Carbon Monoxide, Skeletal Muscle Oxidative Stress, and Mitochondrial Biogenesis in Humans

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Abstract

Given that the physiology of heme oxygenase-1 (HO-1) encompasses mitochondrial biogenesis, we tested the hypothesis that the HO-1 product, carbon monoxide (CO), activates mitochondrial biogenesis in skeletal muscle and enhances maximal oxygen uptake ($\dot{V}O_2$max) in humans. In ten healthy subjects, we biopsied the vastus lateralis and performed $\dot{V}O_2$max tests followed by blinded randomization to air or CO breathing (1hour/day at 100ppm for 5 days), a contralateral muscle biopsy on day 5, and repeat $\dot{V}O_2$max testing on day 8. Six independent subjects underwent CO breathing and two muscle biopsies without exercise testing. Molecular studies were performed by real time RT-PCR, Western blot analysis, and immunochemistry. After $\dot{V}O_2$max testing plus CO breathing, significant increases were found in mRNA levels for nuclear respiratory factor-1 (NRF-1), PGC-1α co-activator, mitochondrial transcription factor-A, and DNA polymerase γ (Polγ) with no change in mtDNA copy number or $\dot{V}O_2$max. Levels of myosin heavy chain I and nuclear-encoded HO-1, superoxide dismutase-2, citrate synthase, mitofusin-1 and -2 and mitochondrial-encoded cytochrome oxidase subunit-I (COX-I) and ATPase-6 proteins increased significantly. None of these responses were reproduced by $\dot{V}O_2$max testing alone, while CO alone increased Tfam and Polγ mRNA, and COX-I, ATPase-6, mitofusin-2, HO-1, and SOD2 protein. These findings provide evidence linking the HO/CO response involved in mitochondrial biogenesis in rodents to skeletal muscle in humans through a set of responses involving regulation of the mtDNA transcriptosome and mitochondrial fusion proteins autonomously of changes in exercise capacity.
Introduction

Carbon monoxide (CO), as a toxic gas, causes tissue hypoxia (36), but direct cellular effects of CO also contribute to adaptive responses (24). Endogenous CO is detectable at nanomolar levels as a by-product of heme catabolism (8), which induces the expression of antioxidant enzymes, including heme oxygenase-1 (HO-1) and mitochondrial superoxide dismutase (SOD2). These responses depend on CO and protect against oxidative stress and thus raise the possibility that near physiological concentrations of CO might be therapeutically useful (29).

CO also stimulates mitochondrial biogenesis, which is activated in part when CO binds to cytochrome c oxidase and boosts mitochondrial hydrogen peroxide production (32, 33). The functionality of this oxidant as a signaling molecule underscores the importance of understanding CO’s physiological roles, as mitochondrial oxidative stress develops both during exercise and in disease states, and mitochondrial DNA (mtDNA) is open to attack by reactive oxygen species (ROS) (3). Oxidative mtDNA damage contributes to the pathogenesis of multiple diseases, such as atherosclerosis (12) and inflammatory organ dysfunction (35), and without bi-genomic coordination of mitochondrial biogenesis, mitochondria cannot adapt to changing physiological conditions (17, 19, 31, 40). New insights into mitochondrial biogenesis have also generated interest in ways to ameliorate mitochondrial pathogenesis. For instance, mitochondrial damage is linked to outcome in sepsis where patients with poor outcomes have greater mitochondrial dysfunction and higher tissue PO2 than those who recover fully (4), and in mice, where metabolic recovery from gram positive sepsis occurs in conjunction with mitochondrial biogenesis (14). Also, interference with mitochondrial biogenesis in doxorubicin cardiac toxicity is abrogated by HO/CO (25, 32).
Mitochondrial biogenesis in various organs is activated by the gaseous signaling molecules, nitric oxide (NO) (23) and CO (33). NO is involved in the heart (22) and the brain (13), while in the heart CO initiates mitochondrial biogenesis through transcriptional activation of the PPAR-γ co-activator PGC-1α, nuclear respiratory factors-1 and -2 (NRF-1 and -2) and mitochondrial transcription factor-A (Tfam) (32). Since CO is simple to administer by inhalation, if it exert similar effects in humans, small amounts might be used to activate mitochondrial biogenesis during training or in rehabilitation from muscle diseases associated with dysfunction of previously healthy mitochondria.

Mitochondrial biogenesis is also clearly necessary for muscle conditioning (15, 16), but the degree to which mitochondrial content determines maximal aerobic exercise capacity is an interesting open question (9, 15). Maximal oxygen uptake ($\dot{V}O_2\text{max}$) may be limited by systemic factors like cardiac output (30) or by peripheral factors like microcirculatory O$_2$ diffusion or mitochondrial oxidative capacity (37, 38). In rodents, inhibition of cytochrome $c$ oxidase causes a dose-dependent decrease in O$_2$ consumption (21), while exercise training increases mitochondrial activity and $\dot{V}O_2\text{max}$ compared with controls, but those gains are eliminated by fixing the respiratory capacity (28).

On this basis, we tested the hypothesis that low level inhaled CO activates mitochondrial biogenesis in human skeletal muscle. Changes in the nuclear regulation of mitochondrial biogenesis, anti-oxidant enzyme expression, and selected nuclear- and mitochondrial-encoded proteins were measured after brief, intermittent periods of CO inhalation. We also analyzed the effects of $\dot{V}O_2\text{max}$ on these parameters and assessed the possibility that $\dot{V}O_2\text{max}$ is affected by CO-induced changes in muscle mitochondrial capacity.
Experimental Procedures

The study was approved by the Duke University Institutional Review Board, and after informed consent, we enrolled 16 healthy non-smoking volunteers (ages 19-39, 4 females, 10 males), who agreed not to deviate from their regular routine of diet and activities. Baseline biopsies of the vastus lateralis muscle were performed with lidocaine anesthesia using a U.C.H. Muscle Biopsy Needle (Popper & Sons, Inc. New Hyde Park, N.Y 11040). Two 30-50 mg muscle samples were obtained: one was snap frozen at -80°C and the other fixed immediately in 10% paraformaldehyde.

Ten subjects were assigned in a double-blind fashion to breathe either 100 ppm CO or air from identical breathing systems for one hour daily on five consecutive days after the first muscle biopsy. These subjects underwent radial arterial line placement and performed $\dot{V}O_2$ max tests on a stationary bicycle using a standard graded-exercise protocol. Blood samples (2-3ml) were drawn at rest, at nine minutes exercise, end of exercise ($\dot{V}O_2$max), and five minutes post-exercise. After the CO exposure on day 5, venous blood was drawn to measure carboxyhemoglobin (HbCO) and a second biopsy performed on the contralateral vastus muscle. Three days after the last CO exposure, the subjects performed a second $\dot{V}O_2$max test with radial arterial line placement and blood samples. To independently evaluate the CO response, six separate subjects underwent the CO exposures without exercise testing between the two muscle biopsies.

RNA extraction and real time quantitative PCR. Total RNA was prepared from muscle biopsies using Trizol reagent (Invitrogen Corp., Carlsbad, CA). One µg of RNA was reverse-transcribed using Random Hexomer primers and a Superscript enzyme (Invitrogen).
Real-time reverse transcriptase (RT)-PCR was performed using an ABI Prism 7000 and gene expression master mix (Applied Biosystems, Foster City, CA) (32). Primers and probes for human NRF-1, NRF-2, PGC-1α and Tfam were from Applied Biosystems and 18S rRNA was used as an internal control. ABI Prism 7000 SDS Software was used to quantify differences in gene expression. The threshold cycle (Ct) was determined in the exponential amplification phase. The amount of transcript was normalized to 18S RNA by subtracting the mean Ct values for each condition. Because of the exponential PCR reaction, a difference of n in Ct values represents a twofold difference in transcript levels. PCR was performed in triplicate.

**Quantitative PCR for mtDNA copy number.** Total and mitochondrial DNA was isolated from muscle specimens using mtDNA Extractor Kit (Wako Chemicals; Richmond, VA). DNA primers were designed to detect COX II and 18S rDNA at a maximum amplicon length of 150 bp: 18S rDNA forward, 5'-GAATTCCCAGTAAGTGCGGTCA -3', and reverse, 5'-TAATGATCCTTCCGCAGGTTC -3'; COXII forward, 5'-ATGGCACAATGCAGCGCAAGTAGG -3', and reverse, 5'-ATTAGTTAGTTTTGTTGAGTGT-3'. The PCR reaction mixture contained 1x platinum SYBR green qPCR SuperMix UDG (Invitrogen), 500 nM of each primer, and 10 ng of total genomic DNA or mtDNA. Real-time PCR conditions were 2 min at 50 °C and 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 60 s at 60°C using the ABI Prism 7000 sequence detector system (Applied Biosystems 7000 SDS software). The Ct values in the linear exponential phase were used to measure the original DNA template copy numbers from a standard curve generated from five 10-fold dilutions of either pure mtDNA or pure nuclear 18S rDNA. Relative values for COX II and 18S rDNA within samples were used to obtain a ratio of mtDNA to nuclear DNA.
**Western analysis.** Muscle proteins were separated by SDS-PAGE and identified by Western analysis (34). Membranes were incubated with validated polyclonal antibodies against human citrate synthase, superoxide dismutase-2 (SOD2), COX-I, ATPase-6, and Akt/pAkt (Protein Kinase B) (Santa Cruz Biotechnology, Santa Cruz, CA), mitofusin-1 and -2, Optic Atrophy-1 (OPA-1), or β-actin (1:5000; Sigma). After three washes in TBST, membranes were incubated at a 1:10,000 dilution of HRP-conjugated goat anti-rabbit IgG (Amersham). Blots were developed with ECL and proteins quantified by densitometry on digitized images from the mid-dynamic range and expressed relative to β-actin.

**MHC isoform analysis.** Muscle was homogenized in 250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris base, pH 6.8, using a glass tissue homogenizer, sonicated, and centrifuged at 1,000 g for 10 min (4°C). The pellet was re-suspended in a wash buffer (175 mM KCl, 0.5% Triton X-100, 2 mM EDTA, 20 mM Tris base, pH 6.8, 4°C) and centrifuged again at 1,000 g for 10 min (4°C). The pellet was re-suspended in the buffer, centrifuged again at 1,000 g, and suspended in 150 mM KCl and 20 mM Tris base (pH 7.0). MHC isoforms were separated via SDS-PAGE using stacking gels of 4% acrylamide-bis and separating gels of 8% acrylamide-bis. Gels were stained (Bio-Rad Silver Plus) and scanned using QuantityOne Software (Bio-Rad).

**Immunofluorescence microscopy.** Four-micron thick paraffin-embedded tissue sections were processed for immunostaining by deparaffinization in xylene and rehydration through a descending series of alcohols, washed extensively in 0.1 M phosphate-buffered saline (PBS), blocked with 10% normal goat serum with avidin and incubated overnight at 4 °C in primary antibody diluted in 10% NGS and biotin. Primary SDHA and COX-I antisera (Invitrogen) were used at a working dilution of 1:100. Sections were washed extensively in PBS, incubated in appropriate secondary IgG, Alexa Fluor 594 red (to detect COX1) or Alexa Fluor 488 green (to
detect SDHA). Conventional fluorescence images were obtained using a Nikon Microphot-FXA fluorescence microscope.

**Statistics.** Grouped data are reported as mean ±SD. The values for the CO control group were compared using the paired t-test. Mean values from the \( \dot{V}O_2 \) max groups were compared within and between groups using two-way ANOVA (SigmaStat, Systat Software). \( P \leq 0.05 \) was accepted as statistically significant.
RESULTS

The characteristics of the subjects are shown in Table 1. HbCO levels measured at baseline and immediately after the last CO exposure in the \( \dot{V}O_2 \) max group showed that mean HbCO levels increased from 1.6 ± 0.2% to 3.3 ± 0.60% (\( P=0.002 \)). In the CO only group, HbCO measurements showed a similar increase following CO exposure (1.2 ± 0.2% to 3.2 ± 0.4%; \( P=0.002 \)).

**Transcriptional activation of mitochondrial biogenesis after inhaled CO and \( \dot{V}O_2 \) max testing.** By real-time RT-PCR, mRNA for two of the major nuclear transcriptional regulators of mitochondrial biogenesis, PGC-1\( \alpha \) and NRF-1, as well as for mitochondrial transcription factor-A (Tfam) and the mtDNA polymerase (Pol \( \gamma \)) was evaluated before and after air or CO with \( \dot{V}O_2 \) max testing, or before and after CO without exercise testing. The summary data, by group pairings (Air + Ex, CO + Ex, or CO alone), are displayed in Fig. 1 for PGC-1\( \alpha \) (panel A), NRF-1 (B), Tfam (C), and Pol \( \gamma \) (D). \( \dot{V}O_2 \) max testing did not significantly affect these four muscle mRNA measurements. In the CO plus \( \dot{V}O_2 \) max group, the levels of all four mRNAs increased significantly and Tfam expression nearly doubled (\( P=0.02 \)). In the CO only group, two of the four muscle mRNA values, Tfam and Pol \( \gamma \), increased significantly.

Fig. 2 summarizes the data from the three groups of subjects for citrate synthase (A), mtDNA copy number (B), and two mitochondrial encoded proteins, COX-I (C) and ATPase-6 (D). None of these four markers increased in the air control group. In the CO plus \( \dot{V}O_2 \) max group, the nuclear-encoded TCA cycle enzyme, citrate synthase, a marker of muscle oxidative capacity, respiratory capacity, and mitochondrial volume density, increased significantly.
However, the mtDNA copy number in all groups remained stable (13% increase in CO plus 
\( \dot{V}O_2 \) max group; \( P=0.072 \)), indicating that mtDNA replication had not been activated at the time 
of the muscle biopsy. To determine if CO had activated mitochondrial protein synthesis, 
mitochondrial-encoded COX-I and ATPase-6 expression was examined, and both protein levels 
increased significantly in the CO plus \( \dot{V}O_2 \) max group. After CO alone, the COX-I and 
ATPase-6 protein levels also increased significantly.

The distribution of specific mitochondrial proteins was assessed by immunofluorescence 
microscopy and staining for succinate dehydrogenase (SDH), a nuclear-encoded mitochondrial 
Complex II protein (green fluorescence), and for the mtDNA-encoded electron transport protein 
COX-I (red fluorescence) (Fig. 3). COX-I staining is more significant in type I muscle fiber, 
while SDH stains both type I and type II fibers. The superimposed images allow assessment of 
co-localization and heterogeneity of these proteins. Fig. 3a-c is representative of normal skeletal 
muscle in one subject prior to CO exposure or \( \dot{V}O_2 \) max testing. Fig. 3b shows modest COX-I 
expression and minimal SDH co-localization (Fig. 3c). Fig. 3d-f shows typical staining in one 
subject in the air plus \( \dot{V}O_2 \) max group. There is a minimal increase in COX-I staining but better 
co-localization with SDH than in the pre-exposure biopsy. At 3 days after the final CO exposure 
in the CO plus \( \dot{V}O_2 \) max group, muscle SDH and COX-I expression are heterogeneously 
enhanced and co-localization within fibers is also heterogeneous (Fig 3g-i). In the CO only 
group, primarily COX-I staining is enhanced (Fig. 3j-l).

**CO and skeletal muscle anti-oxidant enzymes.** CO is known to induce the antioxidant 
enzymes HO-1 and SOD2; both are indicators of the oxidative stress response and are involved 
in mitochondrial biogenesis. Therefore, Western blots for HO-1 and SOD2 were performed in
the muscle tissue before and after inhaled CO, with and without the exercise protocol. A representative Western blot and the mean densitometry data for HO-1 protein are shown in Fig. 4A and 4B respectively. Muscle HO-1 protein levels did not increase in the air \( \dot{V}O_2 \) max group but more than doubled in the CO plus \( \dot{V}O_2 \) max group and increased about two-fold in the CO only group. Fig. 4A also shows a representative Western blot and 4C the mean densitometry data for SOD2, which more than doubled after CO plus \( \dot{V}O_2 \) max, and increased nearly twofold in the CO alone group (P<0.05 for both). These data indicate that CO causes an oxidative stress response in skeletal muscle mitochondria, which is accentuated by \( \dot{V}O_2 \) max testing.

We have demonstrated in the mouse that mitochondrial oxidant generation activates Akt, which phosphorylates nuclear respiratory factor-1 and stimulates mitochondrial biogenesis (33). To evaluate Akt in human muscle, we performed Western blots for phospho- and total Akt, and found a significant increase in phospho-Akt in the CO plus \( \dot{V}O_2 \) max group (P<0.05), and a trend towards Akt phosphorylation in the CO alone group (P=0.05). Subjects in the air \( \dot{V}O_2 \) max group showed no significant increase in Akt activation (Fig. 4D).

**Mitochondrial fusion proteins.** The expression of mitofusin-1, mitofusin-2, and OPA-1 proteins were measured because these proteins are integral to autonomous mitochondrial fusion that occurs in uniting contiguous units (6) (7). These data are displayed relative to the mitochondrial reference protein, porin in Figure 5. Fig. 5A shows representative Western blots and Fig. 5B-5D show the densitometry for each of the proteins. None of the levels changed significantly in the air plus \( \dot{V}O_2 \) max group, but in the CO plus \( \dot{V}O_2 \) max group, the expression
of all three proteins doubled ($P=0.032$, $0.04$, and $0.037$, respectively). In the subjects breathing CO without exercise, the effects, except for mitofusin-2, were not statistically significant.

**Exercise-induced MHC isotype switching.** We measured the ratio of myosin heavy chain isotype proteins (MHC) to determine if CO induces muscle fiber isotype switching from anaerobic to aerobic fibers. Fig. 6 shows a Western blot and densitometry data for MHC Class I and Class II. MHC I protein expression increased significantly only in the CO plus $\dot{V}O_2$ max group ($P=0.038$). There was no significant change in MHC expression after CO alone. These data indicate that the myocyte fiber type switching after the exercise test is augmented by CO.

**Maximal oxygen uptake.** $\dot{V}O_2$ max was measured before and three days after the last CO session, and no significant effect of CO was found (Table 1). There were also no differences detected in the exercise-induced changes in arterial pH or base deficit in the pre- to post-exposure comparison for either the air or the CO group.
Discussion

This is the first appraisal of the effects of inhaled CO on mtDNA transcription, replication, and mitochondrial biogenesis in humans. Using a daily regimen of 1 hour of 100ppm CO breathing for five days after a single \( \dot{V}O_2 \) max test we found increases in muscle mRNA for two key nuclear regulators of mitochondrial biogenesis, PGC-1\( \alpha \), and NRF-1, as well as for mitochondrial transcription factor-A, Tfam, and DNA polymerase, Pol \( \gamma \). Also, CO plus \( \dot{V}O_2 \) max testing increased the muscle mitofusin and OPA-1 protein content coincident with COX-I and ATPase-6 protein expression; the latter is consistent with new mtDNA transcriptional and protein synthetic activity. This regimen also produced mitochondrial oxidative stress and activated the pro-survival kinase, Akt. These responses are consistent with our mouse data, and the CO requirement, as they are not found in air-breathing subjects after \( \dot{V}O_2 \) max testing. In six subjects breathing CO alone, however, not all of these markers increased, indicating that our protocol did not fully activate the mitochondrial biogenesis program in skeletal muscle (33).

A link between CO and mitochondrial biogenesis was first established in the mouse heart, where HO-1/CO enhances mitochondrial H\(_2\)O\(_2\) production and activates Akt (33). Akt phosphorylates the NRF-1 transcription factor, which translocates to the nucleus and activates multiple genes of mitochondrial biogenesis (26). As in the mouse, CO exposures that doubled basal carboxyhemoglobin also increased HO-1 and SOD2 expression, due perhaps to mitochondrial heme release (8) and/or activation of the anti-oxidant response (25).

Although CO is clearly pro-oxidant and cytotoxic (27), it also induces protective or adaptive responses (10, 20). The CO concentration-time product employed here is well under that which produces overt acute or chronic health effects and is one fourth of OSHA’s maximum
eight-hour, time-weighted average. COHb levels increased to only 3%, which does not produce
tissue hypoxia, and our subjects were never symptomatic. Also, the cumulative inhibition of
respiration is highly improbable because nearly five HbCO half lives elapsed between each dose
(36).

Originally, we had intended to test whether CO-related mitochondrial responses are
associated with an increase in maximal aerobic exercise capacity, but discovered an interaction
between CO and the $\dot{V}O_2$ max test. Since the main determinants of human $\dot{V}O_2$ max are
considered to be O$_2$ uptake by the lungs, O$_2$ delivery, O$_2$ diffusion from capillary to
mitochondria, and mitochondrial oxidative capacity (9), we had reasoned that if muscle
mitochondrial mass increased after CO, $\dot{V}O_2$ max might improve. Rodent studies indicate that
mitochondrial density is an important determinant of $\dot{V}O_2$ max (21, 28) and mitochondria may
play a larger role in governing $\dot{V}O_2$ max at altitude (9) or in sustaining sub-maximal exercise
(15). Although the transcriptional regulators of mitochondrial biogenesis were activated and
mtDNA-encoded proteins increased in the CO plus $\dot{V}O_2$ max group, mtDNA copy number did
not increase significantly, and three days later, no increase in $\dot{V}O_2$ max was detected. This
implies that our protocol was not sufficient to increase mitochondrial mass, or that a minor
increase in mitochondrial mass does not enhance maximal aerobic exercise capacity, or that other
study limitations precluded detection of a performance improvement.

The relatively small size of this study also affords a low power to detect small effects.$\dot{V}O_2$ max was measured at a single point after activation of the transcriptional events, and there
may have been insufficient time to complete the assembly of new mitochondria. This could also
account for an apparent discrepancy between increased citrate synthase expression, which is under nuclear control, and the stable mtDNA copy number. Some heterogeneity in nuclear-mitochondrial communication is also evident in the immunohistochemistry (Fig. 3), and may be related to the complex stimulus. Moreover, physiologically CO would not be expected inhibit respiration for three days after the last exposure. Even though, we could not ascertain whether mitochondrial mass limits aerobic capacity in healthy subjects, the approach is novel among human studies because it may be possible to address the question by stimulating mitochondrial biogenesis without exercise training.

It is noteworthy that no significant increases were found in air-control subjects in any of the measured parameters five days after a single exercise test, but earlier transcriptional responses for mitochondrial biogenesis are not precluded. For instance, a single swimming session can increase PGC-1α and NRF-1 levels (2), and endurance exercise will stimulate mitochondrial transcription factor expression and mitochondrial proliferation (2, 16). There is also evidence that high AMP levels during $\dot{\text{V}}\text{O}_2\text{max}$ testing induce mitochondrial enzymes (39) that contribute to endurance adaptation in skeletal muscle (1). However, the addition of CO to a single $\dot{\text{V}}\text{O}_2\text{max}$ test activated a program that allows mitochondria to adjust to stress, and notably much of the effect is attributable to CO alone. These results may imply that CO and exercise recruit parallel transcriptional circuitry with synergy between the two pathways.

A change in mitochondrial fusion protein expression was clearly present in the $\dot{\text{V}}\text{O}_2\text{max}$ plus CO group, but a relatively small shift towards a more aerobic muscle fiber type was found, implying that mitochondrial phenotype may be adjusted before muscle fiber phenotype. Although we did not directly evaluate mitochondrial fusion, mitochondria do exist in inter-
convertible forms: as small isolated organelles and as extended filaments, networks, or clusters connected by junctions. Mitochondria do fuse into electrically-united systems that may facilitate ATP delivery from cell periphery to center and distribute antioxidant enzymes to clusters near the outer membrane where most of the O$_2$ is consumed while distributing $\Delta \psi$ to the core network to distribute ATP production. Further characterization of mitochondrial function is thus desirable, but the small size of the muscle biopsies and the lack of any previous data on the CO effect precluded an extensive analysis of oxidative phosphorylation in this initial study.

In summary, intermittent CO breathing after a single $\dot{V}O_2$ max test induces a mitochondrial oxidative stress response and conjunctively stimulates mitochondrial fusion protein expression and some aspects of mitochondrial biogenesis in human skeletal muscle. Despite the observed transcriptional responses and increases in each of a select group of mitochondrial proteins, maximal aerobic capacity did not change within the limits of a maximum exercise test. Subjects who underwent $\dot{V}O_2$ max testing before CO showed a switch to a more aerobic fiber type that was not detected under either stimulus alone. CO alone did lead to significant increases in mRNA expression for Tfam and Pol$\gamma$ and protein expression for HO-1, SOD2, mitofusin-2, and mitochondrial-encoded COX-I and ATPase-6. These intriguing results raise the prospect that exogenous CO given in optimized, safe, regimens might offer a simple means to hasten recovery in diseases where mitochondrial dysfunction is acquired, for instance, in diabetes (18), sepsis (4), atherosclerosis (5), or COPD (11).
Acknowledgements

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References


Figure Legends

Fig. 1. Carbon monoxide and bi-genomic transcriptional activity in human skeletal muscle. Real-time RT-PCR was performed on muscle biopsy samples of healthy subjects before and after five days of inhaled CO or air exposure and a \( \dot{V}O_2 \) max test, or before and after five days inhaled CO without exercise. Relative levels of mRNA for nuclear-encoded PGC-1\( \alpha \) (A), NRF-1 (B), and mitochondrial-encoded Tfam (C) and Pol \( \gamma \) (D) are shown before and after CO with or without a \( \dot{V}O_2 \) max test. Values are mean ± SD (*\( P<0.05 \) for between group comparisons).

Fig. 2. Nuclear and mitochondrial protein expression and mtDNA copy number. In panel A, expression of skeletal muscle citrate synthase determined by Western blot is shown before and after CO with or without \( \dot{V}O_2 \) max testing compared with porin, the loading control. In B, mtDNA copy number in muscle is shown before and after five daily exposures to air or CO and one \( \dot{V}O_2 \) max test, or before and after CO without exercise. In C and D, mitochondrial-encoded COX-I and ATPase-6 protein are shown respectively relative to porin before and after CO with or without \( \dot{V}O_2 \) max test. Values are mean ± SD (*\( P<0.05 \) between groups).

Fig. 3. Immunofluorescence microscopy of skeletal muscle. COX-I (green fluorescence—mitochondrial encoding) and SDH subunit A (red fluorescence—nuclear encoding) were stained with specific antibodies and the images merged to assess co-localization. Panels a-c: typical SDH and COX-I staining of control muscle and the overlay, respectively. Panels d-f: typical stained muscle sections for SDH, COX-I and the overlay from the air plus \( \dot{V}O_2 \) max group. Panels g-i: muscle sections from the CO plus \( \dot{V}O_2 \) max group stained for SDH, COX- I,
and the overlay. Panels j-k: SDH and COX-I staining and the overlay in a sample of muscle after 5 days of CO breathing.

**Fig. 4. Oxidative stress response and Akt activation in skeletal muscle after CO breathing with or without $\dot{V}O_2\text{max}$ testing.** Western blots for HO-1, SOD2, and phospho- and total Akt were performed on muscle samples before and after five days of inhaled CO, with or without the $\dot{V}O_2\text{max}$ test. Relative protein expression was compared by normalizing HO-1 and SOD2 to $\beta$-actin. Akt activation was determined by the ratio of phospho- to total Akt. (A) Representative Western blots for HO-1 and SOD-2. (B) Densitometry for HO-1 expression relative to $\beta$-actin. (C) Densitometry for SOD-2 expression relative to $\beta$-actin. (D) Akt activation before and after CO exposure. Values are mean ± SD (*$P<0.05$ between groups).

**Fig. 5. Mitochondrial fusion proteins and OPA-1 responses to inhaled CO with or without $\dot{V}O_2\text{max}$ testing.** (A) Western blots of muscle tissue for mFn1, mFn2, and OPA-1 before and five days after inhaled CO with or without the $\dot{V}O_2\text{max}$ test. Relative protein expression was derived by normalizing mFn1, mFn2, and OPA-1 to porin, a stable mitochondrial outer membrane protein. (B) Relative mFn1 expression. (C) Relative mFn2 expression. (D) Relative OPA-1 expression. Values are mean ± SD (*$P<0.05$ between groups).

**Fig. 6. MHC protein expression in skeletal muscle after CO breathing with or without $\dot{V}O_2\text{max}$ testing.** Gels of muscle samples for MHC isoforms from subjects before and five days after inhaled CO with or without the $\dot{V}O_2\text{max}$ test. MHC I was expressed relative to $\beta$-actin, used as a loading control. Values are mean ± SD (*$P<0.05$ between groups).
Table 1: Experimental Groups: Age, HbCO, and $\dot{V}O_2$ max

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<td>ND</td>
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Rhodes et al; Figure 2

A

Citrate Synthase / Porin

B

mtDNA copy number

C

COX-1 / Porin

D

ATPase 6 / Porin

* (P=0.06)
Rhodes et al; Figure 6

A

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</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>MHC I protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
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</tr>
<tr>
<td>CO</td>
<td>0.4</td>
</tr>
<tr>
<td>Ex</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Significant difference.