Carbon monoxide reverses adipose tissue inflammation and insulin resistance upon loss of ovarian function

Eun-Kyung Choi¹, Hyun-Jung Park¹, Ok-Joo Sul¹, Monisha Rajasekaran¹, Rina Yu²,

Hye-Seon Choi*¹

¹Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea
²Department of Food Science and Nutrition, University of Ulsan, Ulsan 680-749, Korea

Address correspondence to Hye-Seon Choi: Department of Biological Sciences, University of Ulsan, Ulsan 680-749, Korea.

E-mail: hschoi@mail.ulsan.ac.kr. TEL: +82-52-259-1545; FAX: +82-52-259-1694.

Running Title: Role of CO in AT inflammation
We hypothesized that carbon monoxide (CO) might suppress chronic inflammation which led to metabolic disturbances. Ovariectomy (OVX) was performed in mice to mimic chronic inflammation secondary to loss of ovarian function. OVX increased fat mass and the infiltration of highly inflammatory CD11c cells into adipose tissue (AT), resulting in a disturbance of glucose metabolism. Treatment of CO attenuated these; CO decreased recruitment of CD11c expressing cells in AT and reduced expression of CD11c in bone marrow-derived macrophages, protecting them from M1 polarization. Up-regulated cGMP and decreased reactive oxygen species were responsible for the inhibitory activity of CO on CD11c expression; knockdown of soluble guanylate cyclase or heme oxygenase-1 using small interfering RNAs substantially reduced this inhibition. Improved OVX-induced insulin resistance (IR) by CO was highly associated with its activity to attenuate AT inflammation. Our results suggest a therapeutic value of CO to treat postmenopausal IR by reducing AT inflammation.

**KEYWORDS:** Loss of ovarian function; Carbon monoxide; CD11c; Adipose tissue; Inflammation; Insulin resistance
Postmenopausal women suffer from an increased incidence of metabolic diseases including bone loss, cardiovascular disease, and insulin resistance (IR) (36). Ovariectomy (OVX) in mice provides a generally accepted animal model of human menopause. Loss of ovarian function is associated with increased visceral fat and chronic inflammation (22, 38), suggesting that increased fat may cause chronic inflammation. However, the molecular and physiological mechanisms underlying obesity and the metabolic perturbations associated with loss of ovarian function are not clearly understood. In contrast, it is well-established that diet-induced obesity causes lipid accumulation in adipose tissue (AT) and leads to cellular stress and inflammation, resulting in IR (44). The increased fat leads to the accumulation of adipose tissue macrophages (ATMs), which play a critical role in chronic inflammation and the development of IR (28). Decreased ATM level or reduced macrophage activity improves systemic glucose homeostasis and insulin sensitivity (26, 52), whereas elevated macrophage infiltration is associated with a reduction in insulin sensitivity in diet-induced obesity (19).

Furthermore, myeloid-specific deletion of IKKβ improves obesity-induced IR (1), emphasizing the critical role of macrophage-induced inflammation. Macrophages are classified into two populations. M1 macrophages which express CD11c specifically are recruited to AT in obesity (28) and produce pro-inflammatory cytokines to lead to the induction of IR, indicating a connection between CD11c expression and IR. In contrast, M2 ATMs which possess high levels of CD163, arginase-1, or IL-10 contribute to tissue repair (11).

Carbon monoxide (CO) is a byproduct of heme oxygenase (HO) (40). Deficiency of HO-1, an inducible isoform of HO, promotes inflammation (37). HO-1 induction in response to enteric bacteria prevents colonic inflammation and HO-1-derived CO enhances bacterial clearance in vivo (32). These results imply that CO may act as a mediator for the cyto-
protective role of HO-1 against inflammation. Controlled amounts of CO have been shown to be therapeutically effective in a variety of pathophysiological conditions (53), although CO impairs oxygen transport when inhaled at high concentrations, leading to tissue hypoxia. Exogenous CO reduces the production of pro-inflammatory cytokines (33) and active inflammation in chronic inflammatory bowel disease (13). More beneficial effects of CO have been demonstrated in several other systems. It improves cardiac function in high fat diet (HFD)-induced metabolic syndrome (24) and protects against OVX-induced bone loss (35). Induction of HO-1 ameliorates IR in the obese mouse model of type 2 diabetes (27), suggesting a possibility of the therapeutic benefit of CO in chronic inflammation.

In the present study, we investigated whether CO protects against OVX-induced IR in mice by reducing AT inflammation.

MATERIALS AND METHODS

Reagents and antibodies. Recombinant mouse M-CSF was obtained from R & D Systems, Inc. (Minneapolis, MN, USA). Tricarbonyldichlororuthenium(II) dimmer ([Ru(CO)3Cl2]2 or CORM-2) was obtained from Sigma Chemical (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide as a stock solution. The inactive form of CORM2 (iCORM2) which was expected not to generate CO was prepared as described (41). Diphenyleneiodonium chloride (DPI), N-acetylcysteine (NAC), 1H-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and 8-bromoguanosine-3’,5’-cyclic monophosphate sodium salt (8-Br-cGMP) were from Sigma Chemical. Cilostazol was generously provided by Otsuka Pharmaceutical Co. (Japan).

Animals and study design. Six-week-old female C57BL/6J mice were subjected to either sham operation or OVX under anesthesia using 2’,2’,2’-tribromoethanol (250 mg/kg, Sigma
Chemical.). CORM2 (5 mg/kg), iCORM2 (5 mg/kg) (which does not release CO), or vehicle was injected intraperitoneally (ip) every day for 12 weeks, starting two days after surgery. The mice were housed in the specific pathogen-free animal facility. All animal care and procedures were conducted according to the protocols and guidelines approved by the University of Ulsan Animal Care and Use Committee (UOUACUC) (2011-007). Food intake and body weight were monitored daily and weekly, respectively. After 12 weeks, mice were fasted for 6 h and killed by CO$_2$ asphyxiation. Blood was collected by cardiac puncture, and tissues were immediately harvested. Blood glucose was measured with a commercially available enzyme assay kit (Asan Pharmacology, Hwa-Seong, Korea). Glucose and insulin tolerance tests were performed on 6 h-fasted mice. Animals were injected with glucose (i.p, 1 mg/g) for glucose tolerance tests, and recombinant human regular insulin (i.p, 0.75 unit/kg; Eli Lilly, Indianapolis, IN) was injected for measuring insulin tolerance. Blood samples were drawn from tail vein at 0, 15, 30, 60, 90 and 120 min after glucose or insulin injection. Serum H$_2$O$_2$ was determined with an Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA).

*Isolation of stromal vascular cells (SVC).* Visceral fat was obtained from the perigonadal adipose tissue depot (51). Visceral fat pads were weighed, rinsed three times in phosphate-buffered saline (PBS), and minced with FACS buffer (PBS with 1% BSA). Tissue suspensions were centrifuged at 500 ×g for 5 min and then treated with type 2 collagenase (1 mg/ml; Sigma Chemical) for 90 min at 37°C with shaking. Cell suspensions were filtered through a 100 µm filter and centrifuged at 500 ×g for 5 min. SVC pellets were incubated with RBC lysis buffer (eBioscience, San Diego, CA) for 5 min, centrifuged at 300 ×g for 5 min, and resuspended in FACS buffer. SVC were incubated with Fc blocker for 20 min at 4°C before staining with fluorescently labeled primary antibodies or control Ig G: FITC-
conjugated anti-F4/80 (BM8, diluted at 1:200) and FITC-conjugated rat IgG2a isotype control, FITC-conjugated anti-CD4 (GK1.5, diluted at 1:100) and FITC-conjugated rat IgG2b isotype control, phycoerythrin (PE)-conjugated anti-CD11c (M418, diluted at 1:100) and PE-conjugated Ameni hamster IgG2a isotype control, PE-conjugated anti-CD11b (M170, diluted at 1:200) and PE-conjugated rat IgG2b isotype control, PE-conjugated anti-CD8a (53-6.7, diluted at 1:100) and PE-conjugated rat IgG2a isotype control (eBiosciences, San Diego, CA, USA), Alexa Flour 647-conjugated anti-CD206 (MR5D3, diluted at 1:10) and their isotype control (AbD Serotec, Oxford, UK). Cells were washed gently twice, resuspended in FACS buffer, and analyzed using a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA).

Detection of intracellular expression HO-1. Intracellular HO-1 was detected by using the fixation/permeabilization buffer (eBioscience) according to the manufacture’s instruction. Briefly, BMM surface-stained with FITC-conjugated anti-F4/80 was followed by intracellular staining with PE-conjugated anti-HO-1 (HO-1-2, diluted at 1:100) and PE-conjugated rat IgG2b served as an isotype control (Enzo Life Sciences, NY, USA).

Cell preparation. Femora and tibiae were removed aseptically and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out with DMEM from one end of the bone, using a sterile 21-gauge needle. The bone marrow was further agitated using a Pasteur pipette to obtain a single-cell suspension, which was washed twice and incubated on plates with M-CSF (30 ng/ml) for 16 hours. Nonadherent cells were then harvested, layered on a Ficoll-Hypaque gradient, and cultured for two more days, by which time large populations of adherent monocyte/macrophage-like cells had formed on the bottoms of the culture plates as previously described (22). The few nonadherent cells were removed by washing the dishes with PBS, and the adherent cells (bone marrow-derived macrophages, BMMs) were harvested and seeded on plates. The adherent cells were analyzed
by FACS and found to be negative for CD3 and CD45R and positive for CD11b. The absence of contaminating stromal cells was confirmed by lack of cell growth in the absence of M-CSF. Additional medium with M-CSF was added and later replaced on day three. After incubation for the recommended times, the cells were analyzed using a FACSCanto II flow cytometer. BMM were transfected with small interfering RNAs (siRNAs) against p47phox (sip47phox), soluble guanylate cyclase (sisGC), heme oxygenase-1 (siHO-1), and scrambled siRNA (scRNA) (Santa Cruz Biotech., Santa Cruz, CA), using Lipofectamine™ RNAiMAX (Invitrogen). Lipofectamine™ RNAiMAX (2 µl) was first diluted in DMEM (50 µl) without serum, mixed with an equal volume of DMEM containing 30 nM of siRNA, and incubated for 20 min. The medium from the cells was removed and the fresh medium without serum was added. The resulting RNAiMAX/siRNA was added directly to the cells, giving a final volume of 700 µl. After 8 h incubation, the cells were replenished with serum-containing medium and cultured for the indicated times for further analysis. Bone marrow cells were obtained from Nrf2+/- (Wild type, WT) and Nrf2−/− (Nrf2-knockout, KO) mice in the background of B6.129X1-Nfe2l2tm1Ywk/J strain which were provided from Jackson Laboratory. The genomic DNA was extracted from the tail of the mice for genotyping by PCR.

cGMP assay. BMM were stimulated with M-CSF with or without CORM2, and 300 µM IBMX (3-isobutyl-1-methylxanthine) (Sigma Chemical) was added to the cells to prevent phosphodiesterase degradation of cGMP 20 min before the end of the exposure. Cell extracts were prepared as described (33). The concentration of cGMP was determined with an EIA kit (Cayman, Ann Arbor, MI).

Quantitative PCR (qPCR). Total RNA from BMMs incubated with M-CSF for the indicated times was extracted with Trizol solution (GIBCO, Life Technol.) and reverse-transcribed with oligo-dT and Superscript I (Invitrogen). qPCR was carried out using SYBR Green 1 Taq polymerase (Qiagen, Hilden, Germany) and appropriate primers on a DNA
Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc.). The specificity of each primer pair was confirmed by melting curve analysis and agarose-gel electrophoresis. GAPDH was amplified in parallel with the genes of interest. Relative copy numbers compared to GAPDH were calculated using $2^{-\Delta\Delta C_t}$. The primer sequences used were as follows: 5’-ctggatagcctttcttgctg-3’ and 5’-gcacactgtgtccgaactc-3’ (CD11c); 5’-cagctgggctgtacaaacctt-3’ and 5’-cattggaagtgaagcgtttc-3’ (iNOS); 5’-tctgtctactgaacctcgggtgatcgt-3’ and 5’-gtatgagatagcaatcggctgacgggttggg-3’ (TNF-α); 5’-ctccaagccaaagtcttagag-3’ and 5’-aggagtgtgatggacatgac-3’ (arginase-1); 5’-tcagttggcaatgccatct-3’ and 5’-agggcggaccagagaga-3’ (sGC); 5’-gccctctgactctcagatg-3’ and 5’-ggattggcctcttg-3’ (HO-1); 5’-gatgttccccattgaggcc-3’ and 5’-gtttcaggtcatcaggccgc-3’ (p47phox); 5’-acccagaagactggtgatgg-3’ and 5’-cacattgagggtagggaac-3’ (GAPDH).

**In vivo phospho-Akt signaling study.** Sham or OVX mice fasted for 16 h prior to i.p. injection of 4 unit/kg insulin (Lilly). After 5 min, mice were anesthetized and white adipose tissues (WAT) were collected and stored in liquid nitrogen. For protein isolation, WAT were homogenized in 1 x RIPA lysis buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS) and fractionated by a 10% SDS-PAGE, followed by electroblotting to visualize the phosphorylated (Thr308) Akt (diluted at 1:1000, Cell Signaling, Beverly, MA, USA) and total forms of Akt (diluted at 1:1000, Cell Signaling). β-actin (1:1000, Sigma Aldrich) was used as loading control. Insulin-induced phosphor-Akt was evaluated by normalization over total Akt signaling, using Image J software.

**Statistical analysis.** Values are expressed as mean ± SEM. Student’s *t*-test was used to evaluate differences between samples of interest and the corresponding controls. Differences between groups were assessed by one-way ANOVA, followed by Bonferroni post-tests. A *P* value of less than 0.05 was considered statistically significant.
RESULTS

CO reduces metabolic perturbation after OVX. To investigate whether CO counteracts the adverse effects of metabolic disorders resulting from loss of ovarian function, we administered CO after OVX in mice. The body weight increase in the OVX mice was reduced by CO treatment at 12 weeks (Fig. 1A, Table 1). Along with body weight, the fat masses of visceral and subcutaneous AT were significantly reduced in the CO-administered mice (Table 1). Neither OVX nor CO treatment provoked a significant difference in daily food intake (Table 1), suggesting that an altered metabolic rate was responsible for the increased fat mass and body weight induced by OVX. Injection of CO also counteracted the elevated levels of blood glucose and serum insulin induced by OVX (Table 1). To confirm that the CO treatment reduced the glucose tolerance resulting from OVX, we measured glucose clearance following an intraperitoneal injection of glucose. In CO-administered OVX mice, the glucose level decreased significantly after glucose injection, and the area under the curve (AUC) for glucose tolerance also decreased compared with that of vehicle-treated mice (Fig. 1B). CO administration did not lead to any change in the sham-treated mice (Table 1). Following intraperitoneal injection of insulin to determine insulin sensitivity, glucose was cleared more effectively in the CO-injected OVX mice than in the controls (Fig. 1C). Since the phosphoinositol 3-kinase (PI3K)-Akt pathway is responsible for the metabolic effects of insulin, we examined the phosphorylation of Thr308 of Akt as an indicator of Akt activity (55) 5 min after insulin injection. The visceral fat of CO-treated OVX mice had significantly greater Akt activity than that of vehicle-treated OVX mice (Fig. 1D). As a control, we showed that Akt activity in sham mice exposed to insulin was higher than in OVX mice.
exposed to insulin, indicating that OVX induces resistance to insulin signaling.

**CO attenuates AT inflammation by decreasing M1 macrophages.** It is well-established that AT inflammation caused by adipose tissue macrophages (ATMs), is responsible for IR in obesity (52) and CO has been shown to be anti-inflammatory *in vivo* and *in vitro* (33). These findings prompted us to see if we could link the cause of the reduced IR observed in CO-injected OVX mice to a reduction in ATMs. To assess whether OVX led to immune cell infiltration, we evaluated numbers of stromal vascular cells (SVC) in visceral fat pads by flow cytometry. A pronounced increase in ATM levels which were indicated by CD11b^{+}F4/80^{+} cells, was found in vehicle-treated OVX mice, whereas fewer ATMs accumulated in CO-administered OVX mice (Fig. 2A). To discriminate between M1 and M2 ATMs by flow cytometry, we used Abs against CD11c and CD206 as specific surface markers of M1 and M2 cells, respectively. The number of CD11c^{+}F4/80^{+} cells was higher in OVX mice than in sham mice, and CO administration reduced their number (Fig. 2B). Conversely CO administration increased CD206^{+}F4/80^{+} cells in the OVX mice (Fig. 2C). In addition, T cells that expressed CD4 and CD8 were more frequent in OVX mice than in sham mice, and CO administration counteracted these effects (Fig. 2D and 2E). These findings indicate that CO acts to decrease inflammation caused by inflammatory immune cells in AT after OVX. Because CD11c is a marker of M1 macrophages which are the type that cause inflammation, we focused on CD11c cells in subsequent work.

**CO decreases CD11c cells by reducing their recruitment to AT as well as by down-regulating CD11c expression via generation of cGMP and scavenging of ROS.** To investigate how CO decreases CD11c cells in the AT of OVX mice, we asked whether it reduces the influx of CD11c-expressing cells. Using CD11b as a marker of monocytes, we classified blood monocytes as CD11c^{+} or CD11c^{-} cells. As shown in Fig. 3A, significant increase in the proportion of CD11c cells among total leukocytes was observed in OVX mice. Treatment
with CORM2 decreased the influx of CD11c-expressing cells into the AT of the OVX mice, suggesting that CO does indeed reduce cell recruitment. A contribution of blood neutrophils to the elevated levels of CD11bCD11c upon OVX was excluded by sorting the Ly6G negative cell population. Next, to examine whether CO also affects the phenotype of resident macrophages, bone marrow-derived precursors were exposed to M-CSF. After a 4 d exposure to M-CSF, ~23% of the total BMM expressed CD11c and F4/80, and CO reduced this percentage (Fig. 3B). Similar pattern was found at mRNA level of CD11c (Fig. 3C). Co-administration of IFN-γ and LPS induced M1 polarization further than M-CSF alone [62% (IFN-γ and LPS) vs. 23% (M-CSF)], but the inhibitory effect of CO was less [17% (IFN-γ and LPS) vs. 43% (M-CSF)]. To analyze other characteristics of the M-CSF-stimulated cells, we examined the transcript levels of specific markers. Administration of CO resulted in decreased transcript levels of CD11c, TNF-α, and iNOS, and increased transcript levels of arginase-1, IL-10, and HO-1 (Fig. 3D), demonstrating that CO opposes the polarization of BMM to M1.

Since CO reduced M-CSF-induced CD11c expression in BMMs, we sought to identify an intermediate in this process. CO binds ferrous heme and there is evidence that it forms complexes with heme proteins such as soluble guanylate cyclase (sGC) (48). We therefore wondered whether the decrease of CD11c expression in response to CO was mediated by sGC. As shown in Fig. 3B, cell permeable 8-Br-cGMP alone reduced CD11c in BMM upon M-CSF stimulation, and a pharmacological inhibitor of GC, ODQ indeed abolished the CO-induced decrease in CD11c expression, whereas a phosphodiesterase inhibitor, cilostazol enhanced it (Fig. 3B). To ascertain whether CO modulates cGMP levels in BMM, we determined the cGMP content of M-CSF-stimulated BMM. As shown in Fig. 4A, cGMP started to increase after 1 h exposure to M-CSF alone, and reached a plateau level after 6 h. Addition of CO elevated cGMP level after 2 h stimulation compared to M-CSF alone, and it
remained elevated for up to 24 h (Fig. 4A). Cilostazol also increased cGMP, and co-treatment with CO produced a level of cGMP higher than that achieved with CO alone (Fig. 4B). To confirm that CO induces CD11c expression by activating sGC, siRNA for sGCβ1 (sisGCβ1) or scRNA was transfected into BMMs. Down-regulation of sGC (Fig. 4C) decreased but did not completely block the effect of CO on CD11c expression after M-CSF stimulation, suggesting that another mediator is implicated in the inhibitory effect of CO on CD11c expression.

We hypothesized that ROS may play a role for the effect of CO to decrease CD11c. Since CO decreased ROS upon RANKL stimulation in osteoclasts (35) that share common precursors with macrophages, we tested whether CO affected M-CSF-induced ROS levels. As shown in Fig. 4D, substantial levels of ROS was generated in BMM upon M-CSF exposure, and exogenous CO opposed this effect. However, exogenously added ROS enhanced CD11c and abolished the inhibitory effect of CO on CD11c expression (Fig. 4D), suggesting an involvement of ROS in the inhibitory effect of CO on CD11c expression. To confirm this idea, we exposed cells to DPI, a selective inhibitor of nicotinamide adenine dinucleotide (NADPH) oxidase and to NAC, a ROS scavenging antioxidant. NAC significantly decreased CD11c expression and attenuated the inhibitory effects of CO on CD11c expression in response to M-CSF stimulation, whereas DPI did not (Fig. 4D). Moreover downregulation of p47phox that is required for the activation of the superoxide producing NADPH oxidase by siRNA did not affect the inhibitory effect of CO on CD11c expression (Fig. 4E), supporting the view that CO is involved in ROS scavenging rather than ROS generation. Because CO induced HO-1 mRNA in BMM exposed to M-CSF stimulation (Fig. 3C), we knocked down HO-1 in BMM. This substantially diminished the inhibitory effect of CO on CD11c expression and ROS level (Fig. 4E), supporting that HO-1 also mediates the effect of CO on CD11c expression BMM. Since HO-1 induction by CO has
reported to be via Nrf2 activation in endothelial cells (21), we also investigated whether Nrf2
is associated with HO-1 induction by CO in BMM. As shown in Fig. 4F, the lack of Nrf2
completely abolished HO-1 induction by CO and diminished the decrease of CD11c
expression by CO, suggesting that HO-1 induction by CO is through Nrf2 and Nrf2
contributes to the effect of CO on decreasing CD11c expression
in BMM. To ascertain whether CO acts through two parallel pathways, we determined the
effect of 8-Br-cGMP on expressions of HO-1 and CD11c. As shown in Fig. 3B and 4G, 8-Br-
cGMP decreased the expression of CD11c, but not that of HO-1 at the protein level. ODQ
also did not change it, indicating that cGMP does not affect HO-1 to attenuate CD11c
expression. Taken together, CO works via two independent pathways, cGMP production and
HO-1 induction to decrease CD11c expression in BMM.

DISCUSSION

We have demonstrated that CO protects against OVX-induced metabolic disturbances in vivo. OVX increased body weight and fat mass, whereas administration of CORM2, but not
iCORM2, opposed these effects. Studies in rats consistently demonstrate that OVX-induced
obesity is in part due to OVX-induced hyperphagia (7). However, our OVX model in mice
did not show hyperphagia under our experimental conditions. Similar pattern was observed in
other studies (38, 51), reflecting the discrepancies due to species and duration of surgery. No
difference in food intake between sham and OVX mice suggested that the obesity in OVX
mice may be caused by altered metabolic rate. Although we have not performed a pair-
feeding experiment, it is likely that CO treatment of OVX mice prevented from increased
adiposity, since CO also did not show a significant difference in food intake, excluding the
possibility that CO affects central nervous system to reduce OVX-induced obesity. In
addition, it has not been clearly demonstrated that CORM crosses blood brain barrier, although released CO gas has been considered to cross the membranes. CO also reduced IR induced by OVX; these are demonstrated by insulin tolerance test (Fig. 1C) and recovered insulin signaling in AT (Fig. 1D). A protective effect of CO on metabolic syndrome has been suggested in several other studies (24, 35). The benefits of CO can be inferred from the reported effects of HO-1 which generates CO along with other byproducts from heme degradation: induction of HO-1 reduces hyperglycemia and lowers oxidative stress and inflammation along with increased levels of cGMP, plasma bilirubin, and ferritin in the Goto-Kakizaki rat model of type 2 diabetes (31), and insulin sensitivity has been improved by increased HO-1 activity in the ob/ob mouse model of T2D (27), supporting our results. However, the role of HO-1 in metabolic disease is controversial. Recently, HO-1 has been reported to play pathological roles in the development of inflammation and metabolic disease using cell type-specific deletion of HO-1 (18) or with extended HO-1 expression (2), suggesting that HO-1 induces inflammatory and metabolic genes rather than suppress them. These confictions are also demonstrated in human studies (3, 43). Similar pattern has been reported in the role of Nrf2 which is an upstream regulator of HO-1 on obesity. Chemical induction of Nrf2 by oltipraz (57) reduces obesity, whereas genetic deletion of Nrf2 decreases obesity (4). It needs to require further investigation to fully elucidate the role of HO-1 in metabolic disease. In contrast, higher levels of inhaled CO are strongly associated with the development of metabolic syndrome and cardiovascular disease (6). Symptoms of CO poisoning start to appear at 20% of carboxyhemoglobin, while death occurs over 50% of carboxyhemoglobin (39). By contrast, mice that received CORM2 injections at 3-100 mg/kg achieved blood carboxyhemoglobin levels of 0.46-2.15% (46), a much lower level that does not have harmful effects in mammals.

We showed that OVX increased macrophages, CD11cF4/80 and CD11bF4/80 cells in AT
along with enlarged visceral fat mass. The cells expressing CD11cF4/80 were ~10% of those expressing CD11bF4/80 in OVX, and similar pattern was found in HFD-induced obesity (23). Increased fat is associated with increased ATM levels (45), which induce IR by generating pro-inflammatory cytokines, suggesting a critical role of macrophages in IR. IR due to HFD-induced obesity was not protected by adipocyte-specific overexpression of HO-1 (17), but was ameliorated by myeloid HO-1 haploinsufficiency (16), demonstrating the importance of macrophages in the development of IR. IR is promoted by pro-inflammatory cytokines (47), but attenuated by anti-inflammatory cytokines (15). The cells that express CD11c, a marker of classically activated M1 macrophages (28), exhibit inflammatory characteristics; they release pro-inflammatory cytokines and contribute to HFD-induced AT inflammation (54). Depletion of CD11c leads to a dramatic decrease in both local and systemic inflammatory markers linked to the development of obesity-associated IR (34), supporting the role of CD11c in inflammation and IR. The elevated CD11c expression in ATMs upon OVX can be explained by the previous findings that OVX increased serum levels of M-CSF (20) and MCP-1 (22). M-CSF drives the expansion and differentiation of macrophages that are of myeloid lineage. In addition, the CD11c expression has been positively associated with M-CSF (9, 30). A reduction in number of CD11c cells is observed in mice lacking functional M-CSF (30). Conversely, M-CSF treatment elevated CD11c cells in vivo (9). Deletion of MCP-1 also reduced CD11c expression along with serum level of M-CSF (22), indicating that M-CSF, MCP-1, or both are necessary for CD11c expression in vivo.

CO counteracted the OVX-induced immune cell infiltration in AT in our studies. It was supported by other findings. CORM3 attenuates the inflammatory response in colon tissue of TNBS-induced colitis by decreasing immune cell recruitment (10). In rodent models of ventilator-induced lung injury, CO prevents neutrophil recruitment, resulting in anti-inflammatory protection (14). Elevated HO-1 level during hypoxic inflammation inhibits
macrophage accumulation and activation, preventing the development of hypoxia-induced pulmonary hypertension (50).

CO increased and decreased the expressions of CD206 and CD11c, respectively at the surface of ATMs (Fig. 2C), suggesting that a microenvironment with elevated CO levels is not favorable for M1 macrophages. Then how CO attenuated CD11c expressing M1 macrophages in AT? CO opposed the increased recruitment of CD11c cells into AT from the blood upon OVX (Fig. 3A). In addition, CO converted the resident macrophage to reduce expression of CD11c along with TNF-α and iNOS in BMMs. How could CO act to decrease CD11c expression in BMM? Addition of 8-Br-cGMP alone decreased CD11c expression (Fig. 3B), indicating a role of cGMP in reducing CD11c. Cilostazol, a selective inhibitor of type 3 phosphodiesterase, when combined with CO, had an additive effect, suggesting that cyclic nucleotides may play roles to decrease CD11c expression. Actually, CO elevated intracellular cGMP levels and decreasing cGMP by knockdown of sGC or ODQ treatment attenuated the inhibitory effect of CO on CD11c expression, implying that CO generates cGMP via sGC. Elevation of cGMP via sGC has been implicated in the therapeutic effects of CO. CO activates sGC by interfering with oxygen binding via a differential heme pivoting and bending mechanism (29). The potential therapeutic benefit of CO in cisplatin-induced nephrotoxicity in vivo was suggested to be due to activation of sGC (49). CO imparts neuroprotection in vivo and in vitro against ischemia-reperfusion injury via activation of sGC (42). Down-regulation of sGC did not completely abolish the inhibitory effect of CO on CD11c expression, implying that another mediator is required for CO activity. OVX induces oxidative stress (22, 25, 35), suggesting that ROS may play a role in enhancement of CD11c expression, resulting in IR. ROS include highly reactive superoxide anion, hydroxyl radical, and stable H₂O₂. Since superoxide is rapidly converted to H₂O₂ in the cell, H₂O₂ has been regarded as the major ROS (12). CORM2 significantly opposed the elevated level of serum
ROS upon OVX (Table 1), suggesting that CO exerted its activities at least partly via reducing oxidative stress. The findings that CO induced HO-1 mRNA (Fig. 3C) and that down-regulation of HO-1, but not that of p47\textsuperscript{phox}, attenuated the inhibitory effect of CO on CD11c expression (Fig. 4E) suggested that the negative effect of CO on CD11c expression was due to ROS scavenging rather than impaired ROS generation. The ability of CO to induce HO-1 could act as a positive-feedback regulator to combat oxidative stress and inflammation efficiently. One of HO-1 products, bilirubin has been reported to increase insulin sensitivity and reduce AT inflammation in obese mice (8), suggesting that HO-1 may boost the activity of CO. Induction of HO-1 by CO has been demonstrated in endothelial cells to activate protein kinase R-like endoplasmic reticulum kinase, leading to Nrf2 activation followed by enhanced HO-1 promoter activity and increased expression of HO-1 (21). Consistently, we found that the ability of CO to decrease CD11c expression was also associated with Nrf2. HO-1 induction by CO disappeared in the Nrf2 deficiency, suggesting that CO induces HO-1 via Nrf2. The absence of Nrf2 also reduced the activity of CO to decrease CD11c expression partly, indicating that CO directs alternative ways to decrease CD11c expression.

We demonstrated that administration of CO attenuated AT inflammation by decreasing M1 macrophages which are represented by expression of enhanced CD11c and decreased CD206; it thus counteracted IR due to loss of ovarian function. CO attenuated recruitment of CD11c-expressing cells in AT. CO activated sGC to generate cGMP and induced HO-1 to reduce ROS, converging in decreased CD11c expression (Fig. 5). Exploiting the protective role of CO in OVX-induced metabolic disturbances could be helpful in the design of novel therapies for postmenopausal syndrome.
This work was supported by the Biotechnology & Medical Technology Development Program of NRF (2012M3A9C3048683) funded by the Korean government. EK Choi and OJ Sul were supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A6A1030318).

DISCLOSURES

The authors have declared that no competing interests exist.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: HSC, EKC. Performed the experiments: EKC, HJP, OJS, MR. Analyzed the data: EKC, OJS, RY, HSC. Wrote the paper: EKC, HSC.
REFERENCES


Fig. 1. CO counteracts the adverse effects of metabolic perturbation induced by OVX. Mice were subjected to OVX or sham surgery and administered vehicle, CORM2 (5 mg/kg), or iCORM (5 mg/kg) for 12 weeks (n=7 for each group). Numerical data are included in Table 1. Body weight change (A), IPGTT, i.p injection of glucose at the does of 1 mg/g body weight (B), IPITT, i.p injection of insulin at the dose of 0.75 units/kg body weight (C) were determined 12 weeks after sham or OVX surgery. Area under curve (AUC) for the IPGTT, IPITT (B, C) were measured and analyzed by 1-way ANOVA followed by Bonferroni post-tests. * P<0.05, ** P<0.01, *** P<0.001; vehicle-treated OVX vs. CO-treated OVX mice. ## P<0.01 compared with vehicle-treated SHAM mice. (D) Representative images western blots of in vivo phosphorylated Akt (T308). Visceral fat was collected after a 16 h fast with or without injection of insulin (i.p, 4 U/kg BW, 5 min) from sham, vehicle-treated OVX, and CORM2-treated OVX mice. β-Actin was used as loading control. Quantification of phosphorylated Akt normalized to total Akt. ** P<0.01; sham vs. OVX mice upon insulin stimulation, OVX vs. CORM2-treated OVX mice upon insulin stimulation. ## P<0.01, ### P<0.001; basal vs. insulin stimulation. Similar results were obtained in 3 independent experiments.

Fig. 2. CO decreases the number of immune cells found in response to OVX in AT. SVC from visceral fat were extracted from vehicle-treated (open bar) and CORM2-treated mice (oblique-lined bar) 12 weeks after sham or OVX surgery (n=6 for each group). SVCs were labeled with conjugated Abs to CD11bF4/80 (A), CD11cF4/80 (B), CD206F4/80 (C), CD4 (E), and CD8 (F), fractionated by FACS, and analyzed by 1-way ANOVA followed by Bonferroni post-tests. (D) Representative results of flow cytometry are shown. * P<0.05, **
\( P < 0.01 \); vehicle-treated OVX vs. CORM2-treated OVX mice. \#\# \( P < 0.01 \), \### \( P < 0.001 \) compared with vehicle-treated SHAM mice. Similar results were obtained in 3 independent experiments.

Fig. 3. CO decreases CD11c expressing cells by reducing recruitment to the AT and protecting against M1 polarization of BMM. (A) The cell population of monocytes expressing Ly6G^CD11b^CD11c^ in the blood of vehicle-treated (open bar) and CORM2-treated mice (oblique-lined bar) 12 weeks after sham or OVX surgery (n=6 for each group) was analyzed by 1-way ANOVA followed by Bonferroni posttests. ** \( P < 0.01 \); vehicle (V)-treated OVX vs. CORM2-treated OVX mice. \### \( P < 0.001 \) compared with vehicle-treated SHAM mice. (B) BMMs were incubated with iCORM (30 \( \mu \)M), cilostazol (CTZ, 10 \( \mu \)M), 8-Br-cGMP (30 \( \mu \)M), or ODQ (10 \( \mu \)M) in the presence or absence of CORM2 (30 \( \mu \)M) upon M-CSF (30 ng/ml) (V) stimulation for 4 d and labeled with conjugated Abs to CD11c and F4/80. # \( P < 0.05 \), \#\# \( P < 0.01 \), \### \( P < 0.001 \) compared with Vehicle (V)-treated cells. *** \( P < 0.001 \); compared to CORM2-treated cells in the presence of CTZ. (C) BMMs were stimulated with M-CSF in the presence or absence (V) of CORM2 (30 \( \mu \)M), 8-Br-cGMP (30 \( \mu \)M), or NAC (3 mM) for 3d, and total RNA was extracted and subjected to qPCR analysis. Expression levels before M-CSF treatment were set at 1.0. * \( P < 0.05 \), ** \( P < 0.01 \) compared with V-treated cells. Similar results were obtained in 3 independent experiments.

Fig. 4. CO decreases CD11c expression by activating sGC and scavenging ROS. cGMP was measured in BMMs upon stimulation of M-CSF (30 ng/ml) with vehicle (V), CORM2 (30 \( \mu \)M), cilostazol (10 \( \mu \)M), or iCORM2 (30 \( \mu \)M) for 0, 1, 2, 6, 12, 24 h (A) or 6 h (B). \### \( P < 0.001 \) compared with 0 h of V-treated cells (A). \# \( P < 0.05 \), \#\# \( P < 0.01 \) compared with V-treated cells (A, B). ** \( P < 0.01 \) compared with cilostazol-treated cells in the presence of
CORM2 (B). (C, E) BMMs were transfected with scRNA, sisGC, sip47phox, or siHO-1. Downregulation of sisGC (C), sip47phox, and siHO-1 (E) was confirmed by RT-PCR and qPCR. The expression level in scRNA-treated cells was at 1. After 8 h of transfection with siRNA, cells were incubated further with M-CSF for 4 d before measuring CD11c-stained cells. *P<0.05, **P<0.01, ###P<0.001 compared with scRNA-transfected cells. *P<0.05, **P<0.01, ***P<0.001 compared with V-treated cells. Numbers above the boxes are ratios of CD11cF4/80-labeled CORM2-treated cells (oblique-lined bar) to V-treated cells (open bar). (D) BMMs were incubated with DPI (20 nM), NAC (3 mM), or H2O2 (300 μM) in the presence or absence of CORM2 (30 μM) upon stimulation of M-CSF (30 ng/ml) (V) for 4 d and labeled with conjugated Abs to CD11c and F4/80. Intracellular levels of ROS upon stimulation with M-CSF for 2 d in the presence or absence of CORM2 (30 μM) or iCORM (30 μM) were determined in BMMs using H2DCFDA. **P<0.01, ###P<0.001 compared with V-treated cells. ***P<0.001 compared with CORM2-treated cells in the presence of DPI. (F) BMMs from WT and Nrf2-KO mice were stimulated with M-CSF in the presence or absence (V) of CORM2 (30 μM) for 2 d (HO-1) or 4 d (CD11c). Total RNA was extracted and subjected to qPCR analysis. *P<0.05 compared with WT cells. *P<0.05, ***P<0.001 compared with V-treated cells. Numbers above the boxes are ratios of CORM2-treated cells to V-treated cells. (G) BMMs were stimulated with M-CSF in the presence or absence (V) of CORM2 (30 μM), 8-Br-cGMP (30 μM), or ODQ (10 μM) for 2 d and labeled with conjugated Abs to HO-1 and F4/80 as described in Materials and Methods. **P<0.01 compared with Vehicle (V)-treated cells. Similar results were obtained in 3 independent experiments.

Fig. 5. Schematic representation of the inhibitory effect of CO on OVX-induced infiltration of M1 macrophages into the AT. CO attenuates AT inflammation by opposing the increase in
M1 macrophage levels provoked by CD11c expression, so reducing IR provoked by loss of ovarian function. CO activates sGC to generate cGMP as well as induces HO-1 via Nrf2 to decrease ROS, resulting in decrease of CD11c expression. The findings in the present study and in other reported studies were marked by bold lines and fine lines, respectively.
Body weight [g] after OVX

Weeks after OVX

** SHAM  OVX  OVX+CORM2

-    +    +     -      +      +      -     +     +

Insulin

FIG 1.
FIG 3.
FIG 4.
Fig. 5

Lean

Obese Adipose Tissue

M2  M1

OVX

Insulin Resistance

M-CSF
C-Fms

Cytoplasm

NOX 2

CO-RMs

ROS

sGC activation

Nrf2

HO-1

Nucleus

ARE

CD11c

CD11c+

GTP

cGMP
Table 1. Metabolic characteristics of SHAM-treated and OVX WT and CORM-treated mice at 12 wk after surgery

<table>
<thead>
<tr>
<th>Variable</th>
<th>SHAM V</th>
<th>SHAM CORM2</th>
<th>OVX V</th>
<th>OVX CORM2</th>
<th>OVX iCORM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased body weight [g]</td>
<td>7.510±0.240</td>
<td>6.270±0.120</td>
<td>10.93±0.770***</td>
<td>8.960±0.330#</td>
<td>10.88±0.420</td>
</tr>
<tr>
<td>Subcutaneous fat [mg]</td>
<td>510.0±43.91</td>
<td>397.0±27.30</td>
<td>1730±176.0**</td>
<td>1275±112.5#</td>
<td>1725±123.7</td>
</tr>
<tr>
<td>Visceral fat [mg]</td>
<td>352.5±48.24</td>
<td>210.0±21.82</td>
<td>1732±151.9***</td>
<td>1291±125.0#</td>
<td>1552±157.1</td>
</tr>
<tr>
<td>Serum H₂O₂ [nmol/ml]</td>
<td>48.15±1.490</td>
<td>46.73±1.970</td>
<td>68.70±1.390***</td>
<td>62.12±2.210#</td>
<td>67.00±2.440</td>
</tr>
<tr>
<td>Blood insulin [ng/ml]</td>
<td>0.6130±0.040</td>
<td>0.4940±0.020</td>
<td>0.8470±0.080**</td>
<td>0.5960±0.010#</td>
<td>0.9630±0.060</td>
</tr>
<tr>
<td>Food intake [g/day]</td>
<td>3.760±0.150</td>
<td>3.535±0.081</td>
<td>3.440±0.080</td>
<td>3.320±0.240</td>
<td>3.540±0.170</td>
</tr>
</tbody>
</table>

The data represent the mean ± S.E. ( n=7 per group)

No significant change between V-treated SHAM mice vs. CORM2-treated SHAM mice.

V (vehicle)-treated SHAM mice vs. V-treated OVX mice; ** P<0.01, *** P<0.001

V-treated OVX mice vs. CORM2-treated OVX mice; # P<0.05, ## P<0.01

No significant change between V-treated OVX mice vs. iCORM-treated OVX mice.