Carbon monoxide-releasing molecules reverse leptin resistance induced by endoplasmic reticulum stress

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Zheng M, Zhang Q, Joe Y, Kim S-K, Uddin MJ, Rhew H, Kim T, Ryter SW, Chung HT. Carbon monoxide-releasing molecules reverse leptin resistance induced by endoplasmic reticulum stress. Am J Physiol Endocrinol Metab 304: E780–E788, 2013. First published February 12, 2013; doi:10.1152/ajpendo.00466.2012.—Leptin, a circulating hormone, regulates food intake and body weight. While leptin resistance represents a major cause of obesity, the underlying mechanisms remain unclear. Endoplasmic reticulum (ER) stress can contribute to leptin resistance. Carbon monoxide (CO), a gaseous molecule, exerts antiapoptotic and anti-inflammatory effects in animal models of tissue injury. We hypothesized that CO could inhibit leptin resistance during ER stress. Thapsigargin or tunicamycin was used to induce ER stress in human cells expressing the leptin receptor. These agents markedly inhibited leptin-induced STAT3 phosphorylation, confirming that ER stress induces leptin resistance. The CO-releasing molecule CORM-2 blocked the ER stress-dependent inhibition of leptin-induced STAT3 phosphorylation. CORM-2 treatment induced the phosphorylation of protein kinase R-like endoplasmic reticulum kinase (PERK), and eukaryotic translation initiation factor-2α and enhanced PERK phosphorylation during ER stress. Furthermore, CORM-2 inhibited X-box binding protein-1 expression, activating transcription factor-6 cleavage, and inositol-requiring enzyme (IRE)1α phosphorylation induced by ER stress. IRE1α knockdown rescued leptin resistance, whereas PERK knockdown blocked CO-dependent regulation of IRE1α. In vivo, CO inhalation normalized body weight in animals fed high-fat diets. Furthermore, CO modulated ER stress pathways and rescued leptin resistance in vivo. In conclusion, the pathological mechanism of leptin resistance may be ameliorated by the pharmacological application of CO.

Because substitution of the Tyr1138 residue of Ob-Rb causes severe obesity in mice, STAT3 represents an important mediator in the regulation of body weight by leptin (2, 8, 15).

Obesity is a condition of excess body fat that in turn may have adverse effects on health. Furthermore, leptin resistance is considered one of the major causes of obesity (16). Leptin therapy could therefore represent an important tool for obesity treatment. Several signaling proteins may contribute to leptin resistance, including suppressor of cytokine signaling 3 (SOCS3) (3, 4), and protein tyrosine phosphatase-1B (PTP1B) (7). Furthermore, obesity-induced insulin resistance and type 2 diabetes can be mediated by endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) signaling pathway (19). Recent studies have shown that ER stress plays a central role in the development of leptin resistance (18), of which the underlying mechanisms remain incompletely understood.

The ER is a major organelle of the eukaryotic cell that functions as the site of protein synthesis, maturation, and folding. Perturbation of these processes causes ER stress, leading to activation of the UPR (22). In mammalian cells, the UPR is a complex signaling network that includes three ER stress sensors: the double-stranded RNA-activated protein kinase-like ER kinase (PERK), the inositol-requiring transmembrane kinase/endonuclease 1α (IRE1α), and the basic leucine-zipper activating transcription factor 6 (ATF6) (27). Among the members of the eukaryotic translation initiation factor (eIF)2α kinase family, PERK remains in the ER membrane and is responsible for the propagation of ER stress signals. The transcription factor IRE1α facilitates the X-box binding protein (Xbp-1) to activate upstream ER stress-responsive genes, such as C/EBP homologous protein (CHOP), an ER stress-induced apoptotic transcription factor. In addition, ER-resident chaperones such as the 78-kDa and 94-kDa glucose-regulated proteins (GRP78 and GRP94), responsible for ER protein folding, are involved in regulating the levels of IRE1α and ATF6 (21).

Carbon monoxide (CO) arises as a reaction product of heme oxygenase (HO) enzymatic activity (EC: 1.14.99.3). HO-1, the inducible form of the enzyme, catalyzes the degradation of heme, generating CO, biliverdin-IX, and ferrous iron (17, 20). Although CO is considered a toxic waste product of heme catabolism, recent studies suggest that this gas may act as a potential physiological and cellular signaling molecule. Furthermore, CO can modulate the pathways that regulate inflammation, apoptosis, and cellular proliferation (5, 6, 25). CO can also modulate STAT3 activation via the phosphotyrosineinositol 3-kinase (PI3K)/Akt and p38 mitogen-activated protein kinase.
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Methods

Reagents. Tricarbonyl dichlororuthenium (II) dimer (CORM-2) was from Sigma-Aldrich (St. Louis, MO). Leptin was from R&D Systems (Minneapolis, MN). Thapsigargin (TG) and tunicamycin (TM) were from Calbiochem (La Jolla, CA). Lipofectamine 2000 was from Invitrogen Life Technologies (Grand Island, NY). Antibodies to phospho-(p)-PERK, PERK, eIF2α, CHOP, Ob-R, STAT3, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to p-STAT3 (Tyr705) and p-eIF2α, were from Cell Signaling Technology (Beverly, MA). The antibody to IRE1α was from Novus Biologicals (Littleton, CO). The small interfering (si)RNA against IRE1α and PERK were from Santa Cruz Biotechnology, Promega, and all other chemicals were obtained from Sigma-Aldrich.

Cell culture. Chinese hamster CHO-K1 cells were obtained from American Type Tissue Collection (ATCC, Manassas, VA) maintained in Ham’s F-12 nutrient mixture supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human neuroblastoma SK-N-AS cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS. Wild-type and IRE1α−/− murine embryo fibroblasts (MEF) cells were obtained from Dr. R. J. Kaufman (University of Michigan) and cultured in DMEM containing 10% FBS and 1 mM nonessential amino acids. Cell cultures were maintained at 37°C in humidified incubators containing 5% CO2 and 95% air.

Generation of Ob-Rb leptin receptor transient and stable-transfected cell lines. The Ob-Rb leptin receptor plasmid DNA was a kind gift from Dr. Sung-Kyu Ju. The Ob-Rb leptin receptor construct was transiently transfected into SK-N-AS and MEF cells using the Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. CHO-K1 cells were transfected with Ob-Rb using Lipofectamine, and then stable transfectants were obtained by selection with hygromycin (12).

Western blot and densitometry analyses. After treatment, cells were harvested and washed twice with ice-cold PBS. Cells were lysed for 20 min in lysis buffer, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, containing protease and phosphatase inhibitor cocktails. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatants were collected. Protein content was measured with a commercially available ELISA kit (R&D Systems), the procedures being performed according to the manufacturer’s protocol.

Statistical analyses. Data were expressed as means ± SD, and t-tests were used to assess significant differences between groups. A P value < 0.05 was considered to represent a statistically significant change.

Results

CO reverses ER stress-induced leptin resistance. ER stress has been reported to induce leptin resistance (11), although the effects of CO on this process are not known. We first confirmed that leptin increased STAT3 phosphorylation in SK-N-AS cells that were transfected with the Ob-Rb leptin receptor (SK-N-AS-Ob-Rb cells). Treatment of Ob-Rb transfected cells with the chemical inducer of ER stress TM induced leptin resistance, as shown by the loss of STAT3 phosphorylation in response to leptin stimulation (Fig. 1A). We next investigated whether CO could rescue leptin resistance induced by UPR-associated ER stress in SK-N-AS-Ob-Rb cells. As shown in Fig. 1A, administration of the CO-releasing molecule (CORM-2) dose-dependently reversed the inhibition of leptin-dependent STAT3 phosphorylation in SK-N-AS-Ob-Rb cells that were exposed to the ER stress agent TM. To validate the effects of leptin on STAT3 phosphorylation, we used the Ob-Rb-transfected CHO-K1 (CHO-K1-Ob-Rb) cells, using IL-6 stimulation as a positive control. In Fig. 1B, leptin increased the levels of STAT3 phosphorylation in a dose-dependent manner like IL-6 stimulation in CHO-K1-Ob-Rb cells. However, CORM-2 or ER stress agents alone or in combination did not significantly affect the expression of the leptin receptor (Fig. 1C).

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Fig. 1. CO reverses endoplasmic reticulum (ER) stress-induced leptin resistance in SK-N-AS-Ob-Rb cells. A: SK-N-AS-Ob-Rb cells were preincubated with CO-releasing molecule (CORM)-2 at indicated concentrations (0–20 
M) for 2 h and then treated with tunicamycin (TM; 2 
µg/ml) or vehicle for 4 h and then stimulated with leptin (10 
µg/ml) for 15 min. B: Chinese hamster ovary (CHO)-K1 cells were treated with IL-6 (25 ng/ml) and leptin (10 
µg/ml); CHO-K1-Ob-Rb cells were incubated with leptin at indicated concentrations (1–10 
µg/ml) for 15 min. C: CHO-K1-Ob-Rb cells were treated with thapsigargin (TG; 1 
µM), TM (2 
µg/ml), CORM-2 (20 
µM), TG (1 
µM) + CORM-2 (20 
µM), or TM (2 
µg/ml) + CORM-2 (20 
µM) for 4 h, and expression levels of Ob-R were analyzed by Western blotting; β-actin served as the standard. D: SK-N-AS-Ob-Rb cells were preincubated with 4-phenylbutyric acid (4-PBA; 5 mM) or CORM-2 (20 
µM) for 2 h and then treated with Tg (1 
µg/ml) or vehicle for 4 h and subsequently stimulated with leptin (10 
µg/ml) for 15 min. A, B, and D: phospho-(p-)STAT3 and STAT3 levels were analyzed by Western blotting. Densitometric analysis of p-STAT3 relative to STAT3 expression is shown; n = 3. ***P < 0.001, **P < 0.01.

CO induces activation of the PERK branch of the UPR in the ER stress response. We induced ER stress with agents that interfere with protein glycosylation (TM) or Ca²⁺ balance (TG). These ER stress-inducing compounds activated the PERK branch of the ER stress response and induced phosphorylation of PERK and its downstream target, the eukaryotic translation initiation factor 2α (eIF2α) (Fig. 2A).

We next examined effects of CO delivered by CORM-2 on ER stress pathways. We found that treatment of SK-N-AS-Ob-Rb cells with CORM-2 activated the phosphorylation of PERK, a major UPR signaling component. CO-induced activation of PERK was followed by the increased phosphorylation of eIF2α. Both TM and TG time-dependently induced the phosphorylation of IRE1α in SK-N-AS-Ob-Rb cells. In contrast, CORM-2 treatment alone had no effect on the phosphorylation of IRE1α (Fig. 2B). TM treatment also activated Xbp-1 expression and ATF6 cleavage in SK-N-AS-Ob-Rb cells. In contrast, CORM-2 treatment alone did not activate the expression of Xbp-1, nor did it stimulate ATF6 cleavage (Fig. 2C). CORM-2 treatment did not affect the expression of the UPR-regulated genes GRP78 and CHOP in these cells (Fig. 2D). These results suggest that CO can potentially modulate the ER stress response specifically through enhanced activation of the PERK pathway.

We next examined the effect of CORM-2 in modulating the response to ER agents. Whereas application of TG or CORM-2 alone stimulated PERK phosphorylation in SK-N-AS-Ob-Rb cells, an additive effect was observed when SK-N-AS-Ob-Rb cells were pretreated with CORM-2 and then exposed to TG (Fig. 3A). In contrast, pretreatment with CORM-2 inhibited the phosphorylation of IRE1α that was induced by TG treatment (Fig. 3B). Pretreatment with CORM-2 inhibited the activation of Xbp-1 expression and ATF6 cleavage in SK-N-AS-Ob-Rb cells exposed to TG (Fig. 3C).

We next determined the relevance of the PERK branch of the UPR in mediating the inhibitory effects of CORM-2 on CORM-2 treatment alone did not significantly modulate STAT3 phosphorylation (Fig. 1A).

Chemical chaperones such as 4-phenylbutyric acid (4-PBA) are known to block ER stress-induced responses (18). Similar to the effects observed with CORM-2 treatment, treatment with 4-PBA recovered leptin-dependent STAT3 phosphorylation in SK-N-AS-Ob-Rb cells exposed to TM (Fig. 1D). These results, taken together, suggest that CO reverses leptin resistance resulting from ER stress.

The up-regulation of CHOP, a transcription factor induced by ER stress, is a well-known indicator of ER stress. The expression levels of CHOP and the phosphorylation status of IRE1α, PERK, and eIF2α were determined in SK-N-AS-Ob-Rb cells by Western blotting and densitometry. The results showed that both CHOP and eIF2α were up-regulated in response to TG treatment, while CORM-2 treatment alone did not significantly modulate the expression of these proteins. These findings provide further evidence for the role of CO in modulating the PERK branch of the UPR and suggest that CO may have potential therapeutic applications in the treatment of ER stress-related diseases.

Fig. 1. CO reverses endoplasmic reticulum (ER) stress-induced leptin resistance in SK-N-AS-Ob-Rb cells. A: SK-N-AS-Ob-Rb cells were preincubated with CO-releasing molecule (CORM)-2 at indicated concentrations (0–20 
µM) for 2 h and then treated with tunicamycin (TM; 2 
µg/ml) or vehicle for 4 h and then stimulated with leptin (10 
µg/ml) for 15 min. B: Chinese hamster ovary (CHO)-K1 cells were treated with IL-6 (25 ng/ml) and leptin (10 
µg/ml); CHO-K1-Ob-Rb cells were incubated with leptin at indicated concentrations (1–10 
µg/ml) for 15 min. C: CHO-K1-Ob-Rb cells were treated with thapsigargin (TG; 1 
µM), TM (2 
µg/ml), CORM-2 (20 
µM), TG (1 
µM) + CORM-2 (20 
µM), or TM (2 
µg/ml) + CORM-2 (20 
µM) for 4 h, and expression levels of Ob-R were analyzed by Western blotting; β-actin served as the standard. D: SK-N-AS-Ob-Rb cells were preincubated with 4-phenylbutyric acid (4-PBA; 5 mM) or CORM-2 (20 
µM) for 2 h and then treated with Tg (1 
µg/ml) or vehicle for 4 h and subsequently stimulated with leptin (10 
µg/ml) for 15 min. A, B, and D: phospho-(p-)STAT3 and STAT3 levels were analyzed by Western blotting. Densitometric analysis of p-STAT3 relative to STAT3 expression is shown; n = 3. ***P < 0.001, **P < 0.01.
We observed that CO inhibited ER stress by downregulating the phosphorylation of the PERK branch of UPR. These results suggest that CORM-2 treatment recovers the ER stress response in SK-N-AS-Ob-Rb cells transfected with siRNA targeting PERK (Fig. 4A). In contrast, pretreatment with TM failed to induce leptin resistance, as evidenced by levels of STAT3 phosphorylation in response to leptin, which were comparable to that observed in cells untreated with TM (Fig. 4C).

CO reversed fatty acid-induced leptin resistance in vitro. Exposure to free fatty acids such as palmitate has been shown to cause ER stress and leptin resistance (1, 14). We therefore tested whether CO could reverse fatty acid-induced ER stress. Treatment with palmitate dose-dependently induced the expression of the ER stress transducer CHOP in SK-N-AS-Ob-Rb cells when applied at doses from 50 to 200 μM in the culture medium (Fig. 5A). Pretreatment with CORM-2 reversed the induction of CHOP expression by palmitate treatment (200 μM; Fig. 5B). Palmitate treatment caused leptin resistance in vitro as evidenced by the dose-dependent inhibition of leptin-inducible STAT3 phosphorylation in SK-N-AS-Ob-Rb cells (Fig. 5C). CORM-2 pretreatment reversed palmitate-induced leptin resistance as evidenced by rescue of leptin-dependent STAT3 phosphorylation (Fig. 5D).

CO reversed high-fat diet-induced leptin resistance by modulating the ER stress response in mice. We next studied the effects of CO application on body weight gain and leptin resistance in vivo. Mice were maintained on a high-fat or normal diet (control) for 16 wk. At the 6th week, mice inhaled CO (250 ppm) or room air for 2 h every day for an additional 10 wk. CO inhalation resulted in reduced body weight in high-fat diet-induced obese mice but did not affect body weight in normal (control) mice fed a regular diet (Fig. 6A). CO treatment also reduced total food intake in obese mice on a high-fat diet (Fig. 6B). To evaluate the effect of CO inhalation...
on fat mass and circulating leptin levels, mice were fed a high-fat or normal diet. The inhalation of CO in diet-induced obese mice decreased epididymal (Fig. 6C) and perirenal fat (Fig. 6D) content and serum leptin levels (Fig. 6E). In contrast to the high-fat group, there was no effect of CO inhalation on the normal-diet group.

**CO reversed high-fat diet-induced leptin resistance by modulating the ER stress response in mice.** We also studied the effects of CO application on ER stress responses in vivo. CO inhalation (250 ppm) resulted in elevated PERK phosphorylation in the hypothalamus of animals on a high-fat diet relative to hypothalamus tissue of air-treated animals on a high-fat diet or normal diet controls (Fig. 7A). IRE1α was activated in the hypothalamus of animals on high-fat diet and reduced in those animals by inhalation of CO (Fig. 7B). Maintenance on a high-fat diet caused leptin resistance in mice as evidenced by reduction of leptin-induced STAT3 phosphorylation. This dietary-induced leptin resistance was rescued by CO inhalation (250 ppm; Fig. 7C).

**DISCUSSION**

In the present study, we have shown that the pharmacological application of CO reverses leptin resistance associated with ER stress in vitro and in vivo. We therefore studied the mechanisms underlying the protective effect of CO against ER stress-induced leptin resistance. Mice that lack the functional long isoform of Ob-Rb (db/db mice) become obese through increases in food intake, demonstrating an essential role of Ob-Rb in the response to leptin. Leptin induces the phosphor-
rylation of STAT3 protein through the long isoform of Ob-Rb. The short isoform of Ob-Rb, which is overexpressed in db/db mice, cannot transduce STAT3 signaling in response to leptin (9, 10, 26). In contrast, both the short and long isoforms of Ob-Rb mice are functional with respect to leptin-induced phosphorylation of MAPKs (4). Substitution of Tyr<sup>1138</sup> in Ob-Rb with a serine residue (Ser<sup>1138</sup>), which disrupts the Ob-Rb-STAT3 signal, also results in marked obesity in mice (2). Taken together, these findings indicate that the leptin-induced STAT3 signal is important in preventing obesity.

Recent studies have shown that ER stress induced by chemical agents or free fatty acids can contribute to leptin resistance (11, 18). Furthermore, 4-PBA, a chemical chaperone that can stabilize protein conformation and improve the protein folding capacity of the ER, can block ER stress-induced leptin resistance (18).

CO, a small gaseous molecule that is produced endogenously by HO activity, can exert physiological roles in cell signaling and homeostasis (17, 20). Application of CO by inhalation at low concentration or by pharmacological delivery with CORMs can confer organ protection in animal models of tissue injury, including ischemia/reperfusion injury and inflammatory lung injury (20).

We (13) have previously shown that CO activated Nrf2 through the phosphorylation of PERK, resulting in the expression of the cytoprotective molecule HO-1. We therefore hypothesized that CO might also inhibit ER stress-induced leptin resistance through amelioration of the ER stress pathway. We observed that CORM-2 reversed ER stress-induced leptin resistance in vitro induced either by chemical agents (i.e., TG, TM) or by treatment with free fatty acid. According to report of Hosoi et al. (11), even though ER stress induces leptin resistance, it does not change the expression level of the leptin receptor (Ob-Rb). Likewise, the effects of CO on downregulation of ER stress-induced leptin resistance were not related to changes in the expression of the leptin receptor (Ob-Rb). To further determine mechanisms underlying the inhibitory effect of CO on leptin resistance induced by ER stress, we studied the effects of CO on the PERK branch of the UPR. We previously demonstrated that CO treatment increased the phosphorylation of PERK. CO-induced PERK activation was followed by eIF2α phosphorylation and increased ATF4 expression (13).

Fig. 4. IRE1 is involved in ER stress-induced leptin resistance in SK-N-AS-Ob-Rb Cells. A and B: SK-N-AS-Ob-Rb cells were transfected with control (si Con) or IRE1α siRNA (si IRE1) (50 nM). A: 36 h after transfection, Western blotting analysis was performed for IRE1α protein expression; β-actin served as the standard. B: 36 h after transfection, cells treated with TM (2 μg/ml, 4 h) and stimulated with leptin (10 μg/ml) for 15 min had Western blotting analysis performed for p-STAT3 and STAT3 expression. C: IRE1<sup>−/−</sup> and IRE1<sup>+/−</sup> murine embryonic fibroblasts (MEF) Ob-Rb cells were preincubated with CORM-2 (20 μM) for 2 h and then treated with TM (2 μg/ml, 4 h), and then expression levels of p-STAT3 were analyzed by Western blotting and quantified by densitometry; n = 3. *P < 0.05.

Fig. 5. CO reverses palmitate-induced leptin resistance in SK-N-AS-Ob-Rb cells. SK-N-AS-Ob-Rb cells were incubated with palmitate at indicated concentrations for 6 h (A), or preincubated with CORM-2 (20 μM) for 2 h (B), and then treated with palmitate (200 μM, 6 h), and expression levels of p-PERK, p-IRE1α, and CHOP were analyzed by Western blotting. C: SK-N-AS-Ob-Rb cells were preincubated with palmitate at the indicated concentrations for 6 h, and then treated with leptin (10 μg/ml) for 15 min. Cell lysates were subjected to Western blotting analysis for p-STAT3 and STAT3 expression. D: SK-N-AS-Ob-Rb cells were preincubated with CORM-2 (20 μM) for 2 h and then treated with palmitate (200 μM/ml, 6 h) and then stimulated with leptin (10 μg/ml) for 15 min. p-STAT3 and STAT3 levels were analyzed by Western blotting.
Unlike its effects on PERK activation, CO did not activate Xbp-1 or ATF6. Instead, CO pretreatment blocked Xbp-1 and ATF6 activation caused by the ER stress response (13). In the current study, CO increased the phosphorylation of PERK and eIF2α to a greater extent than treatment with TM alone. Elevated increases of PERK and eIF2α phosphorylation by CO treatment in the presence of TM may protect protein biosynthesis. Furthermore, CO pretreatment could not block IRE1α phosphorylation by an ER stress inducer under conditions of downregulation of PERK expression by siRNA. Potential mechanisms by which the activation of PERK is affected by CO are still not clear; however, we suggest that CO induced reactive oxygen species (31) might be responsible for the phosphorylation of PERK. We have observed that CO-induced PERK phosphorylation can be inhibited with the use of hemoglobin or the antioxidant NAC (data not shown). These results, taken together, suggest that CO rescues ER stress-induced IRE1α phosphorylation via enhanced phosphorylation of PERK.

We also investigated whether another branch of the UPR, IRE1α, could be involved in ER stress-induced leptin resis-

**Fig. 6.** Inhaled CO reduces body weight, food intake, adipose pads, and serum leptin induced by high-fat diet (HFD). A: after feeding mice HFD or normal diet (ND; control) for 16 wk, mice inhaled CO (250 ppm) for 2 h each day for 10 wk. Inhalation of CO in diet-induced obese mice decreased body weight. In contrast to high-fat group, there was no effect of CO inhalation on the normal diet group. ***P < 0.001. B: food intake was decreased by CO inhalation in the high-fat group. *P < 0.05. C, D, and E: after feeding mice HFD or ND for 8 wk, mice inhaled CO (250 ppm) for 2 h each day for 4 wk. Inhalation of CO in diet-induced obese mice decreased epididymal and perirenal fat content and serum leptin levels. In contrast to HFD group, there was no effect of CO inhalation on ND group; n = 5. ***P < 0.001.

**Fig. 7.** CO reverses HFD-induced leptin resistance by modulating the ER stress response in mice. Mice were fed HFD or ND for 16 wk. Beginning at the 6th week, mice inhaled CO (250 ppm) for 2 h each day for subsequent 10 wk. Western blot analyses were performed for p-PERK (A), and p-IRE1α (B). The corresponding dephospho forms served as standard. C: additionally, mice in each group were stimulated with leptin. p-STAT3 and STAT3 levels were analyzed by Western blotting. Relative protein expression was quantified by densitometry; n = 3. *P < 0.05, **P < 0.01.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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