stress using H_2O_2. In these cells, miR-34a expression was increased by H_2O_2; whereas, it was reduced by CORM2 (Fig. 4H). In addition, CORM2 also efficiently reduced H_2O_2-dependent p53 acetylation through SIRT1 induction in primary hepatocytes (Fig. 4I) or AML12 cells (Supplementary Fig. 5A). Silencing of SIRT1 in H_2O_2 and CORM-treated primary hepatocytes (Fig. 4J) or AML12 cells (Supplementary Fig. 6B) reversed the CO-dependent reduction of cleaved caspase-3, p53 acetylation, and Bax expression. This data suggest that silencing of SIRT1 attenuated the anti-apoptotic functions of CORM through increasing p53 acetylation.

4. Discussion

In the current study, we have identified SIRT1 as a novel therapeutic target of the anti-inflammatory effect of CO, a candidate therapeutic gas, in a hepatic I/R injury model. We demonstrated that induction of SIRT1 in the liver after CO inhalation resulted in protection in a hepatic I/R model. CO, which can be applied by inhalation or by pharmacological delivery using CORMs, continues to show promise as an anti-inflammatory therapeutic in several models of organ I/R injury [38]. For example, inhaled CO conferred tissue protection in rodents subjected to lung I/R injury, as evidenced by reduced markers of apoptosis, which depended on the activation of the MKK3/p38 MAPK pathway [13,14]. An additional mechanism for CO-mediated protection during lung I/R involves downregulation of the pro-inflammatory transcription factor Egr-1 [32]. Several studies demonstrated that pretreatment with CO donor compounds can ameliorate liver transplant-associated I/R injury through increased hepatic HSP70 expression [39], and suppression of inflammatory responses *via* downregulation of the MEK/ERK1/2 signaling pathways [40]. Hepatic I/R injury can be attributed to the infiltration of immune cells and to apoptosis of liver cells. Importantly, we found that CO reduced the inflammatory response by preventing the infiltration of immune cells (Fig. 1D, E). Consistent on our results, CO has also been shown to protect against acute liver injury caused by TNF-α challenge in rodent models [41], and can confer anti-inflammatory protection in hepatic I/R injury models [8,11,12,40]. CO preserved hepatic function *ex vivo* in an isolated perfused liver model subjected to cold ischemia injury, in part by upregulating the p38 MAPK pathway [11]. CO has also been shown to protect against I/R injury during orthotropic rat liver transplantation by downregulating pro-inflammatory mediators, including TNF-α and iNOS expression [40]. Furthermore, the protection afforded by CO in this model was also associated with the modulation of STAT1/STAT3 and inhibition of the MEK/ERK1/2 signaling pathway [8]. HO-1-derived CO can reduce expression of miR-34a to improve the survival of myoblasts after intramuscular transplantation [34]. Based on our data, we suggest a novel mechanism by which CO mitigates murine hepatic I/R injury *via* the induction of SIRT1 (Fig. 1F). SIRT1 is a cytosolic deacetylase that can catalyze the deacetylation of histones and other substrates such as p53 and NF-κB [42,43]. The organ protection conferred by CO-dependent SIRT1 activation was associated with downregulation of the inflammatory response, involving increased NF-κB and p53 deacetylation, and inhibition of pro-inflammatory mediators. Previous studies have demonstrated that knockdown of SIRT1 can result in enhanced activation of LPS-stimulated NF-κB activation and expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [23]. Deacetylation of p53 by SIRT1 can reduce apoptosis in the heart and kidney [44–47]. SIRT1 has also been shown to protect the heart and brain from I/R injury [24,25], and can lead to attenuated inflammation and apoptosis through NF-κB and p53 deacetylation. To date, no studies have assessed the role of SIRT1 in hepatic I/R injury. Therefore, we suggest that the induction of SIRT1 by CO can alleviate inflammation and apoptosis *via* p65 deacetylation (Fig. 3) and *via* p53 deacetylation (Fig. 4). We also demonstrate for the first time that the increase of SIRT1 expression by CO is regulated by miR-34a in the liver (Fig. 4). CO also rescued SIRT1 expression in p53 or miR-34a overexpressing cells. Consistent with our results, SIRT1 expression in the liver is suppressed by miR-34a [26,27] through a miR-34a binding site that occurs in the 3′ untranslated region (UTR) of SIRT1. The miR-34a-dependent downregulation of SIRT1 leads to an increase in acetylated p53 and increased expression of p21 and PUMA, transcriptional targets of p53 that regulate the cell cycle and apoptosis, respectively, ultimately causing apoptosis [27]. Recently, it has been reported that targeting of the miR-34a/SIRT1/p53 pro-apoptotic pathway in primary hepatocytes results in increased SIRT1 expression, decreased acetylation of p53, and reduced apoptosis [28,29].

In summary, our results establish a signaling pathway by which CO can confer anti-inflammatory protection in the liver. CO downregulates miR-34a which results in the activation of the SIRT1 deacetylase, leading to the deacetylation of NF-κB/p65 and p53, and downmodulation of pro-inflammatory gene expression. Our results further validate the potential use of CO as a pharmacological cytoprotective agent against hepatic I/R injury, and identify miR-34a/ SIRT1 as a major therapeutic target of CO action in the liver.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.04.017.

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