FGF21 induced by carbon monoxide mediates metabolic homeostasis via the PERK/ATF4 pathway

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ABSTRACT: The prevalence of metabolic diseases, including type 2 diabetes, obesity, and cardiovascular disease, has rapidly increased, yet the molecular mechanisms underlying the metabolic syndrome, a primary risk factor, remain incompletely understood. The small, gaseous molecule carbon monoxide (CO) has well-known anti-inflammatory, antiproliferative, and antiapoptotic effects in a variety of cellular- and tissue-injury models, whereas its potential effects on the complex pathways of metabolic disease remain unknown. We demonstrate here that CO can alleviate metabolic dysfunction in vivo and in vitro. We show that CO increased the expression and section of the fibroblast growth factor 21 (FGF21) in hepatocytes and liver. CO-stimulated PERK activation and enhanced the levels of FGF21 via the eIF2α–ATF4 signaling pathway. The induction of FGF21 by CO attenuated endoreticulum stress- or diet-induced, obesity-dependent hepatic steatosis. Moreover, CO inhalation lowered blood glucose levels, enhanced insulin sensitivity, and promoted energy expenditure by stimulating the emergence of beige adipose cells from white adipose cells. In conclusion, we suggest that CO acts as a potent inducer of FGF21 expression and that CO critically depends on FGF21 to regulate metabolic homeostasis.—Joe, Y., Kim, S., Kim, H. J., Park, J., Chen, Y., Park, H.-J., Jekal, S.-J., Ryter, S. W., Kim, U. H., Chung, H. T. FGF21 induced by carbon monoxide mediates metabolic homeostasis via the PERK/ATF4 pathway. FASEB J. 32, 2630–2643 (2018). www.fasebj.org

KEY WORDS: hepatic steatosis · metabolic disease · ER stress · ROS · thermogenic genes

ABBREVIATIONS: ALT, alanine aminotransferase; AML, acute myeloid leukemia; AST, aspartate aminotransferase; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; CO, carbon monoxide; CORM, CO-releasing molecule; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; eWAT, epididymal white adipose tissue; FGF21, fibroblast growth factor 21; GGT, glucose tolerance test; H&E, hematoxylin and eosin; HFD, high-fat diet; HSL, hormone-sensitive lipase; IRE1, inositol-requiring enzyme-1; ITT, insulin tolerance test; iWAT, inguinal white adipose tissue; MEF, mouse embryonic fibroblast; mROS, mitochondrial reactive oxygen species; NAC, N-acetyl-L-cysteine; NCD, normal chow diet; OCR, oxygen consumption rate; PERK, protein kinase R-like endoplasmic reticulum kinase; Plin1, perilipin 1A; ppm, parts per million; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; siRNA, negative control siRNA; siRNA, small interfering RNA; TG, triglyceride; Tm, tunicamycin; WAT, white adipose tissue

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Metabolic syndrome and obesity are increasing in prevalence and contribute a burden on public health (1, 2). These conditions may promote the pathogenesis of various metabolic diseases, including glucose metabolism disorders, hypertension, and dyslipidemia, and can represent risk factors for type 2 diabetes and cardiovascular disease (3, 4). The development of insulin resistance may provide a major mechanism for the pathophysiology of the metabolic syndrome. Furthermore, metabolic disease can be associated with other underlying factors, such as nonalcoholic fatty liver disease (5), and mediators, such as proinflammatory cytokines (6) and adiponectin (7, 8). Leptin resistance has been suggested as an alternative mechanism of metabolic syndrome (9). Based on these various mechanisms, the targeting of metabolic homeostasis may represent a potential therapeutic strategy for metabolic syndrome.

Carbon monoxide (CO) is an endogenously produced product of heme oxygenase enzyme activity. When applied exogenously and at a low concentration, CO can provide pleiotropic cyto- and tissue-protective effects in various models of cellular or tissue injury involving anti-inflammatory, antiproliferative, and antiapoptotic effects.
(10, 11). Furthermore, chronic treatment with CO-releasing molecules (CORMs) increases metabolism, resulting in weight loss (12, 13), and increases mitochondrial biogenesis and function (14, 15). CO may mediate metabolic homeostasis via alteration of mitochondrial function, lipid metabolism, and inflammation. Furthermore, CO can protect cellular functions and preserve homeostasis under stress, through the modulation of autophagy, a lysosome-dependent mechanism for the turnover of cellular substrates, such as lipids, protein aggregates, and damaged organelles (16).

Although low CO levels promote the activation of molecular and cellular pathways involved in metabolic homeostasis, high doses of CO can cause acute clinical effects, including nausea, dizziness, and loss of consciousness (13). By this phenomenon, termed hormesis, CO may exert antiatherosclerotic, antilipogenic, anti-inflammatory, and/or antioxidant effects when administered at low, subtoxic doses.

Fibroblast growth factor 21 (FGF21) has emerged as a regulator of development, cell proliferation, and energy metabolism (17–20). FGF21 is an endocrine hormone that is produced predominantly in the liver but also in white adipose tissue (WAT), brown adipose tissue (BAT), pancreas, and skeletal muscle (21). Although FGF21 is expressed in multiple tissues, increases in the circulating levels of FGF21 have been linked to increased hepatic FGF21 synthesis (22). FGF21 is involved in the control of glucose homeostasis, insulin sensitivity, and ketogenesis (8, 20, 23, 24). Moreover, under cold conditions, FGF21 functions to increase the appearance of brown-like adipocytes in WAT depots (25). The expression of FGF21 is induced by peroxisome proliferator-activated receptors (PPARs)-α and -γ in various stresses, such as starvation, ketogenic diet, amino acid deprivation, high-fat diet (HFD) or obesity, low-carbohydrate levels, autophagy deficiency, and mitochondrial stress (26–28). The expression of FGF21 can also be regulated by ATF4, which is a downstream effector protein of the protein kinase R-like endoplasmic reticulum kinase (PERK) branch of the unfolded protein response (29, 30). Among the three known endoplasmic reticulum (ER) stress-sensing pathways: PERK, activating transcription factor 6 (ATF6), and inositol-requiring transmembrane kinase/endoribonuclease 1α (IRE1α), CO can selectively stimulate PERK activation through enhanced reactive oxygen species (ROS) production (31). PERK activation by CO increased elongation initiation factor 2α (eIF2α) phosphorylation and subsequently ATF4 expression (31). Here, we report that ATF4 expression elevated by CO resulted in increased FGF21 levels in hepatocytes and serum. FGF21 increased by CO stimulated the emergence of BATs from WATs. We also demonstrate that CO inhalation ameliorates HFD- and ER stress–induced hepatic steatosis and reduces insulin resistance and triglyceride levels. Therefore, we suggest that CO acts as a potent mediator of metabolic homeostasis by increasing endogenous FGF21 expression, which may have therapeutic implications in nonalcoholic steatohepatitis, obesity, type 2 diabetes, and related metabolic disorders.

**MATERIALS AND METHODS**

**Reagents**

Tunicamycin (Tm), MitoTEMPO, and CORM-2 were from MilliporeSigma (Billerica, MA, USA).

**Cell culture**

Acute myeloid leukemia (AML)12 mouse hepatocytes (CRL-2254; American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37°C in a humidified incubator containing 5% CO2. Protein kinase R-like ER kinase deficient (Perk−/−) or wild-type (Perk+/+) mouse embryonic fibroblasts (MEFs) were cultured in DMEM medium and 1% MEM nonessential amino acid solution (1140-050; Thermo Fisher Scientific). Irelα+/+, Irelα−/−, Atf6a+/+, Atf6α−/−, Atf4+/+, and Atf4−/− mouse hepatocytes were maintained in 199 medium with 1% MEM nonessential amino acid solution and 2.2 μl β-mercaptoethanol.

**Isolation of primary hepatocytes**

Livers were perfused with Ca2+- and Mg2+-free HBSS containing EGTA (2.5 mM) and then digested with a collagenase buffer containing collagenase (0.5 mg/ml, C5138; MilliporeSigma), NaCl (66.7 mM), KCl (6.7 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 50 mM), and CaCl2 (4.8 mM). Digested livers were dissected and then gently teased with forceps until they were in solution. The cell suspensions were filtered through 100-μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were centrifuged for 3 min at 700 rpm and resuspended with HBSS. After the pellet suspensions were centrifuged with 25% Percoll for 5 min at 800 rpm with the brake option off, the pellets were washed with DMEM supplemented with 10% fetal bovine serum, and then, cells were seeded into collagen precoated, 100-mm tissue-culture plates. After 24 h, nonadherent cells were removed by aspiration, and fresh medium was added.

**Animal experiments**

All experiments with mice were approved by the Animal Care Committee of the University of Ulsan. C57BL/6 mice (6 wk old, male) were purchased from Orient Bio (Seongnam, Korea). The mice were maintained under specific pathogen-free conditions and given access to food and water ad libitum. To produce mice with diet-induced obesity, 6-wk-old Fgf21+/+ and Fgf21−/− mice (6 mice/group) were fed an HFD (D12492; Research Diet, New Brunswick, NJ, USA) or normal chow diet (NCD) for 16 wk. Starting in wk 8, the mice were subjected to inhalation of CO gas [250 parts per million (ppm)] in air (Coregas, Yenora, Yenora, Australia) for 2 h/d for the remaining 8 wk. To model ER stress–induced hepatosteatosis, 6-wk-old C57BL/6 mice were pretreated with or without CORM-2 (20 mg/kg) for 6 h, followed by stimulation with Tm (1 mg/kg). Tissue harvest was performed after 24 h. Heterozygous elf2α−/−/fTg mice and elf2AN/A mutant mice were kindly provided by Dr. S. H. Back (University of Ulsan).

**RT-PCR**

Total RNA from cells was isolated with Trizol reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions. In
brief, total RNA (2 μg) was used to synthesize the first strand cDNA with oligo (dT) primer (Bioneer, Daejeon, Korea) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The synthesized cDNA was subjected to the PCR-based amplification. The PCR products were detected on 2% agarose gels. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal control. The following primers were used: mGAPDH forward, 5'-AGGCGCGGTCTGAGATGGTGC-3', reverse, 5'-TGCCTGGTTCCACCACTCT-3'; mFGF2 forward, 5'-ACAGATGAGCCAAGACACTG-3', reverse, 5'-GTCCCTCAGCAGCAGTCTC-3'; mPERK forward, 5'-ACTTGGGTGCTGCTCTCT-3', reverse, 5'-TGGCTGGGTAGGTTAGTG-3'; mATF4, forward, 5'-GCTGCGCCATTGGCTCG-3', reverse, 5'-GTTTCCAGGTCATTGCCTTG-3'; and mTFAM forward, 5'-CACCCAGGCCAGCTCAGACTA-3', reverse, 5'-ATTAGGAGGTCTCGCTCCA-3'.

**Real-time quantitative RT-PCR**

Total RNA was prepared using Trizol reagent (Thermo Fisher Scientific); 2 μg of total RNA was used to synthesize the first-strand cDNA with oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (Promega), according to the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) was performed with SYBR Green qPCR MASTER Mix (2×, USB production; Thermo Fisher Scientific) on an ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Real-time PCR primer pairs are listed in Supplemental Table 1.

**Western blot analysis**

Harvested liver tissues and cells were lysed in RIPA buffer containing phosphatase and protease inhibitors. Equal amounts of cell lysates was measured by BCA protein assay reagent (Thermo Fisher Scientific). Lysates were boiled in sample buffer containing β-mercaptoethanol for 5 min. Proteins were then subjected to SDS-PAGE and transferred to PVDF membranes (SigmaMillipore). After blocking with 5% skim milk in PBS, membranes were incubated with appropriate dilutions of antibodies at 4°C overnight as follows: anti-α-tubulin (Cell Signaling Technology, Danvers, MA, USA), anti–phospho-PERK (Cell Signaling Technology), anti–PERK (Cell Signaling Technology), anti–phospho-eIF2α (Cell Signaling Technology), anti–eIF2α (Cell Signaling Technology), anti–ATF4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti–phospho-AMPK (Cell Signaling Technology), anti–AMPK (Cell Signaling Technology), and anti–PGC1α (Abcam, Milton, Cambridge, United Kingdom), anti–COX III (Abcam), and anti–COX IV (Cell Signaling Technology). Membranes were then washed with 0.05% PBS-Tween 20 and incubated with 1/5000 dilution of horseradish peroxidase–conjugated secondary antibodies at room temperature for 1 h. Immunoreactivity was detected with an enhanced chemiluminescent detection system (GE Healthcare Life Sciences, Little Chalfont St Giles, United Kingdom). Chemiluminescence signals were read with an Azure Biosystems C300 analyzer (Azure Biosystems, Dublin, CA, USA).

**Hematoxylin and eosin staining**

To detect the pathologic changes, portions of the liver were fixed in 10% neutral-buffered formalin solution and then dehydrated in graded alcohol, embedded in paraffin, and sectioned into 4-μm–thick sections. Tissue sections were mounted on regular glass slides, deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E).

**Small interfering RNA transfection**

Small interfering RNAs (siRNAs) against mouse Fgf21 (sc-39485) and Perk (sc-36214) were purchased from Santa Cruz Biotechnology, and negative control siRNA (scRNA; AM4611) was purchased from Ambion (Austin, TX, USA). AML12 cells (7 × 10^5) were transfected with siRNAs with Lipofectamine 2000 (Thermo Fisher Scientific) for 24 h.

**Hepatocellular damage assay**

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, as indicators of hepatocellular injury, were measured with the EnzyChrom Alanine Transaminase assay kit and the EnzyChrom Aspartate Transaminase Assay Kit (Bio Assay Systems, Hayward, CA, USA).

**Measurement of triglycerides**

Hepatic triglycerides (TGs) were assessed with a TG colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI, USA). Briefly, liver tissues (50 mg) were homogenized in 200 μl diluted standard diluents. After centrifugation for 10 min at 10,000 g, supernatants were obtained. Before assaying, tissue samples required dilutions of ≥1.5, and 10 μl supernatant was used for the assay.

**Oxygen consumption rate measurement**

Oxygen consumption rate (OCR) was measured with an XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA), based on the fluorometric detection of O2 and pH levels. The day before the assay, the cells were seeded in XF24 microplates at 10,000 cells/well in 250 μl of complete culture medium and were maintained at 37°C in a humidified incubator containing 5% CO2; the cells were fully confluent on the day of the assay. The culture medium in the XF24 microplate was replaced with 525 μl/well of standard assay medium (Seahorse Bioscience). The cells were equilibrated in that assay medium for 1 h at 37°C without CO2. The Seahorse cartridge was incubated in the calibration solution (Seahorse Bioscience) overnight before loading oligomycin (75 μl) in port A, FCCP (75 μl) in port B, and a combination of rotenone and antimycin A (75 μl) in port C (XF Cell Mito Stress Test Kit; Seahorse Bioscience). Following the calibration and equilibration of the loaded cartridge, OCR was periodically measured with a sequence of 3-min mix, 2-min wait, and 3-min measurement. Baseline rates were measured, as well as rates after addition of oligomycin, FCCP, and the combination of rotenone and antimycin A. The averages of the 3 measurements of each experiment were used for data analyses (the first reading of baseline was dropped for analysis). OCR is reported as picomoles per minute or percentage relative to the basal level of the individual experiment. All experiments were performed at least hexaplicates.

**Glucose tolerance test and insulin tolerance test**

Glucose tolerance tests (GTTs) were performed in overnight-fasted mice with an intraperitoneal injection of glucose (1 g/kg). An insulin tolerance test (ITT) was conducted in 4-h–fasted mice with an intraperitoneal injection of insulin (1 U/kg). Blood glucose levels were measure with SD Codefree blood glucose meter (SD Biosensor, Suwon, South Korea).

**Mitochondrial ROS measurement**

AML12 cells were treated with CORM2 (20 μM) or CO gas (250 ppm) for 2 h in the absence or presence of Mito-Tempo
Fgf21 expression was increased by CO through increased ROS production

We next investigated the mechanisms by which CO can increase FGF21 expression. Application of CORM2 or exposure to CO gas increased FGF21 expression in AML12 cells or primary hepatocytes (Fig. 2A, B and Supplemental Fig. 2A, B). CORM2 (20 μM, 1 h treatment) stimulated the increase of FGF21 mRNA expression in AML12 cells (Fig. 2A), which was confirmed by qRT-PCR analysis (Fig. 2B). CO exposure (0–5 h, 250 ppm) increased FGF21 expression at 5 h in primary hepatocytes (Supplemental Fig. 2A). Increased FGF21 gene expression was confirmed by qRT-PCR analysis (Supplemental Fig. 2B). Knockdown of FGF21 using siRNA against Fgf21 (si-Fgf21), prevented up-regulation of FGF21 expression by CORM2 treatment, compared with control siRNA transfected cells (Fig. 2C). These results indicate that FGF21 expression is transcriptionally increased by CO. We also determined whether the increase of FGF21 by CORM2 or CO gas involved mitochondrial ROS (mtROS) generation, using the specific mitochondria-targeted, antioxidant mitoTEMPO (Fig. 2D, E and Supplemental Fig. 2C, D). The increase of FGF21 expression with CORM2 was reduced by mitoTEMPO. We also treated CORM2 or CO-stimulated AML cells with NAC, a general ROS inhibitor. As shown in Fig. 2F, G and Supplemental Fig. 2E, F the increase of FGF21 expression by CORM2 or CO was inhibited by NAC treatment. To determine whether the administration of CORM2 or CO gas could affect mtROS production, we next performed flow cytometry using MitoSox. As expected, treatment with CORM2 (Fig. 2H) or CO gas (Supplemental Fig. 2G) increased mtROS at 2 h in AML12 cells. Moreover, pretreatment with NAC or mitoTEMPO significantly decreased ROS production in the presence of CORM2 (Fig. 2H) or CO gas (Supplemental Fig. 2G). The mitochondrial complex I inhibitor rotenone (10 μM) was used as a positive control for mtROS production. These results suggested that ROS production serves an important role in up-regulating FGF21 expression by CO.

PERK activation by CO mediates the regulation of FGF21 expression

We next explored the molecular mechanisms by which FGF21 expression was regulated by the PERK pathway. In our previous study (31), we reported that CO activated PERK but not other eIF2α kinase family proteins, such as hemin-regulated inhibitor kinase, general control of amino acid biosynthesis kinase, or protein kinase R. We also demonstrated that CO induced ATF4 expression via the PERK-mediated eIF2α pathway (31). Based on our studies, we assessed whether FGF21 expression was dependent on PERK activation. As shown in Fig. 3A, FGF21 gene expression was increased by CORM2 treatment in MEF cells isolated from Perl+/− mice, but not Perl−/− mice. These results were also confirmed by qRT-PCR analysis (Fig. 3B).

Furthermore, knockdown of PERK using siRNA also
Figure 1. The protective effects of CO on HFD-induced metabolic syndrome are mediated by FGF21. A, B) Male, 6-wk-old C57BL/6 mice (6 mice/group) were fed an NCD or HFD for 16 wk. After 8 wk, animals were subjected to inhaled CO (250 ppm) for 2 h/d for 8 wk. The average body weight of Fgf21+/+ (A) and Fgf21−/− (B) mice was measured every 2 d. C, D) GTT (C) and ITT (D) were determined after 16 wk of NCD and HFD feeding. E) H&E staining of liver tissues was performed in Fgf21+/+ and Fgf21−/− mice. Scale bars, 50 μm. F–I) Liver weight (F), triglyceride (G), serum ALT (H), and AST (I) levels were investigated after 16 wk of NCD and HFD feeding. Data are presented as means ± SEM (n = 6). NS, not significant. **P < 0.01, ***P < 0.001.
prevented the increase of FGF21 gene expression in response to CORM2 (Fig. 3C). PERK activation by CO is associated by eIF2α phosphorylation and increases in ATF4 expression (31). To further identify the mechanism by which CO-induced activation of the PERK pathway stimulates FGF21 expression and the involvement of eIF2α and ATF4, we first compared the levels of FGF21 expression between control heterozygous eIF2α^{S/A} / Fg mice and eIF2α^{A/A} mutant mice after CO inhalation. The levels of FGF21 in serum were increased by CO inhalation in

Figure 2. CO enhances the expression of FGF21 via ROS production. A, B) AML12 cells were treated with CORM2 (20 µM) for various times (0, 0.5, 1, 3, and 6 h), and then, the FGF21 mRNA levels were measured by RT-PCR (A) and qRT-PCR (B). C) AML12 cells were transfected with scRNA and siRNA against Fgf21, followed by the administration of CORM2 (20 µM) for 0.5 and 1 h. D, E) AML12 cells were pretreated with MitoTEMPO (100 µM) for 1 h, and then, cells were treated with CORM2 (20 µM) for another 2 h. Then, the mRNA expression of FGF21 was determined by RT-PCR (D) and qRT-PCR (E). F, G) AML12 cells were transfected with NAC (3 mM) for 30 min, followed by the administration of CORM2 (20 µM) for another 2 h. Then, the mRNA expression of FGF21 was detected by RT-PCR (F) and qRT-PCR (G). H) To assess the production of mtROS, AML12 cells were pretreated with NAC (3 mM) for 30 min and MitoTEMPO (100 µM) for 1 h, and then were treated with CORM2 (20 µM) for another 2 h. mtROS was detected with MitoSOX Red (5 µM) for 50 min and measured by flow cytometry. Rotenone (10 µM) was used as a positive control for mtROS production. Data are presented as means ± SEM (n = 3). NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001.
control heterozygous elf2a<sup>S/A</sup>/fTg mice, but not in elf2a<sup>A/A</sup> mutant mice (Fig. 3D). Next, we assessed FGF21 expression in the livers or in primary hepatocytes from control heterozygous elf2a<sup>S/A</sup>/fTg mice and elf2a<sup>A/A</sup> mutant mice. The mRNA expression of Fg21 in response to CORM2 treatment was greatly elevated in the liver tissues of control heterozygous elf2a<sup>S/A</sup>/fTg mice, compared with those of elf2a<sup>A/A</sup> mutant mice (Fig. 3E). The increase of FGF21 by CO inhalation in liver tissue from control heterozygous elf2a<sup>S/A</sup>/fTg mice was investigated by qRT-PCR (Fig. 3E). In addition, FGF21 expression was markedly up-regulated by CO treatment in primary hepatocytes isolated from control heterozygous elf2a<sup>S/A</sup>/fTg mice, compared with those of elf2a<sup>A/A</sup> mutant mice (Fig. 3G). ATF4 expression is mediated by the PERK-mediated elf2a pathway (32) and regulates FGF21.
To verify whether the increase of FGF21 expression by CO was also dependent on ATF4 expression, we assessed the levels of FGF21 expression in hepatocytes isolated from Atf4+/+ and Atf4−/− mice after CORM2 treatment. The expression of FGF21 in response to CORM2 was greatly increased in hepatocytes from Atf4+/+ mice, compared with Atf4−/− mice (Fig. 3H). We next investigated whether the other two known ER stress–sensing pathways, namely the IRE1α and the ATF6 pathways, could regulate the expression of FGF21 in response to CO, using hepatocytes isolated from Ire1α+/+, Ire1α−/−, Atf6+/+, and Atf6−/− mice. The ability of CO to induce the expression of Fgf21 mRNA was not affected by genetic deficiency of IRE1α and ATF6 (Fig. 3I, J). Thus, CO increases FGF21 expression through the PERK-eIF2α-AFT4 pathway.

Figure 4. CO protects against ER stress-induced hepatic steatosis by induction of FGF21 expression. A) 6-wk-old Fgf21+/+ and Fgf21−/− mice were pretreated with or without CORM2 (20 mg/kg) or RuCl2 as control for ruthenium, for 6 h, followed by Tm (1 mg/kg) challenge. After 24 h, the mice were euthanized, and liver tissues were extracted and analyzed by H&E staining. B–D) Liver triglyceride (B), serum ALT (C), and AST (D) levels were measured after stimulation with Tm. E) Serum FGF21 levels were detected by ELISA. F, G) The mRNA expression of FGF21 in liver tissues was measured by RT-PCR (F) and qRT-PCR (G). Data are presented as means ± SEM (n = 3). NS, not significant. ***P < 0.001.
Induction of FGF21 expression by CO ameliorates ER stress-induced hepatic steatosis

Obesity induces ER stress which in turn can promote hepatic steatosis (35). We previously reported that CO ameliorates ER stress-induced hepatic steatosis (36, 37). Thus, we investigated whether CO-induced FGF21 expression alleviates hepatic steatosis in Fgf21+/+ and Fgf21−/− mice challenged with ER stressors. As shown in Fig. 4A, administration of Tm, an inhibitor of N-linked glycosylation that causes accumulation of misfolded protein in the ER, induced hepatic injury in Fgf21+/+ mice. CORM2 treatment protected against Tm-induced hepatic injury in Fgf21+/+ mice but was ineffective in Fgf21−/− mice. Furthermore, liver TG, ALT, and AST levels were increased by Tm in both Fgf21+/+ and Fgf21−/− mice (Fig. 4B–D). CORM2 treatment reduced the levels of liver TG, ALT, and AST in Fgf21+/+, but not in Fgf21−/−, mice.

The expression of FGF21 is induced by ER stress through the PERK-eIF2α-ATF4 pathway (30). Tm treatment induced FGF21 in the serum of Fgf21+/+ mice as an adaptation to ER stress. The addition of CORM2 further increased the FGF21 levels >2.5 times relative to Tm treatment alone (Fig. 4E). FGF21 levels were accordingly not increased in Fgf21−/− mice by either treatment. To investigate the mRNA expression of FGF21 in the liver after Tm and CORM2 treatment in Fgf21+/+ and Fgf21−/− mice, we performed RT-PCR (Fig. 4F) and qRT-PCR (Fig. 4G). CORM2 greatly elevated the expression of FGF21 mRNA in the Tm- and CORM2-treated group. CORM2 promoted recovery from ER stress by alleviating proinflammatory cytokine production (i.e., IL-6, IL-1β, and TNF-α), and that was dependent on the presence of FGF21 (Supplemental Fig. 3A). In addition, we found that Tm-induced hepatocyte damage in the liver was alleviated by administration of rmFGF21 (Supplemental Fig. 3B). Furthermore, the levels of ALT and liver TGs increased in response to Tm were reduced by application of exogenous FGF21 (Supplemental Fig. 3C, D). These data indicate that exogenous FGF21 reduces liver damage caused by Tm and thus has an important role in the regulation of ER stress-induced hepatic steatosis.

CO regulates lipid metabolism and energy expenditure through the induction of FGF21 expression

To assess directly whether induction of FGF21 expression by CO inhalation is responsible for a protective role against metabolic dysfunction, we investigated metabolic changes in Fgf21+/+ or Fgf21−/− mice fed with HFD. Histologic analysis revealed that the size of adipocytes were increased by HFD and were decreased by CO treatment in Fgf21+/+ mice but not in Fgf21−/− mice (Fig. 5A). We also found that CORM2 treatment increased the OCR in the AML12 mouse hepatocyte cell line, relative to the control groups (Fig. 5B). Genetic interference of FGF21 prevented the increase of OCR elicited by CORM2 treatment in those cells. The measurement of OCR may represent an increase in energy expenditure or mitochondrial function. To further study the mechanisms by which CO can alter mitochondrial function or mitochondrial biogenesis, we examined the effect of CORM2 on the expression of components of mitochondrial respiratory chain subunits (COX III, COX IV), and cellular mtDNA levels using wild-type or FGF21-deficient AML12 cells (Supplemental Fig. 4A–D) and primary hepatocytes from Fgf21+/+ or Fgf21−/− mice (Supplemental Fig. 4E–H). CORM2 treatment resulted in increased expression of COX III and COX IV protein and increased mtDNA levels in control (scRNA) AML12 cells (Supplemental Fig. 4B, C) and wild-type primary hepatocytes (Supplemental Fig. 4F, G). In AML12 cells transfected with siFGF21, knockdown of FGF21 was validated in Supplemental Fig. 4A. In FGF21 knockdown cells, CORM2 treatment failed to increase COX III and COX IV protein expression and mtDNA levels, relative to CORM2-treated control (scRNA transfected) cells (Supplemental Fig. 4B, C). Furthermore, in primary hepatocytes deficient in FGF21 (Fgf21−/−) (Supplemental Fig. 4E), CORM2 treatment failed to increase COX III and COX IV protein expression and mtDNA levels, relative to CORM2-treated wild-type (Fgf21+/+) hepatocytes (Supplemental Fig. 4F, G).

We also investigated the effects of CORM2 on mitochondrial functions by measuring the expression levels of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1α), mitochondrial transcription factor-A (TFAM), and nuclear respiratory factor-1 (NRF-1), protein factors critical in the regulation of mitochondrial biogenesis. As shown in Supplemental Fig. 4D, H, CORM2 increased the PGC1α, TFAM, and NRF-1 mRNA levels in AML12 cells transfected with scRNA and in primary hepatocytes from Fgf21+/+ mice but not in corresponding FGF21-deficient (siFGF21-transfected or Fgf21−/−) control hepatocytes. These data suggest that CORM2 regulates mitochondrial biogenesis and function via FGF21 increase. These findings indicate that CO-induced FGF21 expression has an important role in energy balance in HFD-induced metabolic syndrome, via regulating mitochondrial functions.

We also investigated whether the regulation of energy balance by CO-induced FGF21 involved alteration of fatty acid metabolism. We first measured the various adipose tissue weights after CO inhalation in Fgf21+/+ or Fgf21−/− mice fed with HFD. As shown in Fig. 5C–E, the weight of epididymal WAT (eWAT) or inguinal WAT (iWAT) was increased with HFD, and was decreased by CO in Fgf21+/+ mice, but not in Fgf21−/− mice (Fig. 5C, D). CO also increased BAT weight in NCD-fed wild-type (Fgf21+/+) mice. The BAT weight was unchanged in NCD-fed Fgf21−/− mice or in either Fgf21+/+ or Fgf21−/− mice fed an HFD (Fig. 5E). Gene expression analysis revealed that CO inhalation elicited a marked increase in mRNA levels of lipolytic enzymes in eWAT, including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), perilipin 1A (Plin1), and PPARγ in either NCD- or HFD-fed Fgf21+/+ mice, but not in Fgf21−/− mice (Fig. 5F). To investigate liver lipolysis, we measured the mRNA expression of PGC1α, ATGL, HSL, lipolysis-stimulated lipoprotein receptor, Plin1, CPT1b, and PPARα in liver tissues (Supplemental Fig. 5). CO increased the mRNA expression levels of lipolytic enzymes in Fgf21+/+ mice regardless of diet (NCD.
Figure 5. CO regulates lipolysis through the induction of FGF21 expression. A) H&E staining of epididymal WAT from Fgf21+/+ and Fgf21−/− mice that had been fed a NCD or HFD for 16 wk with CO inhalation. B) OCR was measured in AML12 cells transfected with scRNA and siRNA against Fgf21 in the presence of CORM2 (20 μM) and RuCl3 as control for ruthenium to detect energy expenditure and mitochondrial function. C–E) The weight of eWAT (C), iWAT (D), and brown fat (E) was investigated after 16 wk of NCD and HFD feeding with CO inhalation. F) The lipolysis-related genes (ATGL, HSL, Plin1, and PPARγ) from Fgf21+/+ and Fgf21−/− eWAT were measured by qRT-PCR. Data are presented as means ± SEM (n = 6). NS, not significant. *P < 0.05; **P < 0.01, ***P < 0.001.
CO-mediated induction of thermogenic genes is regulated by FGF21 expression

The regulation of metabolic homeostasis is associated with white adipocyte metabolism as well as the browning of white adipose depots. FGF21 is a critical regulator of the thermogenic activity of adipose tissue. Therefore, we investigated whether the induction of thermogenic genes by CO required FGF21 expression. CO inhalation resulted in a dramatic increase in the expression of genes normally associated with the browning of iWAT, such as uncoupling protein-1 (UCP1) (Fig. 6A), PGC1α (Fig. 6B), PRDM16 (Fig. 6C), Cidea (Fig. 6D), and Cited (Fig. 6E) in Fgf21+/+ mice. In contrast, there were no significant changes in the expression of those genes in iWAT of Fgf21−/− mice. Therefore, we conclude that the increase in the browning of iWAT by CO is dependent on FGF21 expression. We present a scheme in Fig. 6F for the role of CO in the control of metabolic homeostasis. CO increases PERK activation via mtROS production. Subsequently, FGF21 expression induced by CO via the PERK-ATF4 pathway regulates lipolysis and thermogenesis.

DISCUSSION

In the current study, we demonstrated that CO induces FGF21 expression, which, along with previous studies (12), provides a mechanism by which CO regulates metabolic homeostasis. Although CO can be poisonous when inhaled in air at excess of 1500 ppm (13), CO can exert various therapeutic effects in preclinical models of lung...
injury and disease, either as an endogenous mediator or in response to exogenous inhalation or pharmacologic delivery (38, 39). Here, we demonstrate that CO-dependent hormesis includes an antiobesity effect at a low dose (250 ppm) via increasing FGF21 expression. Moreover, we show that CO induces FGF21 expression in primary hepatocytes as well as in metabolic tissues, such as liver and WAT.

The increase of FGF21 expression by CO is associated with alleviating metabolic disorders, including improving glucose tolerance and insulin sensitivity and in ameliorating hepatic steatosis and adipose tissue dysfunction. Notably, increased FGF21 expression was required for the effects of CO on metabolic disease during HFD feeding in vivo. FGF21 expression is induced by ER stress via ATF4 overexpression and ATF4-induced promoter activity (40), activation of CHOP-dependent (34) and IRE1α-XBP1 pathways (41) and stimulation of the PERK-eIF2α-ATF4 axis (30). In this study, we demonstrated that the mechanism by which CO-induced FGF21 expression primarily involves stimulation of the PERK-eIF2α-ATF4 axis. CO, however, failed to induce the IRE1α-mediated Xbp1 pathway or protease-mediated ATF-6 cleavage, as shown in our previous report (31). Our present results are consistent with selective PERK activation by CO, which excludes the IRE1α and ATF6 pathways. We also demonstrated here that PERK activation by CO requires up-regulation of mtROS production. Both NAC and the mitochondria-targeting antioxidant MitoTEMPO inhibited CO-induced FGF21 expression. Another report (16) has shown that mitochondria-derived ROS were required for CO-dependent induction of autophagy. We also demonstrate that CO up-regulated other mitochondrial functions, including the expression of electron transport chain complexes (COX III, COX IV) and mitochondrial biogenesis (as evidenced by increased mtDNA copy number and increased gene expression of mitochondrial biogenesis factors) and that those effects were diminished in hepatocytes genetically deficient in FGF21. The mechanism for FGF21 to enhance mitochondrial function remains unclear. However, there are some reports that FGF21 enhances mitochondrial functions in human dopaminergic neurons (via the Sirt1-dependent regulation of PGC-1α) (42) and in skeletal muscle cells (43).

These results, taken together, suggested that CO targets mitochondrial functions, in a fashion that requires the presence of FGF21. Additionally, a recent article reported that FGF21 expression is increased by application of an ER stressor compound in hepatocytes (40). FGF21 is induced as a compensatory mechanism by various stresses, such as glucose starvation, cold shock, and autophagy deficiency (25, 44, 45). ER stress exerts an important pathogenic mechanism in various metabolic disorders, including the development of nonalcoholic fatty liver disease (35, 46, 47). Recombinant FGF21 protein could ameliorate hepatic liver injury that was caused by typical ER stress (41). Therefore, the reduction of ER stress by CO may result from FGF21-induced suppression of lipid accumulation and inflammation. In other reports, FGF21 was shown to prevent increased hepatic fat accumulation and enhanced hepatic inflammation in response to alcohol, endotoxin, or HFD (48–50). Our study indicated that CO-induced FGF21 expression attenuates liver damage and hepatic lipid accumulation resulting from ER stress. We also showed that CO can ameliorate ER stress-induced liver injury as well as HFD-induced metabolic disorders. Several previous reports have suggested that CO prevents HFD-induced obesity. However, the mechanisms for the preventive effects of CO on metabolic disorders remain unknown.

This study is the first, to our knowledge, to establish the critical role of FGF21 in the salutary effects of CO on metabolic disorders, using mice genetically deficient in FGF21. FGF21, as a potential therapeutic for metabolic disease, such as diabetes and obesity (51, 52), can increase thermogenesis. Specifically, FGF21 stimulates the browning of white fat in response to cold or stimulation with adrenergic agonists (25). Because FGF21 is linked with energy expenditure (25), thermogenesis increased by FGF21 may provide a mechanism for weight loss. Interestingly, CO-induced FGF21 expression enhanced the transcription of thermogenic genes. In mice genetically deficient in FGF21, CO failed to increase gene expression related to the browning of WAT. Therefore, we conclude that CO promotes increased thermogenic expression via induction of FGF21 expression.

In summary, CO protects against HFD-induced weight gain in wild-type Fgf21+/− mice. The changes in body weight induced by CO were accompanied by improved metabolic status via FGF21 induction. Thus, CO-induced FGF21 expression counteracts metabolic dysfunction in ER stress or HFD-induced pathologies through up-regulation of mitochondrial function and biogenesis, the decrease of white adipocyte size, and the browning WAT. Finally, CO activates the PERK-ATF4 pathway via enhanced ROS production, which enhances FGF21 expression.

Completed and ongoing clinical trials to date for CO therapy in humans have, in general, demonstrated the safety of low-dose CO application in humans, although further trials will be needed to confirm efficacy. It is, therefore, premature to speculate that CO will prove to be an effective therapeutic in humans for metabolic disease applications (53). Nevertheless, our findings that an increase of FGF21 by CO improves metabolic dysfunction suggest that a low-dose application of CO may represent a potential therapeutic strategy for the amelioration of metabolic diseases.

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AUTHOR CONTRIBUTIONS

Y. Joe, S. Kim, H. J. Kim, J. Park, and Y. Chen wrote the protocol, designed the study, and participated in data acquisition and interpretation; H.-J. Park and S.-J. Jekal contributed technical assistance and suggestions; Y. Joe,
S. W. Ryter, U. H. Kim, and H. T. Chung designed the study and were responsible for interpreting the data, drafting the article, and obtaining final approval of this version; and all authors contributed to the final version of the manuscript.

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