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The HO-1/CO System and Mitochondrial Quality Control in Skeletal Muscle

Heath G. Gasier[1], Hagir B. Suliman[1,2], and Claude A. Piantadosi[1,2,3]

[1]Department of Anesthesiology, [2]Department of Pathology; and [3]Department of Medicine, Duke University Medical Center, Durham, NC

GASIER, H.G., H.B. SULIMAN, and C.A. PIANTADOSI. The HO-1/CO system and mitochondrial quality control in skeletal muscle. Exerc. *Exerc. Sport Sci. Rev.*, Vol. 50, No. 1, pp. 49–55, 2022. *Inducible heme oxygenase (HO)–1 catalyzes the breakdown of heme to biliverdin, iron, and carbon monoxide (CO). CO binds to cytochrome c* oxidase and alters mitochondrial redox balance and coordinately regulates mitochondrial quality control (MQC) during oxidant stress and inflammation. The hypothesis presented is that the skeletal muscle HO-1/CO system helps modulate components in the MQC cycle during metabolic stress. **Key Words:** exercise, fission, fusion, mitochondrial biogenesis, mitophagy, obesity

Key Points

- Heme oxygenase (HO)–1 activity increases cellular carbon monoxide (CO) production, which initiates mitochondrial redox-sensitive signals that integrate mitochondrial biogenesis, mitochondrial dynamics (fission and fusion), and mitophagy, that is, mitochondrial quality control (MQC).
- HO-1/CO system induction stimulates skeletal muscle MQC similar to exercise and preserves skeletal muscle mitochondrial morphology and function during nutrient excess.
- The mechanisms by which the HO-1/CO system in skeletal muscle regulates MQC in exercise and diet-induced obesity are under investigation.

INTRODUCTION

Mitochondria are known for matching energy production to demand, but they also are critical in maintaining cellular redox balance, calcium homeostasis, and heme synthesis. These activities are all essential for cell survival and met through an interconnected mitochondrial network that continuously undergoes fusion and fission (dynamics) to exchange mitochondrial DNA (mtDNA) and other contents, and prevent the accumulation of mitochondrial damage or mtDNA mutatations by promoting mitophagy (1). Mitochondria are not produced *de novo* but are regenerated by coordinated nuclear and mitochondrial genomic transcription and translation (1). Collectively, these tightly regulated interrelated processes comprise the mitochondrial quality control (MQC) cycle, ensuring mitochondrial mass, morphology, and distribution rapidly adapt to maintain cellular homeostasis. Altered MQC has been reported in inflammation, aging and sarcopenia, cancer, diabetes, cardiovascular, and neurodegenerative diseases (2). Because of this, basic research has been dedicated to understanding how MQC processes are regulated in health, affected by stress, and adapt to disease with the goal of identifying targeted therapies.

We have focused on understanding how MQC is regulated by the heme oxygenase (HO)–1 and carbon monoxide (CO) system during oxidant stress and inflammation. Much of the new information has been generated from cardiac tissue and cells (3–6); however, early data from skeletal muscle suggest that the control mechanisms of the MQC cycle are similar (7,8). This led us to hypothesize whether the HO-1/CO system regulates skeletal muscle MQC during metabolic stress, that is, exercise and nutrient excess. We now know that low-dose inhaled CO is capable of activating skeletal muscle mitochondrial fusion and biogenesis in healthy adults and may increase aerobic exercise capacity ($\dot{V}O_{2max}$) when combined with exercise training. Our goal here is to provide clinicians and researchers with a deeper understanding of the functionality of the HO-1/CO system and its role in skeletal muscle.

THE HO-1/CO SYSTEM AND MYOCARDIAL MQC

Heme is composed of four pyrroles (porphyrin) attached to a central iron atom and is generated from the turnover of heme proteins or formed in mitochondria from glycine and succinyl-CoA (9) (Fig. 1). Heme proteins include those that bind oxygen for delivery and storage (hemoglobin and myoglobin), enzymes activated by oxygen binding (peroxidases, catalases, oxygenases, and

Address for correspondence: Heath G. Gasier, PhD, RD, Department of Anesthesiology, Center for Hyperbaric Medicine and Environmental Physiology, Duke University Medical Center, Durham, NC 27710 (E-mail: heath.gasier@duke.edu).

Accepted for publication: September 24, 2021.

Associate Editor: Stephen E. Alway, Ph.D., F.A.C.S.M.

0091-6331/5001/49–55

Exercise and Sport Sciences Reviews

DOI: 10.1249/JES.0000000000000277

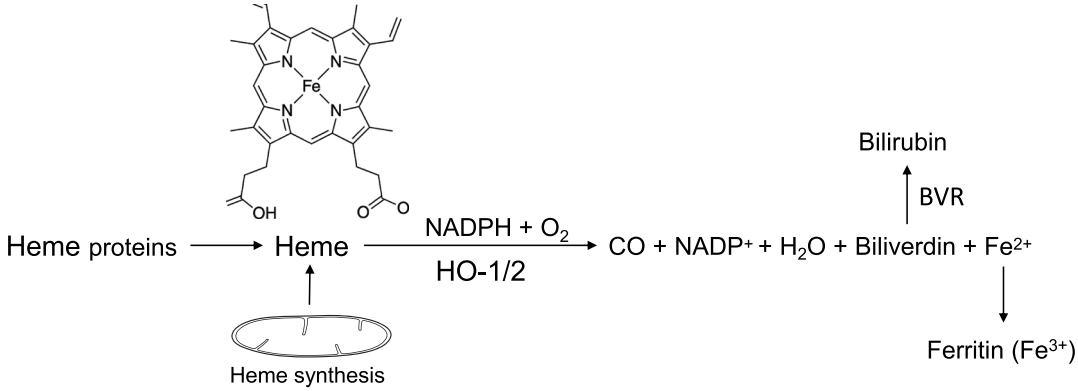

Figure 1. Heme clearance pathway. Free heme released from heme proteins or synthesized by mitochondria is enzymatically degraded by HO-1/2 in the presence of NADPH and O_2 to CO, biliverdin, and ferrous iron (Fe^{2+}). Biliverdin is further reduced to bilirubin by biliverdin reductase, and Fe^{2+} is stored as ferritin (Fe^{3+}).

monooxygenases), and protein electron carriers (cytochromes) (10). In humans, ~85% of the total heme is found in hemoglobin (9). Free heme is a strong pro-oxidant and promotes inflammation, becoming toxic if not adequately catabolized.

The HO enzymes are the main mechanism for heme detoxification (10). There are two main HO isoforms, HO-1 and HO-2, and a third (HO-3) that is a pseudogene derived from HMOX2 mRNA (10,11). HO-2 is constitutively expressed in most tissues, whereas HO-1, also called heat shock protein 32, is induced by stress. HO-1 and HO-2 catalyze the NADPH-dependent oxidation of heme to equimolar amounts of biliverdin, ferrous iron (Fe^{2+}), and CO (10). Biliverdin is rapidly reduced to bilirubin by biliverdin reductase, and Fe^{2+} is stored in ferritin (Fe^{3+}). CO is transported by hemoglobin and exhaled in the lungs, but in the cell, it serves several functions, including activation of stress-induced kinases and redox-sensitive transcription factors that promote adaptation to cellular stress (12).

Cellular CO binds to cytochrome *c* oxidase at the a_3 heme, which slows enzyme turnover and increases the cellular partial pressure of oxygen (PO_2) (4) (Fig. 2). The lower turnover increases the reduction state of cytochrome bc_1 and improves filling of the Q cycle. Because the rate of electron transfer from semiquinone to ($Q\cdot^-$) to oxygen is proportionate to the product of $Q\cdot^-$ and [O_2], superoxide ($\cdot Q^-$) production increases. Mitochondrial superoxide dismutase (SOD2) helps catalyze the dismutation of $\cdot Q^-$ to hydrogen peroxide (H_2O_2), which provides a redox signal for activating MQC by two mechanisms (13). First, H_2O_2 oxidizes cysteine residues in phosphatase and tensin homolog (PTEN), $Cys^{71\ \&\ 124}$, and protein tyrosine phosphatase 1B (PTP1B), Cys^{215}, inhibiting their phosphatase activity (14,15). In a phosphoinositide 3-kinase (PI3K)–dependent manner, protein kinase B (PKB or Akt) phosphorylates nuclear respiratory factor 1 (NRF1), leading to its nuclear translocation (4). In addition, H_2O_2 oxidizes Cys^{151} in Kelch-like ECH-associated protein 1 (Keap1), which inhibits nuclear factor erythroid 2-related factor 2 (Nrf2) ubiquitination and degradation, allowing its nuclear transport (16). Moreover, Akt phosphorylates glycogen synthase kinase 3β (GSK3β), repressing its inhibitory effect on Nrf2 nuclear import independently of Keap1 (17). In the nucleus, Nrf2 binds to antioxidant response elements (AREs) in the promoter regions of HMOX1, SOD2, and NRF1 genes, stimulating mRNA transcription and translation (6). Thus, H_2O_2 production and HO-1 protein levels increase. The accumulation of nuclear NRF1 leads to its promoter binding of multiple genes, including mitochondrial transcription factors A, B1, and B2 (TFAM, TFB1M, and TFB2M), and with its coactivator, peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), resulting in mRNA transcription and mtDNA transcription and replication (4,18).

NRF1 also induces the mitophagy proteins PTEN-induced kinase 1 (PINK1), a serine/threonine kinase, and PARK2 (Parkin), an E3 ubiquitin ligase (5). PINK1 is localized to the inner mitochondrial membrane in functional mitochondria, where it is ubiquitinated and degraded; however, when mitochondrial membrane potential ($\Delta\psi m$) falls (depolarization), PINK1 is stabilized in the outer mitochondrial membrane (19). PINK1 autophosphorylates and recruits and stimulates Parkin ligase activity, ubiquitinating outer membrane proteins that are then phosphorylated by PINK1, initiating mitophagy. PINK1-Parkin interferes with mitochondrial dynamic regulation by two ways. First, Parkin ubiquitinates the outer mitochondrial membrane fusion GTPase, mitofusion 2 (MFN2), which is then phosphorylated by PINK1 and degraded by proteasomes (20). Second, PINK1 is involved in activating dynamin-related protein 1 (Drp1) activity, a mitochondrial fission GTPase, promoting mitochondrial fission and mitophagy. During oxidant stress, the HO-1/CO system is required to activate PINK1-Parkin–dependent mitophagy (5). Collectively, HO-1/CO regulates mitochondrial redox-sensitive pathways that coordinate cardiac mitochondrial biogenesis and mitophagy to maintain a healthy and stable mitochondrial population.

EXERCISE-INDUCED SKELETAL MUSCLE HO-1/CO SYSTEM AND MQC

In 1997, Essig *et al.* (21) first reported the presence of HO-1 and HO-2 in rat skeletal muscle (plantaris and tibialis anterior) and that muscle contractions (running and nerve stimulation) induce HO-1 mRNA expression. Others established that HO-1 mRNA expression is higher in type I versus type II fibers, reflective of the greater myoglobin and mitochondrial content, and is induced by exercise in a time-, intensity-, and fiber type–dependent manner (22,23). More specifically, low-intensity and long-duration running exercise leads to a rapid and robust elevation in HO-1 mRNA expression in type I and type II rich muscle fibers (red gastrocnemius), whereas high-intensity and shorter-duration exercise sustains HMOX1 gene transcription in type II rich muscle fibers (white gastrocnemius) (22). A similar time course in skeletal muscle HMOX1 transcription rate and mRNA

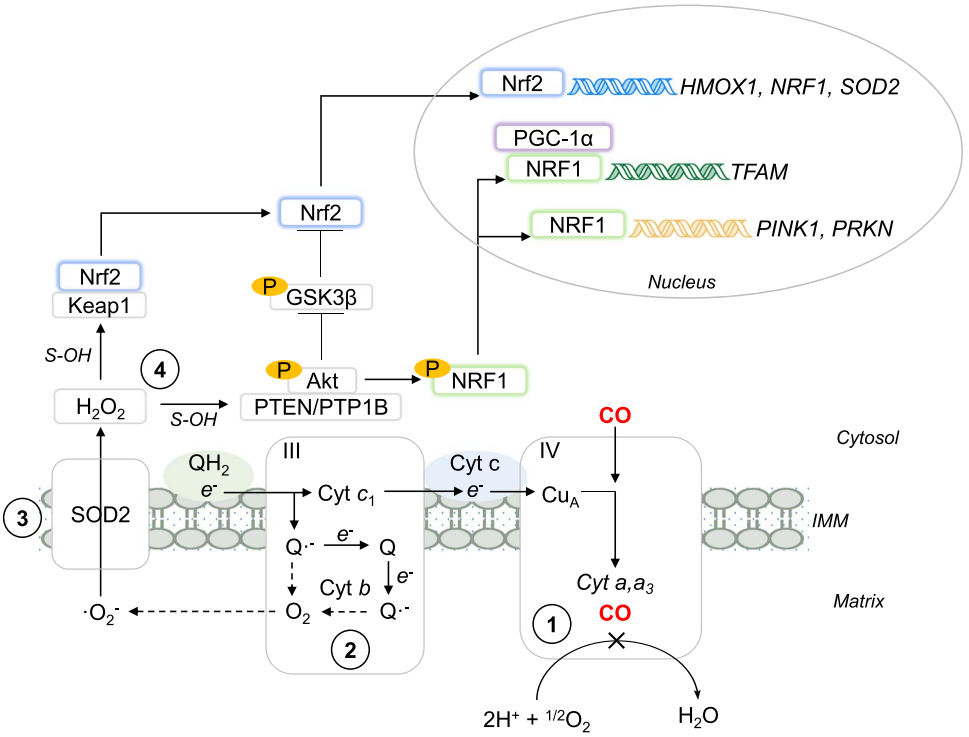

Figure 2. Overview of carbon monoxide (CO)-dependent transcriptional activation of mitochondrial quality control (MQC). 1) CO binding to heme a_3 of cytochrome *c* oxidase slows enzyme activity and reduction of $^{1/2}O_2$ to H_2O. 2) Electrons fill the Q cycle, increasing the reduction state of cytochrome bc_1 and oxygen availability. This leads to increased $\cdot O_2^-$ production (dotted lines). 3) Mitochondrial superoxide dismutase (SOD2) catalyzes the dismutation of $\cdot O_2^-$ to H_2O_2, which acts as an oxidant-signaling molecule. 4) H_2O_2 oxidizes thiols of Keap1, permitting Nrf2 nuclear translocation and transcriptional activation of HMOX1, NRF1, and SOD2. H_2O_2 also oxidizes thiols in PTEN or PTP1B, activating protein kinase B (Akt), NRF1 phosphorylation and nuclear translocation, and activation of mitochondrial transcription factor A (Tfam), PTEN-induced kinase 1 (PINK1), and Parkin (PRKN) transcription. Akt phosphorylates GSK3β, repressing the inhibitory effect on Nrf2 nuclear translocation. Q, quinone (oxidized); O·–, semiquinone; QH_2, ubiquinol (reduced).

expression was reported in young healthy adults after 5 d of exhaustive one-legged knee extensor exercise (24). In addition, rats with a high intrinsic running capacity have significantly more (~2-fold) HO-1 protein in skeletal muscle than those with a low intrinsic running capacity, and aerobic exercise training increases skeletal muscle HO-1 mRNA and protein content (25). Moreover, skeletal muscle HO-1 deletion compromises mitochondrial adaptations to aerobic exercise training and reduces endurance exercise capacity (25). These data indicate skeletal muscles contain HO enzymes, aerobic exercise induces HO-1 mRNA transcription and translation, and levels of each are increased with aerobic exercise training.

Essig *et al.* (21) postulated that the signal for exercise-induced skeletal muscle HO-1 activation is the oxidants produced from mitochondrial respiration, or from heme. The mechanisms by which oxidant production may link the HO-1/CO system to MQC in skeletal muscle are shown in Figure 3. Exercise-induced oxidant production increases phosphorylation, thus, activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) at Thr^{286} and its downstream target 5′-AMP-activated protein kinase (AMPK)α at Thr^{172} (26). AMPK also is activated directly by S-glutathionylation of $Cys^{299\ \&\ 304}$ of the AMPKα subunit, and indirectly by slowed respiration and increased AMP-to-ADP ratio (27,28). Upon activation, AMPK regulates Nrf2 activation by four mechanisms: 1) AMPK directly phosphorylates Nrf2 at Ser^{40} (29); 2) AMPK activates protein kinase C (PKC), which phosphorylates Nrf2 at Ser^{40} (30); 3) AMPK phosphorylates p62 at Ser^{349} (Ser^{351} in mice), which promotes Keap1 degradation and Nrf2 stabilization (29,31); and 4) AMPK phosphorylates GSK-3β at Ser^{9} via Akt, preventing Nrf2 phosphorylation ($Ser^{334–338}$ in Neh6 domain) and $SCF^{\beta\text{-}TrCP}$–mediated degradation (17,32). Nrf2 then translocates to the nucleus where it binds to the ARE and transcriptionally activates HMOX1 and NRF1 gene expression (33). Skeletal muscle contraction–generated H_2O_2 and NO may also stabilize Keap1 independently of AMPK and promote Nrf2 nuclear transport and ARE binding (33); however, this has not been directly tested.

To reflect HO-1 activity, the production of endogenous CO should vary, but this has not been examined in skeletal muscle. When carboxyhemoglobin (HbCO, baseline 1%) was used as a surrogate of HO-1 activity and CO production, 2 h of intermittent cycling exercise increased HbCO nearly twofold, from 1.1 ± 1.6% to 2.1 ± 1.6%, (34). Cellular CO stimulates soluble adenylyl cyclase activity, thus, increasing the concentration of cyclic adenosine monophosphate (cAMP) (35,36). CO also binds to skeletal muscle soluble guanylyl cyclase (sGC) and increases cyclic guanosine monophosphate (cGMP) (36). In both cases, protein kinase A (PKA) is activated and translocated to the nucleus, where it phosphorylates cAMP-responsive element-binding protein (CREB) at Ser^{133} (36–38). Upon phosphorylation, CREB binds to the cAMP response element (CRE) in the PGC-1α promoter, increasing its mRNA transcription (39). In coordination with estrogen-related receptor (ERR)α, PGC-1α promotes MFN2 transcription (40) and coactivates NRF1 bound to the TFAM gene promoter (18). These muscle contraction–induced redox-sensitive signals

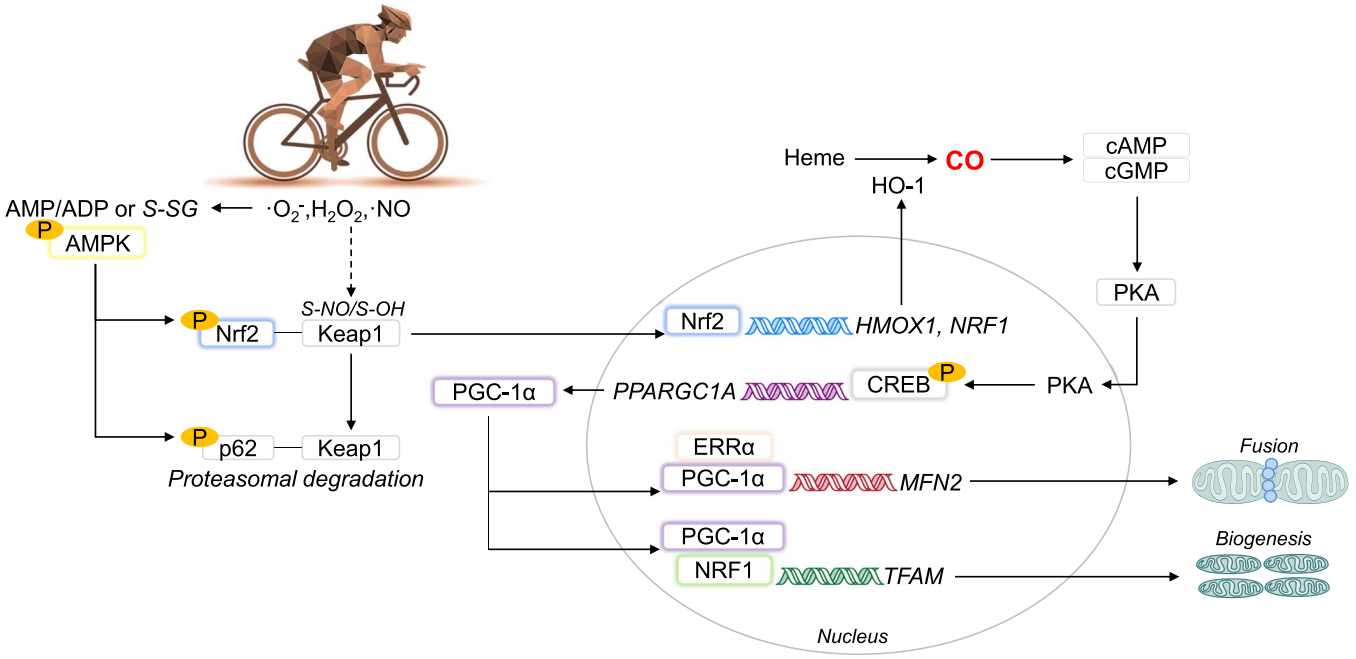

Figure 3. Proposed role of the Heme oxygenase (HO)–1/carbon monoxide (CO) system in how exercise activates the skeletal muscle mitochondrial quality control (MQC). Exercise increases oxidant production ($\cdot O_2^-$, H_2O_2, and ·NO) to regulate Nrf2 liberation from Keap1 and nuclear import by AMPK-dependent (black arrow) and possibly AMPK-independent mechanisms (dotted black arrow). In the nucleus, Nrf2 stimulates NRF1 and HO-1 mRNA transcription and translation that increases CO production, activation of cAMP and cGMP, and PKA. PKA is translocated to the nucleus where it phosphorylates CREB that activates peroxisome proliferator-activated receptor gamma coactivator-1α mRNA transcription (PPARGC1A) and translation (PGC-1α). In coordination with ERRα and NRF1, PGC-1α stimulates mRNA transcription and translation of mitochondrial fusion protein 2 (MFN2) and mitochondrial transcription factor A (TFAM), promoting mitochondrial fusion and biogenesis.

increase mitochondrial content and promote an elongated mitochondrial morphology (33,41).

SKELETAL MUSCLE HO-1/CO SYSTEM AND MQC IN OBESITY

In 2003, Bruce *et al.* (42) provided the first evidence that the skeletal muscle HO-1/CO system may be involved in obesity and comorbid conditions by showing a 55% reduction in HO-1 mRNA expression in obese subjects with type 2 diabetes. In the same subjects, HO-1 mRNA increased ~70-fold after a hyperinsulinemic-euglycemic clamp procedure. Others reported marked improvements in weight control and glycemia, and a reduction in oxidative stress in Zucker diabetic fatty rats up to 4 months after HO-1 induction by hemin (36). These observations were accompanied by a decrease in soleus muscle nuclear factor-kappa B (NF-κB), c-Jun N-terminal kinase (JNK), and activator protein 1 (AP-1) protein expression, suggestive of reduced inflammation. In addition, AMPK protein expression was increased whereas PTP1B and GSK-3β levels were reduced. Because the HO-1/CO system integrates Nrf2- and NRF1-mediated anti-inflammatory cytokine expression and mitochondrial biogenesis (12), it is easy to speculate that MQC is influenced by hemin treatment.

When nutrient availability exceeds energy demand, skeletal muscle mitochondria undergo Drp1-mediated fission and become punctate and fragmented (Fig. 4A), resulting in reduced efficiency and a lowered capacity to produce ATP (7,43). In addition, PINK1 protein expression and conjugated microtubule-associated protein 1A/1B-light chain 3 (LC3 II), required for autophagosomal fusion to lysosomes, increase, and this is accompanied by reduced mitochondrial number and mass, indicating mitophagy (43,44). Logically, induction of the HO-1/CO system should preserve MQC and function during overfeeding, so we performed experiments whereby we delivered CO to C2C12 myoblasts using water-soluble CO releasing molecule-3 (CORM-3) and rats by low-dose inhaled CO (7). CORM-3 contains tricarbonylchloro(glycinato)ruthemium (II), releases 1 CO·mol^{-1}, and has a half-life of 1 min (45). When cells were incubated in high glucose and high fat, CORM-3 (20 μM) treatment attenuated mitochondrial oxidant production and Drp1 regulated fission and fragmentation (Fig. 4B). PINK1 protein levels decreased, respiratory capacity increased, and a modest improvement in coupling efficiency was observed in some of the experiments. These changes are expected because elongated mitochondria have a higher respiratory capacity and possibly improved efficiency than fragmented mitochondria (46). In a different experiment, obesity-prone rats were fed a high-fat and high-sucrose diet for 10 wk and remained sedentary, received low-dose inhaled CO (250 ppm (8.9 mM) twice per week), underwent moderate running exercise training three times per week, or received CO and performed exercise training (7). The combination of CO and exercise promoted a mitochondrial population that were larger, more complex, and had well-defined cristae compared with sedentary obese (Fig. 4C and D), CO, or exercise groups. ADP-stimulated respiration in the presence of NADH-linked substrates and whole-body energy expenditure were also greater when CO and exercise were combined. Collectively, these data suggest the HO-1/CO system influences skeletal muscle MQC and bioenergetics in cell and rat models of diet-induced obesity.

LOW-DOSE INHALED CO, MQC, AND $\dot{V}O_{2MAX}$

Mitochondrial respiratory capacity contributes to $\dot{V}O_{2max}$. Because the HO-1/CO system activates mitochondrial biogenesis, inhaling low-dose CO should increase $\dot{V}O_{2max}$. To test this hypothesis, our group conducted two independent investigations

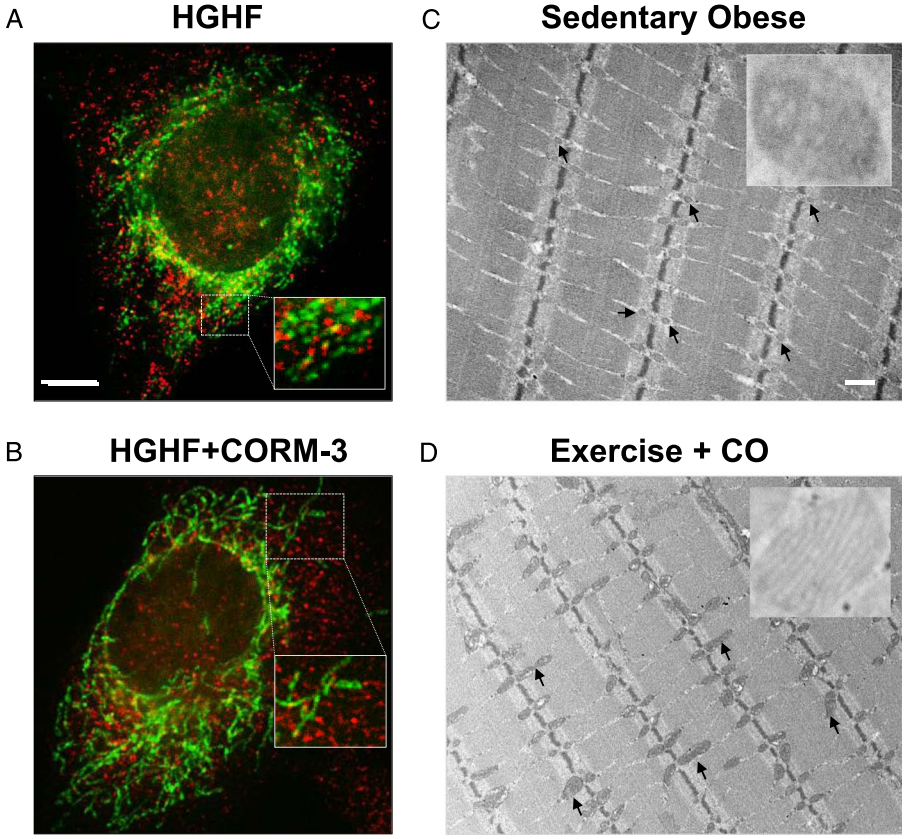

Figure 4. Heme oxygenase (HO)–1/carbon monoxide (CO) regulates mitochondrial dynamics in obesity. A and B. C2C12 myoblasts were incubated with high glucose (15.6 mM) and high fat (0.250 mM palmitate) (HGHF) for 4 h (7). Cells were pretreated 30 min before adding HGHF with DMSO (A) or 20 μM CORM-3 (B). Cells were stained with cytochrome c (green) and Drp1 and imaged at ×40 magnification with an epifluorescence microscope (scale bar, 5 μm). Nutrient excess caused Drp1-mediated mitochondrial fragmentation (A), whereas CORM-3 maintained an elongated/tubular mitochondria network (B). C and D. Obesity-prone rats were fed a high-fat and high-sucrose diet for 10 wk and remained sedentary (C) or underwent 3 h·wk^{-1} of moderate running exercise training and treated with inhaled CO at 250 ppm (five 1-h treatments before and two 1-h treatments during the experiment) (D) (7). Soleus muscle mitochondria were imaged using transmission electron microscopy at ×12–×15,000 magnification (×60,000 insets) (scale bar, 0.5 μm). Mitochondria from sedentary obese rats were small (black arrows) and had disorganized cristae (inset) (C). In contrast, mitochondria from rats that received CO and underwent running exercise training were larger with increased complexity (black arrows) and had clear and defined cristae (inset) (D). The morphological changes were significantly greater than CO or exercise training alone (images not shown).

whereby healthy recreationally active adults breathed CO for 1 h over five consecutive days (8,47). In the first study, the dose was 100 ppm (3.57 mM) and raised the HbCO to ~3.3% (47). This led to increased PGC-1α, NRF1, Tfam, and mtDNA polymerase γ (POLG; the catalytic subunit), and MFN1 and 2, and OPA1 mRNA expression, indicative of transcriptional activation of mitochondrial biogenesis and fusion. These changes were accompanied by increased protein expression of citrate synthase and ATPase, but not in mtDNA copy number. Moreover, $\dot{V}O_{2max}$ (cycling) was unchanged, leading to a second study whereby the dose was increased to 200 ppm (7.14 mM) in healthy recreationally active adults, the National Institute for Occupational Safety and Health upper limit for CO exposure (8). In addition, $\dot{V}O_{2max}$ testing was completed in hypobaric hypoxia (simulated altitude of 4572 m) using a single-leg cycling exercise protocol with the intent of shifting the limitation in exercise capacity from systemic oxygen delivery to tissue diffusion and mitochondrial respiratory capacity. This level of CO raised HbCO to ~5.5% and led to complete induction of the mitochondrial biogenesis transcriptional program and increased MFN2 and OPA1 mRNA expression, responses that were accompanied by increased mitochondrial volume density. In addition, myoglobin content and the number of capillaries per muscle fiber increased. Despite improvement in the peripheral components that are achieved with aerobic exercise training, $\dot{V}O_{2max}$ was unchanged. These data imply that low-dose inhaled CO is capable of stimulating skeletal muscle mitochondrial biogenesis and fusion control, and other components of the oxygen transport system, but does not augment $\dot{V}O_{2max}$.

The null result of inhaled CO on $\dot{V}O_{2max}$ has been reported by others (48,49). In recreationally active adults who inhaled a single dose of CO (~1145 ppm) daily for 10 d, submaximal and maximal $\dot{V}O_2$ did not increase (48). This dose raised HbCO to ~4.4%. When healthy and moderately trained subjects inhaled a bolus of CO five times per day for 3 wk, a dose that raised HbCO to ~5%, $\dot{V}O_{2max}$ did not improve despite an increase in hemoglobin (Hb) mass of ~5% (49). The authors did, however, report a significant relation between changes in $\dot{V}O_{2max}$ and Hbmass (r = 0.70). In contrast, when trained soccer players inhaled a single bolus of CO that raised HbCO to ~4.5% daily before running exercise training over 4 wk, Hbmass, $\dot{V}O_{2max}$, and running economy improved by ~3.7%, 2.7%, and 4% (medium effect size), respectively (50). These data suggest aerobic exercise training is needed with CO to increase $\dot{V}O_{2max}$.

CONCLUSION AND PERSPECTIVE

HO-1 is a stress-mediated enzyme that produces the endogenous gas molecule CO. CO binding to heme within cytochrome *c* oxidase increases extramitochondrial H_2O_2 levels that activate antioxidant and anti-inflammatory DNA binding transcription factors that modulate MQC to support cellular energy demand and prevent cell death. Although much of the foundational research has been completed in nonskeletal muscle cells and tissue, emerging data support a role of the HO-1/CO system in regulating skeletal MQC during oxidant and inflammatory stress, that is, muscle contractions and nutrient excess. In exercise, oxidant and energy availability regulates skeletal muscle Nrf2 translocation to the nucleus where it can activate mRNA transcription of HO-1 and NRF1. HO-1 catalyzes the breakdown in heme, which increases during exercise (25), increasing CO production and PKA-mediated PPARGC1A transcriptional activation. In coordination with NRF1 and ERRα, PGC-1α promotes mitochondrial fusion and biogenesis. Inhaled low-dose CO also transcriptionally activates skeletal muscle mitochondrial fusion and biogenesis and increases myoglobin content and capillarization. In diet-induced obesity, HO-1/CO system induction shifts the mitochondrial dynamic balance toward fusion, resulting in an elongated and tubular mitochondrial network that is linked to increased mitochondrial respiration. Aerobic exercise training, however, is necessary to increase resting and maximal $\dot{V}O_2$.

Production of this review was possible because of the progress in the field of HO-1/CO biology; still, our understanding of the skeletal muscle HO-1/CO system in exercise and obesity is incomplete. For instance, it remains unknown whether the HO-1/CO system controls mitophagy in exercise and obesity. Clearly, damaged or poorly functioning mitochondria need to be segregated by fission and ubiquitinated for mitophagy, or fuse with healthy neighboring mitochondria. In this instance, mitochondrial mass changes are due to complementation versus mitophagy and biogenesis. Measuring mitophagy in tissue is difficult; thus, single time-point measurements of activation and repressor control mRNA and proteins, or immunofluorescence of colocalized proteins specific to mitochondria and autophagosomes or lysosomes are used as proxies. To overcome this shortcoming, the use of transgenic mouse models, for example, mt-Keima or *mito*-QC, should advance our understanding of the HO-1/CO systems' role in mitophagy (51). More research also is needed to determine efficacious CO dosing protocols, which is limited to HbCO in the 6% to 10% range, and how exercise training and fitness level influence the HO-1/CO system responsiveness to stress and exogenous CO. With this information, testing low-dose inhaled CO for complications apart from respiratory illnesses (52,53), for example, obesity and metabolic diseases, may be possible.

Acknowledgments

This study was supported by the Uniformed Services University of the Health Sciences (HGG) and NIH HL135239-03 (CAP).

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