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

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POST MENOPAUSAL 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 proinflammatory 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 cytoprotective 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 proinflammatory 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 macrophage colony-stimulating factor (M-CSF) was obtained from R & D Systems (Minneapolis, MN), Tricarbonyldichlororuthenium(II) dimmer ([Ru(CO)₂Cl₃]₂ or CORM2) was obtained from Sigma Chemical (St. Louis, MO) 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). Diphenylsulfoxonium chloride (DPI), N-acetylcysteine (NAC), 1H-oxadiazolo[4,3-a]quinazolin-1-one (ODQ), and 8-bromo-guanosine-3',5'-cyclic monophosphate sodium salt (8-Br-cGMP) were from Sigma Chemical. Cilostazol was generously provided by Otsuka Pharmaceutical (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 (which does not release CO; 5 mg/kg), or vehicle was injected intraperitoneally (ip) every day for 12 wk starting 2 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 (2011-007). Food intake and body weight were monitored daily and weekly, respectively. After 12 wk, mice were fasted for 6 h and euthanized by CO2 asphyxiation. Blood was collected by cardiac puncture, and tissues were harvested immediately. Blood glucose was measured with a commercially available enzyme assay kit (Asan Pharmacology, HwaSeong, South Korea). Glucose and insulin tolerance tests were performed on 6-h-fasted mice. Animals were injected with glucose (1 mg/g ip) for glucose tolerance tests, and recombinant human regular insulin (0.75 U/kg ip; Lilly, Indianapolis, IN) was injected for measuring insulin tolerance. Blood samples were drawn from the tail vein at 0, 15, 30, 60, 90, and 120 min after glucose or insulin injection. Serum H2O2 was determined with an Amplex Red hydrogen peroxide/peroxidase assay kit (In Vitrogen, Carlsbad, CA).

Isolation of stromal vascular cells. 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 fluorescence-activated cell sorting (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. Stromal vascular cells (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 IgG: 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-CD11b (M170, diluted at 1:200) and PE-conjugated rat IgG2b isotype control, PE-conjugated anti-CD8α (53-6.7, diluted at 1:100) and PE-conjugated rat IgG2a isotype control (eBiosciences, San Diego, CA), and Alexa Fluor 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 FACSscan 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 manufacturer’s instructions. Briefly, bone marrow-derived macrophages (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).

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 h. Nonadherent cells were then harvested, layered on a Ficoll-Hypaque gradient, and cultured for 2 more days, by which time large populations of adherent monocyte/macrophage-like cells had formed on the bottoms of the culture plates, as described previously [22]. The few nonadherent cells were removed by washing the dishes with PBS, and the adherent cells (BMM) 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 3. After incubation for the recommended times, the cells were analyzed using a FACSscan II flow cytometer. BMM were transfected with small-interfering (si)RNAs against p47phox (sip47phox), soluble guanylate cyclase (sisGC), heme oxygenase-1 (sHO-1), and scrambled siRNA (scRNA) (Santa Cruz Biotechnology, 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 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 of 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) mice in the background of the B6.129X1-Nfe2l2tm1gdk/J strain, which were provided from The 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 nM 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. Total RNA from BMMs incubated with M-CSF for the indicated times was extracted with Trizol solution (GIBCO Life Technologies) and reverse-transcribed with oligo-dT and Superscript I (Invitrogen). Quantitative PCR (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). 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 with GAPDH were calculated using 2−ΔΔCt. The primer sequences used were as follows: 5′-ggatgttccccattgaggccg-3′ and 5′-gcacactgtggcaactg-3′ (CD11c), 5′-gttgctggtattctcaaatcctgggactttg-3′ and 5′-tgtatagagacaaatcctggctggttgg-3′ (TNFα), 5′-ctccagactctggctggagttg-3′ and 5′-aggggagacggagacttgag-3′ (GSC), 5′-cttcattaagtctcgagagtcg-3′ and 5′-aggagacgtgctggaggttg-3′ (arginase-1), 5′-tcgttaggcagactctcaagagtc-3′ and 5′-aggggagacggagagcttgag-3′ (iNOS), 5′-tcggagccgcctctgcagc-3′ and 5′-tggctcgagcttgatcctg-3′ (ho-1), 5′-actin (1:1,000; Sigma-Aldrich) was used as loading control. Insulin-induced phosphorylated Akt was evaluated by normalization over total Akt signaling using ImageJ software.

Statistical analysis. Values are expressed as means ± SE. 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 posttests. A P value of <0.05 was considered statistically significant.
Fig. 1. Carbon monoxide (CO) counteracts the adverse effects of metabolic perturbation induced by ovariectomy (OVX). Mice were subjected to OVX or sham surgery and administered vehicle, tricarbonyldichlororuthenium(II) dimmer (CORM2; 5 mg/kg), or the inactive form of CORM2 (iCORM2) (5 mg/kg) for 12 wk (n = 7 for each group). Numerical data are included in Table 1. Body weight change (A), intraperitoneal (ip) glucose tolerance test (IPGTT) and ip injection of glucose at the dose of 1 mg/g body wt (B), and ip insulin tolerance test (IPITT) and ip injection of insulin at the dose of 0.75 U/kg body wt (C) were determined 12 wk after sham or OVX surgery. Area under the curve for the IPGTT and IPITT was measured and analyzed by 1-way ANOVA, followed by Bonferroni posttests. *P < 0.05, **P < 0.01, and ***P < 0.001, vehicle-treated OVX vs. CO-treated OVX mice. No significant change between V-treated sham mice and CORM2-treated sham mice. **P < 0.01 compared with vehicle-treated sham mice.

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 wk (Fig. 1A and 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 at the dose of 1 mg/g body wt (B), and ip insulin tolerance test (IPITT) and ip injection of insulin at the dose of 0.75 U/kg body wt (C) were determined 12 wk after sham or OVX surgery. Area under the curve for the IPGTT and IPITT was measured and analyzed by 1-way ANOVA, followed by Bonferroni posttests. *P < 0.05, **P < 0.01, and ***P < 0.001, vehicle-treated OVX vs. CO-treated OVX mice. No significant change between V-treated sham mice and CORM2-treated sham mice. **P < 0.01 compared with vehicle-treated sham mice.

Table 1. Metabolic characteristics of sham and OVX WT and CORM2-treated mice 12 wk after surgery

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>V</th>
<th>CORM2</th>
<th>iCORM</th>
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<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>
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<tr>
<td>Subcutaneous fat, mg</td>
<td>510.0 ± 43.91</td>
<td>397.0 ± 27.30</td>
<td>1.730 ± 176.0**</td>
<td>1.275 ± 112.5##</td>
</tr>
<tr>
<td>Visceral fat, mg</td>
<td>352.5 ± 48.24</td>
<td>210.0 ± 21.82</td>
<td>1.732 ± 151.9***</td>
<td>1.291 ± 125.0##</td>
</tr>
<tr>
<td>Serum H2O2, nmol/ml</td>
<td>48.15 ± 1.900</td>
<td>46.73 ± 1.970</td>
<td>68.70 ± 1.300***</td>
<td>62.12 ± 2.210#</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>99.85 ± 4.140</td>
<td>110.2 ± 3.720</td>
<td>135.6 ± 3.450**</td>
<td>113.1 ± 6.220#</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>
</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>
</tr>
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</table>

Data represent means ± SE; n = 7/group. OVX, ovariectomy; WT, wild type; CORM2, tricarbonyldichlororuthenium(II); V, vehicle; iCORM, inactive form of CORM2. No significant change between V-treated sham mice and CORM2-treated sham mice. **P < 0.01 and ***P < 0.001, V-treated sham mice vs. V-treated OVX mice; #P < 0.05 and ##P < 0.01; V-treated OVX mice vs. CORM2-treated OVX mice. No significant change between V-treated OVX mice and iCORM-treated OVX mice.
of insulin, we examined the phosphorylation of Thr308 of Akt. The 3-kinase-Akt pathway is responsible for the metabolic effects of insulin. Since the phosphoinositol 3-kinase-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 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 whether CO could reduce the cause of the reduced IR observed in CO-injected OVX mice to a reduction in ATMs. To assess whether CO led to immune cell infiltration, we evaluated numbers of SVCs 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 countered these effects (Fig. 2, E and F). 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 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 downregulating CD11c expression via generation of cGMP and scavenging of reactive oxygen species. 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, a 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 CD11b+CD11c 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-day exposure to M-CSF, ~23% of the total BMM expressed CD11c and F4/80, and CO
Fig. 3. CO decreases CD11c-expressing cells by reducing recruitment to the AT and protecting against M1 polarization of bone marrow-derived macrophages (BMM). A: the cell population of monocytes expressing Ly6G-CD11b+CD11c+ in the blood of V-treated (open bars) and CORM2-treated mice (hatched bars) 12 wk after sham or OVX surgery (n = 6 for each group) was analyzed by 1-way ANOVA, followed by Bonferroni posttests. **P < 0.01, V-treated OVX vs. CORM2-treated OVX mice; ###P < 0.001 compared with vehicle-treated SHAM mice. B: BMM were incubated with iCORM (30 μM), cilostazol (CTZ; 10 μM), 8-bromoguanosine-3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP; 50 μM), or 1H-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM) in the presence or absence of CORM2 (30 μM) upon macrophage colony-stimulating factor (M-CSF; 30 ng/ml) (V) stimulation for 4 days and labeled with conjugated Abs to CD11c and F4/80. #P < 0.05, ##P < 0.01, and ###P < 0.001 compared with V-treated cells; **P < 0.01 compared with CORM2-treated cells in the presence of CTZ. C: BMM were stimulated with M-CSF in the presence or absence (V) of CORM2 (30 μM), 8-Br-cGMP (30 μM), or N-acetylcysteine (NAC; 3 mM) for 3 days, and total RNA was extracted and subjected to quantitative PCR (qPCR) analysis. Expression levels before M-CSF treatment were set at 1.0. *P < 0.05 and **P < 0.01 compared with V-treated cells. Similar results were obtained in 3 independent experiments. NS, not significant.

Reduced this percentage (Fig. 3B). A similar pattern was found at the mRNA level of CD11c (Fig. 3C). Coadministration of IFNγ and LPS induced M1 polarization further than M-CSF alone [62 (IFNγ and LPS) vs. 23% (M-CSF)], but the inhibitory effect of CO was less [17 (IFNγ 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, TNFα, and iNOS and increased transcript levels of arginase-1, IL-10, and HO-1 (Fig. 3C), demonstrating that CO opposes the polarization of BMM to M1.

Since CO reduced M-CSF-induced CD11c expression in BMM, 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 sGC (48). Therefore, we wondered whether the decrease in 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, i.e., 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 of exposure to M-CSF alone and reached a plateau level after 6 h. The addition of CO elevated cGMP level after 2 h of stimulation compared with M-CSF alone, and it remained elevated for ≥24 h (Fig. 4A). Cilostazol also increased cGMP, and coinubation 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 trans ferrated into BMM. Downregulation 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 reactive oxygen species (ROS) may play a role for the effect of CO to decrease CD11c further. 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 precursors with macrophages, we tested whether CO affected M-CSF-induced ROS levels. As shown in Fig. 4D, substantial levels of ROS were 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 decreased CD11c expression significantly 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 genera-
Fig. 4. CO decreases CD11c expression by activating soluble guanylate cyclase (sGC) and scavenging reactive oxygen species (ROS). cGMP was measured in BMM upon stimulation of M-CSF (30 ng/ml) with vehicle (V), CORM2 (30 µM), CTZ (10 µM), or iCORM2 (30 µM) for 0, 1, 2, 6, 12, 24 (A), or 6 h (B). **P < 0.01 compared with 0 h of V-treated cells (A). *P < 0.05 and **P < 0.01 compared with V-treated cells (A and B). ***P < 0.01 compared with CTZ-treated cells in the presence of CORM2 (B). C and E: BMM were transfected with scRNA, small interfering (si)sGC, sip47phox, or siHO-1 (heme oxygenase-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 1. After 8 h of transfection with siRNA, cells were incubated further with M-CSF for 4 days before CD11c-stained cells were measured. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with scRNA-transfected cells. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with V-treated cells. Numbers above the bars are ratios of CORM2-treated cells to V-treated cells (open bars). D: BMM were incubated with diphenyleneiodonium chloride (DPI; 20 µM), 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 days and labeled with conjugated Abs to CD11c and F4/80. Intracellular levels of ROS upon stimulation with M-CSF for 2 days in the presence or absence of CORM2 (30 µM) or iCORM (30 µM) were determined in BMM using H2DCFDA. ###P < 0.01 and ####P < 0.001 compared with V-treated cells. ***P < 0.001 compared with CORM2-treated cells in the presence of DPI. F: BMM from WT and Nr2f2-knockout (KO) mice were stimulated with M-CSF in the presence or absence (V) of CORM2 (30 µM) for 2 (HO-1) or 4 days (CD11c). Total RNA was extracted and subjected to qPCR analysis. #P < 0.05 compared with WT cells; *P < 0.05 and ***P < 0.01 compared with V-treated cells. Numbers above the bars are ratios of CORM2-treated cells to V-treated cells. G: BMM 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 days and labeled with conjugated Abs to HO-1 and F4/80, as described in MATERIALS AND METHODS. **P < 0.01 compared with V-treated cells. Similar results were obtained in 3 independent experiments.
tion. 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 of BMM. Since HO-1 induction by CO has been reported to be via Nr2 activation in endothelial cells (21), we also investigated whether Nr2 is associated with HO-1 induction by CO in BMM. As shown in Fig. 4F, the lack of Nr2 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 Nr2, and Nr2 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 Figs. 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. A 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 increased adiposity, since CO also did not show a significant difference in food intake, excluding the possibility that CO affects the 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; this is 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 generate 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 type 2 diabetes (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 rather than suppresses inflammatory and metabolic genes. These confictions are also demonstrated in human studies (3, 43). A similar pattern has been reported in the role of Nr2, which is an upstream regulator of HO-1 on obesity. Chemical induction of Nr2 by oltipraz (57) reduces obesity, whereas genetic deletion of Nr2 decreases obesity (4). Further investigation is required 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, whereas death occurs at >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 and CD11cF4/80 and CD11bF4/80 cells in AT and enlarged visceral fat mass. The cells expressing CD11cF4/80 were ~10% of those expressing CD11bF4/80 in OVX, and a similar pattern was found in HFD-induced obesity (23). Increased fat is associated with increased ATM levels (45), which induces IR by generating proinflammatory cytokines, suggesting a critical role for macrophages in IR. IR due to HFD-induced obesity is not protected by adipocyte-specific overexpression of HO-1 (17) but is ameliorated by myeloid HO-1 haploinsufficiency (16), demonstrating the importance of macrophages in the development of IR. IR is promoted by proinflammatory 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 proinflammatory 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 finding that OVX increased serum levels of M-CSF (20) and monocyte chemoattractant protein-1 (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 levels 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. But then how did CO attenuate 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 TNFα and iNOS in BMM. How could CO act to decrease CD11c expression in BMM? The 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 in decreasing 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). Downregulation 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 the 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 downregulation of HO-1, but not that of p47phox, 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 the HO-1 products, bilirubin, has been reported to increase insulin sensitivity and reduce AT inflammation in vivo (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 partly reduced the activity of CO to decrease CD11c expression, 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 the expression of enhanced CD11c and decreased CD206; thus it counteracted IR due to the 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.

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