Pretreatment with carbon monoxide releasing molecules suppresses hepcidin expression during inflammation and endoplasmic reticulum stress through inhibition of the STAT3 and CREBH pathways

Da-Yong Shin1, Jihwa Chung1, Yeonsoo Joe1, Hyun-Ock Pae3, Ki Churl Chang4, Gyeong Jae Cho5, Stefan W. Ryter6 and Hun-Taeg Chung1,2

1School of Biological Sciences, 2WCU, University of Ulsan, Meta-Inflammation Basic Research Laboratory, Ulsan, Republic of Korea,
3Department of Microbiology and Immunology, Wonkwang University School of Medicine, Iksan, Republic of Korea
4Department of Pharmacology and 5Department of Anatomy, School of Medicine, and Institute of Health Sciences, Gyeongsang National University, Jinju, 660-751, Korea.
6Pulmonary and Critical Care Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA.

*Correspondence to: Hun-Taeg Chung, M.D., Ph.D.
School of Biological Sciences, University of Ulsan, Ulsan 680-749, Republic of Korea
Tel: +82-52-259-2392, Fax: +82-52-259-2740
E-mail: chung@ulsan.ac.kr

Left running head: SHIN et al.
Right running head: Suppressive effects of CO on hepcidin expression
Article Type: Regular Article
Keywords: Carbon monoxide, hepcidin, inflammation, ER-stress, IL-6, CREBH, STAT-3, anemia
Abbreviations

CO, carbon monoxide;
CORM-2, CO-releasing molecule-2 (tricarbonyl dichlororuthenium dimer);
CORM-3, CO-releasing molecule-3 (tricarbonylchliro(glycinate)ruthenium);
CREBH, cyclic AMP response element-binding protein H;
ER, endoplasmic reticulum;
HO-1, heme oxygenase-1;
HCYS, homocysteine;
TM, tunicamycin;
TG, thapsigargin;
OSM, oncostatin M;
LIF, leukemia inhibitory factor;
BMP-2, bone morphogenetic protein 2;
UPR, unfolded protein response;
XBP-1, X-box binding protein-1;
STAT-3, signal transducer and activator of transcription-3;
SOCS-3, suppressor of cytokine signaling-3.
Abstract

The circulating peptide hormone hepcidin maintains systemic iron homeostasis. Hepcidin production increases during inflammation and as a result of endoplasmic reticulum (ER) stress. Elevated hepcidin levels decrease dietary iron absorption and promote iron sequestration in reticuloendothelial macrophages. Furthermore, increased plasma hepcidin levels cause hypoferremia and the anemia associated with chronic diseases. The signal transduction pathways that regulate hepcidin during inflammation and ER stress include the IL-6-dependent signal transducer and activator of transcription-3 (STAT-3) pathway, and the unfolded protein response-associated cyclic AMP response element-binding protein-H (CREBH) pathway, respectively. We show that carbon monoxide (CO) suppresses hepcidin expression elicited by IL-6 and ER-stress agents by inhibiting STAT-3 phosphorylation and CREBH maturation, respectively. The inhibitory effect of CO on IL-6-inducible hepcidin expression is dependent upon the suppressor of cytokine signaling (SOCS)-3 protein. Induction of ER stress in mice resulted in increased hepatic and serum hepcidin. CO administration inhibited ER stress-induced hepcidin expression in vivo. Furthermore, ER stress caused iron accumulation in splenic macrophages, which could be prevented by CO. Our findings suggest novel anti-inflammatory therapeutic applications for CO, as well as therapeutic targets for the amelioration of anemia in the hypoferremic condition associated with chronic inflammatory and metabolic diseases.

Introduction

Hepcidin, a circulating peptide hormone synthesized by the liver, functions as a master regulator of iron homeostasis.1-3 Elevated levels of hepcidin can block the release of iron from macrophages, impair intestinal iron absorption, and cause hypoferremia leading to iron deficiency anemia.4,7 Hepcidin plays a major role in the anemia of inflammation observed in patients with a variety of disorders, including infection, arthritis, inflammatory bowel disease, trauma, cancer, and organ failure.1,8 Interleukin-6 (IL-6) represents the principle regulator of hepcidin expression associated with the anemia of inflammation.8 In humans, IL-6 can increase hepcidin levels and decrease serum iron levels. IL-6 treatment also induces hepcidin expression in vivo, and in primary hepatocytes and hepatoma cell lines. The critical role of IL-6 in hepcidin expression is illustrated by experiments showing that anti-IL-6 antibodies block hepcidin mRNA expression in vivo as well as in hepatocytes stimulated by LPS.9 Furthermore, IL-6 deficient mice display impaired hepcidin induction and thereby do not display low serum iron in response to pro-inflammatory stimuli.9 Taken together, these observations suggest a relationship between IL-6 and the expression of hepcidin in inflammation. IL-6 plays a central role in the regulation of the acute-phase response (APR) in hepatocytes.10 Upon exposure to pro-inflammatory stimuli, IL-6 is released and binds to a complex of the IL-6 receptor (IL-6R)-α and gp130.11 The IL-6 ligand-receptor interaction results in the activation of Janus kinases (JAKs) which in turn activate the signal transducer and activator of transcription protein-3 (STAT-3) by tyrosine (Y705) phosphorylation. Phosphorylated STAT-3 subsequently translocates into the nucleus where it regulates the transcription of target genes including hepcidin.12 The transcriptional activity of STAT-3 is regulated by
serine (S727) phosphorylation. Additional factors appear to regulate hepcidin expression, since IL-6 knockout mice maintain some hepcidin responsiveness to LPS. For example, IL-1 has been shown to stimulate hepcidin expression in both mouse hepatocytes and human Huh7 cells.\textsuperscript{13-14}

Recent studies indicate that the unfolded protein response (UPR) associated with endoplasmic reticulum (ER) stress can induce APR proteins, including C-reactive protein (CRP), serum amyloid P component (SAP), and hepcidin, through the activation of the transcription factor cyclic AMP response element-binding protein-H (CREBH).\textsuperscript{15} CREBH binds and trans-activates the hepcidin promoter. ER stress can induce hepcidin-dependent hypoferremia and splenic iron sequestration in mice.\textsuperscript{16} These studies suggest linkages between the ER-stress associated UPR, innate immunity, and iron homeostasis.

Carbon monoxide (CO) arises endogenously as the by-product of the cytoprotective heme oxygenase (HO) enzyme system, which also generates equimolar biliverdin-IX\textsubscript{α} and ferrous iron during the oxidative catabolism of heme. CO, when applied at low concentration, can exert anti-inflammatory, anti-proliferative and anti-apoptotic effects in a variety of models of cellular injury.\textsuperscript{17} Previously, we have shown that exogenous CO can activate anti-inflammatory signaling through the UPR. Specifically, CO selectively activated one branch of the UPR, the protein kinase R-like endoplasmic reticulum kinase (PERK), resulting in increased activation of Nrf-2, a master regulator of the stress response, and downstream expression of the cytoprotective protein heme oxygenase-1 (HO-1).\textsuperscript{18} Concomitantly, CO suppressed the other two branches of the UPR involving the inositol-requiring transmembrane kinase/endonuclease-1\textsuperscript{α} (IRE1\textsuperscript{α}), and the activating transcription factor 6 (ATF-6).\textsuperscript{18-19} However, the precise mechanisms and signaling pathways involved in CO-dependent anti-inflammatory and cytoprotective effects remain to be fully elucidated.

Since both pro-inflammatory and ER stress responses can induce hepcidin expression, we investigated whether the modulation of cytokine and UPR-dependent signaling pathways by therapeutic gases such as CO, could attenuate overproduction of hepcidin. Specifically, we found that CO could inhibit both IL-6 and ER stress-induced hepcidin expression. We therefore investigated the underlying mechanisms for this homeostatic function of CO on iron metabolism.

### Methods

#### Reagents

Tricarbonyl dichlororuthenium (II) dimer (RuCO, CORM-2), thapsigargin (TG), tunicamycin (TM), homocysteine (Hcys), 4-phenylbutyric acid (4-PBA), and ruthenium chloride were from Sigma-Aldrich (St. Louis, MO). Tricarbonylchloro (glycinate) ruthenium (II) (CORM-3) was kindly contributed by Dr. Haksung Kim (Kangwon National University, Korea). CORM-3 was first synthesized by Motterlini’s group and its properties are well documented.\textsuperscript{20} CORM-3 is a novel water-soluble ruthenium-based carbonyl CO carrier, which was developed for \textit{in vivo} applications. CORM-3 is stable in water at 37°C and at acidic pH for over 24 h, and liberates CO rapidly in physiological solutions and biological fluids. Moreover, \textit{in vivo} studies have demonstrated that CORM-3 specifically delivers CO to cells and tissues, mimicking the biologic activities associated with CO gas inhalation.\textsuperscript{21}

Recombinant human and mouse IL-6, and oncostatin M (OSM) were from R&D systems
(Minneapolis, MN). Recombinant LIF and BMP-2 were from Sigma-Aldrich. The antibody to hepcidin was from Abcam Inc. (Cambridge, MA). According to manufacturer's datasheet for hepcidin antibody (Abcam; ab30760), this antibody detects the mature cleaved form of hepcidin. The immunogen is a synthetic peptide conjugated to KLH, derived from within residues 50 to the C-terminus of human hepcidin-25. This antibody recognized a band with an approximate molecular size of 32 kDa, when tested against human recombinant hepcidin. Antibodies to total STAT-1 and -3, and CREBH, phospho-ERK, total ERK were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-STAT-1 and -3, phospho-SMAD 1/5/8 were purchased from Cell Signaling (Beverly, MA). Antibodies to Flag were purchased from Sigma Aldrich. All other chemicals were from Sigma-Aldrich.

**Animals**

All experiments with mice were approved by the Animal Care Committee of the University of Ulsan. Seven-week-old male C57BL/6 wild type mice and C3H were purchased from ORIENT (Pusan, Korea). The mice were maintained under specific pathogen-free conditions at 22°C and given access to food and water *ad libitum*. CORM-3 was freshly dissolved in distilled water before each experiment, and administered to C57BL/6 mice (10 mg/kg/day, *i.v.*). The control mice received same amounts of distilled water. Three hours after CORM-3 treatment, mice were injected with recombinant mouse IL-6 (mIL-6, 25 μg/kg *i.p.*) or with tunicamycin (1.5 mg/kg, *i.p.*) dissolved in 0.5% v/v DMSO/saline solution. The control mice received the same amounts of 0.5% DMSO/saline solution. After 24 h, CORM-3 was administrated again. At 48 h post-injection, mice were sacrificed by cervical dislocation, and then serum (from venous blood collected from the orbital sinus), as well as spleen and liver tissue were obtained for experiments.

**Cell culture**

Cell cultures were grown at 37°C in humidified incubators containing an atmosphere of 5% CO2. The human hepatocellular carcinoma cell line, HepG2, was purchased from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin solution. Human hepatocytes (Huh7) were maintained in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin solution.

**Immunoblot analysis**

Following experimental treatments, cells were harvested and washed twice with ice-cold PBS. Cells were lysed with 1X RIPA buffer containing phosphatase and protease inhibitors. Serum sample obtained from mice were diluted in 1X PBS. Equal amounts of cell lysates and serum were measured with the BCA protein assay reagent (Pierce Biotechnology Inc., Rockford, IL). The samples were diluted with 2X sample buffer containing β-mercaptoethanol, and then equal amounts of protein were separated on 6%-15% SDS-PAGE gels followed by transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBS-T) for 20 min and incubated overnight with antibodies to hepcidin (1:1000), CREBH (1:500) and SOCS-3 (1:1000) in PBS-T containing 1% nonfat milk. The blots were developed with a peroxidase-conjugated secondary antibody and reacted proteins were visualized using the ECL Plus Western Blotting Detection System.
RNA isolation and reverse-transcription polymerase chain reaction

Total cellular RNA was isolated from the HepG2 cells and liver tissue of mice using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, 2 μg of extracted RNA was reverse-transcribed into first strand complementary DNA (cDNA) using M-MLV reverse-transcriptase and oligo (dT) 15 primer (Promega, Madison, WI). cDNA was amplified using primers specific for human hepcidin, mouse hepcidin, and spliced XBP-1 using PCR. Gene expression data from RT-PCR was quantified relative to GAPDH. Primer sequences and reaction conditions are provided in the Supplementary Information.

Real time quantitative RT-PCR (qRT-PCR)

Total cellular RNA was extracted using TRIzol reagent according to the manufacturer’s instructions (Invitrogen, San Giuliano Milanese, MI, Italy). cDNA was generated by reverse transcription. Real-time PCR was performed using SYBR Green qPCR Master Mix (2X) (USB products, Affymetrix) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Primer sequences are provided in the Supplementary Information.

Plasmid, siRNA transfection

pCMV-Flag/wtSTAT3, or pCMV-Flag/dnSTAT3(R382W), pCMVT2B-Flag were kind gifts from Dr. Jeong-Woo Park (University of Ulsan). Plasmids were purified by Hispeed Plasmid Maxi Kit (Qiagen). Cells were transfected with plasmid (4 μg DNA concentration per well) for 12 h by the lipofectamine method according to the manufacturer’s protocol (Invitrogen) and then restored to fresh media containing 10% FBS for 36 h.

Predesigned siRNAs against human STAT-3, SOCS-3 and CREBH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with siRNA (50 nM or 100 nM) for 12 h by the lipofectamine method according to the manufacturer’s protocol (Invitrogen) and then restored to fresh media containing 10% FBS for 36 h. The interference of STAT-3 and SOCS-3, CREBH expression was confirmed by Western blotting using anti-STAT-3, SOCS-3 and CREBH antibodies. Corresponding scrambled siRNAs were used as transfection controls.

Serum iron and hepcidin analysis.

Serum iron was determined using a commercially available Iron Assay Kit (BioVision, Mountain View, CA). Mouse hepcidin ELISA kit was purchased from USCN Life Science Inc. (Wuhan, China). Serum iron and hepcidin levels were analyzed according to the manufacturer’s instructions.
Iron staining of paraffin-embedded sections

To evaluate iron deposition in the liver and the spleen, mouse tissues were immersion-fixed in 4% formalin, embedded in paraffin wax, sectioned, and stained with Perls’ Prussian Blue for iron content using the Iron Stain Kit (Sigma-Aldrich, St. Louis, MO, USA).

Tissue non-heme iron analysis.

Spleen samples were dried at 65°C for 24 h and weighed. Samples were digested in acid-digestion mixture (3 M hydrochloric acid/10% trichloroacetic acid, Sigma Aldrich) at 65°C for 24 hours, and then 100 μl of each acid extract was then incubated with 300 μl of ferene (3 mM, Sigma Aldrich) chromogen reagent. The absorbance at 592 nm was measured in a Spetramax M2 spectrophotometer. A calibration curve was constructed, using serial dilutions of a solution of 45 mM ferrous ammonium sulfate (Sigma). Results were expressed in mmol/gm dry weight of spleen tissue.

Statistical analysis

Data were expressed as mean ± S.D. Statistical analysis was performed by paired nonparametric T-tests and calculated using a one-tail P value in GraphPad Prism (GraphPad Software, La Jolla, CA). A P value of <0.05 was considered to represent a statistically significant change.

Results

CO inhibits hepcidin expression by IL-6 and ER stress inducers.

IL-6 acts as a major regulator of the APR in hepatocytes. In response to pro-inflammatory stimuli, macrophages secrete IL-6, which induces hepcidin expression in vitro and in vivo.9,23 Consistent with these observations, we found that IL-6 treatment induced the time-dependent expression of hepcidin in HepG2 cells, beginning at 12 h, with a maximum at 18 h post-treatment (Figure 1A). To investigate whether the anti-inflammatory potential of CO can suppress the induction of hepcidin by IL-6, HepG2 cells were treated with IL-6 in the absence or presence of the CO-releasing molecule (CORM)-2. CORM-2 treatment markedly inhibited the IL-6-dependent induction of hepcidin expression in these cells (Figure 1A, Supplemental Figure 1A). Previous reports have shown that agents that trigger ER stress can induce hepcidin expression in vivo, resulting in hypoferremia and splenic iron sequestration.15-16 We sought to confirm whether three well-characterized inducers of the ER stress response (i.e., tunicamycin, thapsigargin and homocysteine) could induce hepcidin expression in vitro in our hepatoma cell line model. Treatment of HepG2 cells with these ER stress activators markedly increased the level of hepcidin (Figure 1B-D). Next, we examined whether CO treatment could modify hepcidin expression induced by these ER stress agents. Pretreatment with CORM-2 significantly inhibited ER stress-induced expression of hepcidin protein (Figure 1B-D) and mRNA (Supplemental Figure 1B). To confirm that the effect of CORM-2 on hepcidin expression was attributable to its CO releasing effect rather that an off-target effect, HepG2
cells were pretreated with CORM-2 or ruthenium chloride (RuCl₂), and then treated with tunicamycin to induce hepcidin. Despite the fact that both compounds contain ruthenium, RuCl₂ did not have a suppressive effect on hepcidin expression induced by tunicamycin (Figure 1E). We also conducted similar experiments to confirm that the effect of CO on hepcidin expression induced by IL-6 or ER-stress inducers could also be observed in the hepatocyte cell line Huh7 (Supplemental Figure 1C, D).

Oncostatin M (OSM) and leukemia inhibitory factor (LIF), members of the IL-6-related cytokine family, play important roles in immune and inflammatory responses. Oncostatin M is known as an alternate cytokine able to regulate hepcidin gene expression. Also, LIF can regulate hepcidin expression. In the anemia of multiple myeloma, increased levels of bone morphogenetic protein-2 (BMP-2) stimulate hepcidin expression. Therefore, we also investigated whether CO could down-regulate hepcidin expression elicited by these inflammatory mediators. Treatment of HepG2 cells with OSM, LIF, or BMP-2, resulted in a significant increase of hepcidin protein levels at 18 h. Pretreatment with CO greatly attenuated the increased expression of hepcidin in response to these factors (Supplemental Figure 1E). We investigated whether CORM-2 could inhibit SMAD signaling, since CORM-2 down-regulated BMP-2-induced hepcidin (Supplemental Figure 1H). CORM-2 was not effective in reducing BMP-SMAD signaling, when assessed with p-SMAD 1/5/8 antibody (Supplemental Figure 1F). We also tested whether CO could down-regulate another BMP-2 target gene, Id2. Treatment with BMP-2 (24 h) increased the gene expression of Id2, whereas CO pretreatment markedly down-regulated the expression of Id2 induced by BMP-2 (Supplemental Figure 1G). These data suggest that CO could suppress BMP-2 signaling through a SMAD-independent pathway. We hypothesized that CO can inhibit BMP-2 signaling through inhibition of ERK1/2 activation. It is well documented that BMP-2 signaling activates ERK1/2 phosphorylation to modulate SMAD signaling. CO suppressed ERK1/2 activation under our experimental conditions (Supplemental Figure 1H).

CO attenuates IL-6-induced STAT-3 and STAT-1 activation.

IL-6 can induce hepcidin though the activation of STAT-3. To test whether the inhibition of hepcidin by CO is due to its regulation of the STAT-3 pathway, we examined STAT-3 tyrosine phosphorylation (Y705) in response to IL-6 treatment. Phospho-STAT-3 was significantly increased after IL-6 treatment, with an apparent maximum at 30 minutes post-treatment (Figure 2A). Pretreatment with CORM-2 decreased the IL-6-dependent activation of STAT-3, as assessed by Y705 phosphorylation (Figure 2B) and serine phosphorylation (Supplemental Figure 2A). Similar to STAT-3, STAT-1 activation was also inhibited by CO pretreatment (Supplemental Figure 2B).

To examine the involvement of STAT-3 in hepcidin expression, we transfected cells with empty plasmid, or expression constructs encoding wild-type STAT-3, or STAT-3 dominant negative mutant (STAT-3 DN) which is mutated in the DNA binding domain. In HepG2 cells transfected with wild-type STAT-3, or subjected to mock transfection, treatment with recombinant IL-6 induced hepcidin expression. In contrast, recombinant IL-6 treatment failed to induce hepcidin in STAT-3 DN-transfected cells. Furthermore, CO pre-treatment could not effectively down-regulate hepcidin in STAT-3 DN transfected cells (Figure 2C). To confirm transfection efficiency of the STAT-3 DN mutant expression construct, we probed with Flag antibody because the vector was tagged with Flag (Supplemental Figure 2C). Similar results were shown in cells transfected with STAT-3 siRNA (Supplemental Figure 2D).
efficiency of STAT-3 siRNA was confirmed in Supplemental Figure 2E. These results suggest that CO inhibits the IL-6-dependent induction of hepcidin through inhibition of STAT-3 activation.

The suppressors of cytokine signaling (SOCS) proteins act as negative regulators of cytokine-dependent signal transduction. Once expressed, SOCS can down-regulate JAK/STAT-dependent signaling pathways. SOCS proteins contain a variable amino-terminal region, a central Src-homology 2 (SH2) domain and a novel conserved carboxy-terminal motif termed the SOCS box.29 We hypothesized that CO-mediated inhibition of IL-6-induced activation of the JAK/STAT signaling pathway involves the induction of SOCS-3. CORM-2 induced SOCS-3 protein expression in the absence or presence of IL-6 (Figure 2D). Also, we confirmed that CORM-2 induced SOCS-3 gene expression (Supplemental Figure 2F). To investigate the involvement of SOCS-3 in the inhibition of IL-6 induced STAT-3 activation, we transiently transfected HepG2 cells with SOCS-3 siRNA, and confirmed siRNA efficiency (Supplemental Figure 2G). Cells transfected with SOCS-3 siRNA showed greater induction of phopho-STAT-3 in response to IL-6, relative to cells transfected with scramble siRNA. Furthermore, CO was ineffective at inhibiting STAT-3 activation in SOCS-3-siRNA-infected cells (Figure 2E). Next, we investigated whether the reduced inhibitory effect of CO on STAT-3 activation under these conditions could affect hepcidin levels. In the SOCS-3 siRNA transfected cells, a significant increase of hepcidin expression was observed after treatment with recombinant human IL-6. CORM-2 was ineffective at reducing hepcidin expression which was slightly upregulated by IL-6 (Figure 2F). These data demonstrate that SOCS-3 is required for the inhibitory effect of CO on the STAT-3 pathway.

**CO inhibits hepcidin expression via suppression of CREBH activation.**

Several studies have reported that CREBH is a key transcription factor for constitutive and inducible expression of hepcidin in vitro and in vivo.15 The activation of CREBH during ER stress requires its cleavage to a truncated form which mediates the expression of APR genes, including hepcidin.23, 30 We therefore sought to determine whether the inhibition of hepcidin expression afforded by CO could be mediated in part by its modulation of CREBH activation. We first confirmed whether IL-6 could induce the cleavage of CREBH19, resulting in the production of its active form (CREBH-N) in HepG2 cells. As shown in Figure 3A, IL-6 induced the time-dependent cleavage of CREBH to form the activated product CREBH-N, which began to appear at 6 h post-treatment (Figure 3A). The cleaved CREBH-N was translocated to the nucleus (Figure 3B). CREBH-N was exclusively detected in the nuclear fraction, marked with the nuclear protein Lamin A/C, after 12 h of IL-6 treatment (Figure 3B).

Pre-treatment of HepG2 cells with CORM-2 decreased IL-6-induced cleavage of CREBH (Figure 3C). We also analyzed the effect of CORM-2 on CREBH mRNA expression. The increased CREBH gene expression stimulated by IL-6 was decreased after pre-treatment of HepG2 cells with CORM-2 (Figure 3D). To confirm the requirement for CREBH in IL-6 dependent hepcidin expression, we transiently transfected HepG2 cells with CREBH siRNA or the corresponding control (scramble) siRNA. The siRNA-dependent inhibition of CREBH was confirmed in Supplemental Figure 2H, and resulted in the attenuated expression of hepcidin mRNA levels (Figure 3E) and protein levels (Figure 3F) compared with the control siRNA transfected group. In CREBH siRNA transfected cells, CORM-2 pretreatment did not significantly modulate hepcidin expression compared with the IL-6 treated group (Figure 3F). To further explore the mechanisms by which CO inhibits ER stress-induced hepcidin...
expression, we examined the effects of CO on the activation of CREBH. As expected, ER stress by TM treatment induced the cleavage of CREBH and increased the amount of CREBH-N (Figure 4A). TM treatment promoted the translocation of CREBH-N to the nucleus (Figure 4B). The increased formation of CREBH-N by ER stress was markedly reduced by pretreatment with CORM-2 (Figure 4C). CORM-2 also inhibited CREBH gene expression in response to TM (Figure 4D).

Similar to the results observed with IL-6, transfection with CREBH siRNA attenuated TM-inducible expression of hepcidin mRNA (Figure 4E) and protein levels (Figure 4F, G) compared with the control siRNA transfected group. In CREBH siRNA transfected cells, CORM-2 pretreatment did not significantly modulate hepcidin expression compared with the TM treated group (Figure 4G). These results, taken together, suggest that CO can exert inhibitory effects on IL-6-dependent and ER stress-induced hepcidin expression by IL-6 or ER stress inducers through inhibition of CREBH cleavage and activation.

**CO inhibits hepcidin expression and regulates iron homeostasis in the ER-stressed mice.**

We next asked whether the observed inhibitory effects of CO treatment on hepcidin expression in vitro could also be demonstrated in vivo. C57BL/6 mice and C3H mice were pre-treated with CORM-3, and then injected 3 hours later with either recombinant mIL-6 or TM to cause inflammatory or ER stress, respectively. C57BL/6 mice were sacrificed at the 48 h time point, and then blood was collected for the analysis of serum iron and hepcidin, and liver tissue was collected for the analysis of hepatic hepcidin mRNA levels. After the same experimental procedure, the spleen tissue of C3H mice was collected for iron staining and non-heme iron analysis. While CORM-2 is soluble in organic solvents such as DMSO, CORM-3 dissolves in water. To eliminate potential off-target effects of DMSO, we used CORM-3 in mice. Mice injected with mIL-6 displayed enhanced hepcidin expression (Figure 5A, Supplementary Figure 3A). CORM-3 pretreatment blocked the expression and release of hepcidin in both hepatic tissue (Figure 5A) and serum (Figure 5B, and Supplementary Figure 3B). We also investigated the effect of CORM-3 in TM-injected mice. We first confirmed the occurrence of ER stress in TM-injected mice by assessing the splicing of XBP-1 mRNA. TM injection caused the increased accumulation of the spliced form of XBP-1 mRNA in hepatic tissue. Under these conditions, CORM-3 significantly down-regulated the levels of serum hepcidin protein (Figure 5B, and Supplementary Figure 3C), and hepatic hepcidin mRNA (Figure 5C).

Because hepcidin is an iron regulatory hormone which degrades the iron exporter ferroportin, we checked the serum iron level and intracellular iron accumulation in the spleen. Serum iron levels were significantly lower in ER-stressed mice, whereas CORM-3 pre-treatment completely restored the levels of serum iron to control values (Figure 5D). Intracellular iron accumulation in the spleen was also assessed by Perls' Prussian blue stain. Because staining capacity varies with mouse strain, we used the C3H strain, which is more susceptible to Prussian Blue staining. Iron accumulation in splenic macrophages, as detected by blue staining, was increased in ER-stressed mice, whereas CORM-3 pre-treatment blocked the accumulation of iron (Figure 5E). To quantify the non-heme iron content in spleen, an acid extract of the spleen was reacted with ferene chromogen reagent. Non-heme iron content was increased approximately 2 fold compared with control mice. In contrast, CORM-3 pretreated mice displayed an attenuated increase in non-heme iron content (Figure 5F).
These results suggest that CO inhibits the increase of hepcidin expression, and therefore could be used to regulate iron metabolism in ER-stressed mice.

**Discussion**

In the current study we demonstrate a novel anti-inflammatory function of CO, which involves modulation of intracellular iron homeostasis, through the down-regulation of hepcidin expression in hepatoma cell lines. The anemia of inflammation, a complication of common inflammatory disorders, is largely triggered by excess production of hepcidin by the liver.¹,⁶,⁸ Hepcidin binds to the iron export protein ferroportin, resulting in its endocytosis and degradation.¹ As a consequence, the efflux of cellular iron is inhibited, resulting in iron accumulation in macrophages, enterocytes, and hepatocytes.¹ The regulation of hepcidin during inflammation is primarily mediated by IL-6, though other pro-inflammatory mediators may play a role.⁹ Thus, the regulation of hepcidin may represent an important therapeutic target for controlling the pathological consequences of inflammation.³¹ The anti-inflammatory potential of CO has been extensively characterized in vitro and in vivo, though the signaling and effector pathways are not yet fully understood.¹⁷ Exogenous CO, when applied at low concentration, inhibited the LPS-dependent production of the pro-inflammatory cytokines IL-1β, TNFα, and macrophage inflammatory protein-1β in cultured RAW.264.7 macrophages, and also inhibited cytokine levels in the serum of endotoxin-challenged mice. These effects were found to be mediated by selective up-regulation of p38β MAPK.³² CO inhalation was also shown to inhibit IL-6 production in LPS stimulated macrophages, by modulating the c-Jun-NH₂-terminal kinase pathway.³³ In addition to MAPK pathways, recent studies reveal additional molecules that function as downstream mediators of CO-dependent anti-inflammatory effects, including stress proteins, such as HO-1 and the peroxysome proliferator-activated receptor-γ (PPAR-γ).¹⁸, ³⁴ The endogenous production of CO is inextricably linked to intracellular and systemic iron homeostasis and metabolism. CO can bind to heme iron centers of hemoproteins, resulting in gain-of-function (i.e., guanylate cyclase) or loss-of-function (i.e., hemoglobin, cytochrome p-450).³⁵ The evolution of CO during the enzymatic degradation of heme by heme oxygenases is stoichiometrically related to heme-iron release.³⁶ Interestingly, genetic deletion of HO-1, the enzymatic source of CO, results in aberrant tissue iron deposition in experimental mice, though the role of hepcidin in this phenomenon has not been tested.³⁷ In this study we show for the first time that exogenously applied CO can also impact iron metabolism during pro-inflammatory states by inhibiting the expression of the circulating hormone hepcidin. We also show that inflammatory responses associated with ER stress may also be modulated by CO. In vivo, the protection afforded by CO translated to significant inhibition of splenic iron deposition.

In the current study we find that the down-regulation of cytokine-induced hepcidin expression by CO involves the inhibition of STAT-3 phosphorylation. Activated STAT-3 binds to the hepcidin promoter at a target sequence (TTCTTGGA), located -64 bp upstream of the transcriptional start site, and this represents a major mechanism for IL-6-dependent regulation of hepcidin.³⁸ Interestingly, STAT-3 was previously found to mediate the cytoprotective effects of CO in endothelial cells subjected to high oxygen stress.³⁹ The endoplasmic reticulum (ER) is a cellular organelle responsible for the biosynthesis, folding, assembly and modification of proteins.⁴⁰ The aberrant accumulation of unfolded proteins, starvation, hypoxia, toxins, viral infections and increased demand on the
biosynthetic machinery can cause perturbations in the ER lumen commonly referred to as ER stress. Under these conditions, the ER activates a complex response system known as the unfolded protein response (UPR).41

In our previous study, we have shown that CO inhibits ER stress-induced CRP expression at the transcriptional and translational level. CO induced phosphorylation of the PERK branch of the UPR, but inhibited activation of IRE1α, ATF6 and CREBH.19 Here, we find that CO also can down-regulate hepcidin through impairment of CREBH activation during ER stress. CREBH is a regulated intramembrane proteolysis (RIP)-regulated liver-specific transcription factor that is cleaved upon ER stress and required to activate expression of acute phase proteins such as CRP and SAP.42 CREBH directly binds to at least one cyclic-AMP-responsive element consensus sequence on the hepcidin promoter.16 Hepcidin induction in response to the UPR during ER stress was found to be defective in CREBH knockout mice.16 With respect to IL-6-dependent stimulation of hepcidin expression, both STAT-3 and CREBH pathways are involved, consistent with the fact that IL-6 treatment also induces biochemical markers of the ER stress response.19 Taken together, these studies point to additional therapeutic targets that are modulated by CO.

It is well known that tissue hypoxia affects hepcidin expression. It has been demonstrated that acute hypoxia can reduce the level of hepcidin mRNA in HepG2 cells cultured at 2% O2 and in vivo in mice housed in hypobaric hypoxia chambers simulating an altitude of 5,500 m.43 It has been also demonstrated that chronic hypoxia reduced the expression of hepcidin mRNA in HepG2 cells cultured at 1% O2 and in liver from rats placed in atmospheric chambers containing 10% O2.44 However, the molecular mechanisms by which hypoxia can reduce hepcidin expression remain unexplained. A major mechanism of CO toxicity is tissue hypoxia due to binding of CO to hemoglobin, myoglobin, and other hemoproteins, and inhibition of cytochrome-dependent electron transport. In this regard, CO might reduce hepcidin expression, at least in part, through its induction of hypoxia.

We also examined the effect of CO on alternative pathways to hepcidin expression. Bone morphogenetic proteins (BMPs) are cytokines belonging to the TGF-β superfamily. Binding of BMP to complexes of receptors causes phosphorylation of type I receptors. Activated BMP type I receptors subsequently phosphorylate the BMP-responsive SMAD 1/5/8. These proteins form complexes with a common mediator SMAD-4, which translocates to the nucleus to regulate transcription of BMP responsive genes. BMPs have been found to have a previously unexpected role in iron metabolism through inducing hepcidin expression in HepG2 and in primary murine hepatocytes.45-47 In addition to the SMAD pathway, non-SMAD-dependent pathways of BMP signaling include extracellular signal-regulated protein kinases (ERK1/2).27 A recent paper demonstrating cross-talk between the BMP/hemojuvelin and ERK1/2 pathways, showed that ERK1/2 activation by holotransferrin provoked increased levels of phospho-Smad1/5/8.48 Our data suggest that CO regulates hepcidin expression in part via ERK1/2 regulation. A previous study reported that ERK1/2 activation was significantly attenuated in the presence of CO in human airway smooth muscle cells.49

In conclusion, the current study demonstrated that the down-regulation of cytokine-induced (i.e., IL-6) hepcidin expression by CO involves two distinct and separable mechanisms: the inhibition of STAT-1/3 activation through a SOCS-3 dependent mechanism, and the inhibition of CREBH maturation (see Fig. 6 for diagram). Furthermore, we have shown that CO can exert a suppressive effect on ER stress-induced hepcidin expression in vitro and in the mouse model.

The results of this study support the notion that CO, one of the biologically important gases, could potentially be used in therapeutic strategies designed to control inflammation. This
promise stems largely from animal modeling studies which highlight protective effects of inhaled CO in organ injury models. The pharmacological application of CO, using CORMs and related compounds may provide a feasible alternative to the application of inhaled gas. The principle advantage of using CORMs involves the delivery of CO to tissues without excessive carboxyhemoglobinemia. Determination of the safety and feasibility of pharmacological CO application in humans, however, await further experimentation and clinical trials.

Acknowledgements

This work is supported by grant from the Korea Research Foundation Grant funded by the Korean government (MOEHRD) (BRL-2011-0087350)

Authorship Contributions

Da-Yong Shin, performed research, analyzed data, and wrote the paper
Jihwa Chung, performed research, designed research
Yeonsoo Joe, performed research, designed research
Hyun-Ock Pae, performed research, designed research
Ki Churl Chang, designed research, performed research
Gyeong Jae Cho, performed research, designed research
Stefan W. Ryter, designed research, wrote the paper
Hun-Taeg Chung, designed research, wrote the paper.

Disclosure of Conflicts of Interest

The authors declare that they have no competing interests.

References


40. Kaufman, R.J. Stress signaling from the lumen of the endoplasmic reticulum:


**Figure 1. CO inhibits hepcidin expression by IL-6 and ER stress inducers.** HepG2 cells were treated with IL-6 (10 ng/ml) (A) or TM (10 mg/ml) (B) or TG (10 mM) (C) or homocysteine (1 mM) (D) for the indicated periods of time. The expression levels of hepcidin were analyzed by Western blotting (*Upper panels*). Cells were pre-incubated for 3 hours with CORM-2 (20 µM) and were then exposed for 18 hours to IL-6, TM, TG, or homocysteine (*Lower panels*). (E) HepG2 cells were treated with CORM-2 or RuCl₂ and treated with TM for 18 hours. (A-E) β-actin served as the standard. Values are means ± SEM from 3 independent experiments. *P < 0.05 ; **P < 0.01 ; ***P < 0.001.
Figure 2. CO attenuates IL-6-induced STAT-3 activation. (A) HepG2 cells were treated with IL-6 (10 ng/ml) in a time-dependent manner, and then total cell lysates were probed with antibody for tyrosine phosphorylated or total STAT-3. Cells were pre-incubated for 2 hours with CORM-2 (20 µM) and were then exposed for 30 minutes to IL-6 (B, D). (C) HepG2 cells were transiently transfected with STAT-3 dominant negative (DN) vector or STAT-3 wild-type vector or empty vector and exposed to 10 ng/ml of IL-6 for 18 h in the presence or absence of CORM-2. Cells were transiently transfected with siRNA against human SOCS-3. After transfection, the cells were incubated with CORM-2, and then exposed to IL-6 for 30 min (E), or for 18 hours (F). Values are means ± SEM from 3 independent experiments. *P < 0.05 ; **P < 0.01 ; ***P< 0.001.

Figure 3. CO inhibits IL-6-induced hepcidin expression via suppression of CREBH activation. (A) HepG2 cells were incubated with IL-6 for increasing time intervals. (B) To detect the cleavage and translocation of CREBH, cell lysates were divided into cytoplasmic and nuclear fractions, and each fraction was probed with CREBH-N antibody. Lamin A/C served as a marker for nuclear fractions. (C) Cells were pre-incubated for 3 hours with CORM-2 (20 µM) and then treated with IL-6 for 12 hours. CREBH-N was analyzed by Western blotting. (D) CREBH mRNA levels were analyzed by RT-PCR. (E, F) HepG2 cells were transiently transfected with scramble siRNA or CREBH siRNA, and pre-incubated for 3 hours with or without 20 µM of CORM-2, and then treated with IL-6 for 18 hours to induce hepcidin expression (F). To analyze HAMP mRNA level, cells were incubated with IL-6 for 4 hours (E). β-Actin served as the standard for Western blots. GAPDH served as the standard of mRNA. Values are means ± SEM from 3 independent experiments. *P<0.05 ; **P < 0.01 ;

Figure 4. CO inhibits ER stress-induced hepcidin expression via suppression of CREBH activation. (A) HepG2 cells were treated with TM (10 mg/ml) for the indicated times. (B) To detect cleavage and translocation of CREBH, cell lysates were divided into cytoplasmic and nuclear fractions after 12 h of TM treatment, and each fraction was probed with CREBH-N antibody. Lamin A/C served as a marker for nuclear fractions. (C) Cells were incubated in the absence or presence of CORM-2 (20 µM) and treated with TM for 12 hours to detect CREBH cleavage. (D) CREBH mRNA levels were analyzed by RT-PCR. (E, F, G) HepG2 cells were transiently transfected with SC siRNA or CREBH siRNA. TM and TG were treated for 5
hours (E) or 18 hours (F), and hepcidin expression was analyzed for mRNA (using RT-PCR) and protein levels. (G) Transfected cells were treated with CORM-2 or DMSO, and incubated for 18 h to induce hepcidin expression. β-Actin served as the standard for Western blots. GAPDH served as the standard of mRNA. Values are means ± SEM from 3 independent experiments. *P < 0.05 ; **P<0.01.

Figure 5. CO inhibits ER stress-induced hepcidin expression and controls iron homeostasis in the ER stressed mouse. C57BL/6 mice and C3H mice were pre-injected with CORM-3 (10 mg/kg/day) and then injected 3 hours later with either recombinant mIL-6 (25 µg/kg) or tunicamycin (1.5 mg/kg). Mice were sacrificed at the 48 hour time point. (A) Hepatic hepcidin gene expression was analyzed by RT-PCR in IL-6 injected C57BL/6 mice. (B) Hepcidin protein levels were detected from serum of IL-6 or TM injected C57BL/6 mice by mouse hepcidin ELISA. (C) Hepatic hepcidin gene expression was analyzed by RT-PCR in TM injected C57BL/6 mice. (D) Serum iron levels of C57BL/6 mice were determined using an Iron assay kit. (E) Iron accumulation in splenic macrophages of C3H mice were assessed by staining with Perls’ Prussian Blue. (F) Non-heme iron content was assessed in spleen tissue of C3H mice. 10 mice were used in each group. Values are means ± SEM from 3 independent experiments. *P<0.05; **P < 0.01 ; ***P < 0.001; (U: unspliced, S: spliced).

Figure 6. Schematic of proposed pathways. Down-regulation of cytokine-induced (i.e., IL-6) hepcidin expression by CO involves two distinct and separable mechanisms: the inhibition of STAT-1/3 activation through a SOCS-3 dependent mechanism, and the inhibition of CREBH maturation. CO can exert a suppressive effect on ER stress-induced hepcidin expression in vitro and in the mouse model. CO also regulates hepcidin expression in part via ERK1/2 regulation induced by BMP signaling.
Figure 1
Figure 3
Figure 4
[Figure 5]
Pretreatment with carbon monoxide releasing molecules suppresses hepcidin expression during inflammation and endoplasmic reticulum stress through inhibition of the STAT3 and CREBH pathways

Da-Yong Shin, Jihwa Chung, Yeonsoo Joe, Hyun-Ock Pae, Ki Churl Chang, Gyeong Jae Cho, Stefan W. Ryter and Hun-Taeg Chung