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# Effects of carbon monoxide (CO) delivery by a CO donor or hemoglobin on vascular hypoxia inducible factor 1 $\alpha$ and mitochondrial respiration

Chad E.N. Reiter, Abdu I. Alayash\*

Laboratory of Biochemistry and Vascular Biology, Division of Hematology, Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA), Bethesda, MD 20892, United States

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## ABSTRACT

**We examined carbon monoxide (CO) delivery by carbon monoxide-releasing molecule 2 (CORM-2) or hemoglobin (Hb) on cellular oxygen sensing and mitochondrial respiration in bovine aortic endothelial cells (BAECs). CORM-2 reduced hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and endothelin-1 (ET-1) expression in normoxic and hypoxic cells, but while Hb alone significantly reduced HIF-1 $\alpha$  stabilization in hypoxic cells, CO delivered by Hb (Hb-CO) had no effect on HIF-1 $\alpha$  stabilization. CO dose-dependently increased basal oxygen consumption and reduced overall mitochondrial respiratory capacity. Hb-CO increased basal oxygen consumption but did not alter respiratory capacity. Together, CO reduced ET-1, and, at low doses, had no effect on endothelial mitochondria oxygen consumption. CO ligation to Hb may be developed further as non-vasoactive oxygen therapeutic without compromising mitochondrial function.**

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## 1. Introduction

CO is an endogenously produced natural byproduct of heme catabolism by the enzyme heme oxygenase. The primary known molecular target of CO is the heme iron center of hemoproteins, such as Hb and mitochondrial cytochromes, in which high and prolonged exposure to CO results in classical CO-poisoning [1]. Emerging research has shown that low levels of CO can act as an important signaling molecule with broad physiological effects on the vasculature. CO was found to play a key role in various inflammatory and cardiovascular events, and many of these signaling pathways have become attractive targets for drug development. Besides its beneficial antioxidant effects, CO was shown to have additional vasodilatory effects similar to nitric oxide (NO), the penultimate vasoactive molecule produced by the vascular system. Activation of soluble guanylyl cyclase (sGC), increases in cGMP content, and direct activation of calcium-activated potassium channels have been shown to be involved in CO-induced vasodilation [2,3]. The development of CO-releasing molecules (CORMs), which are designed to deliver CO locally in a controlled manner,

have been at the forefront of potential therapeutic applications [4,5]. The CO-releasing properties of first generation CORMs were reported to be at  $t_{1/2} \geq 1$  min in vivo, ex vivo, and in vitro biological models [6]. The kinetics of CO release from Hb is more complex, particularly when Hb is free in solution. Additionally, Hb exists in an equilibrium between two different conformational states: the oxygenated R (relaxed) and deoxygenated T (tense) states. It has been estimated that the rate-limiting step for CO delivery is the R-state off-rate, giving an overall  $t_{1/2}$  for the reaction of approximately 30–40 s [7].

The presence of cell-free Hb in circulation as result of hemolytic anemias or when Hb is used as oxygen-carrying blood substitutes can present numerous serious complications including an immediate raise in systemic and pulmonary blood pressure due to removal of NO and subsequent tissue-damaging oxidative toxicity [8]. The use of NO donating compounds or nitrite have been advocated as possible countermeasures that can be infused with Hb to control hemodynamic imbalances in anemic patients or in patients exposed to hemoglobin-based oxygen carriers (HBOCs) [9]. NO-induced oxidation of free Hb and possible heme loss motivated the search for safer HBOCs. HBOCs carrying CO is currently being developed as a strategy to reduce toxicity, maintain oxygen delivery, and promote vasodilation. CO is known for its cell signaling as well as for its antioxidant and vasodilatory actions [1,4]. Hb-CO derivatives have been manufactured with the notion that transfusion of these molecules will perform dual functions as CO and oxygen delivery agents, once CO is released from Hb [7]. Hb conjugated to polyethylene glycol and ligated to CO was recently shown to reduce myocardial infarcts in rats [7].

*Abbreviations:* CO, carbon monoxide; CORM-2, CO-releasing molecule-2; Hb, hemoglobin; Hb-CO, hemoglobin-ligated with CO; BAEC, bovine aortic endothelial cells; HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; ET-1, endothelin-1; HBOC, Hb-based oxygen carrier; NO, nitric oxide; OCR, oxygen consumption rate; PHD-2, proline hydroxylase-2

\* Corresponding author. Address: Laboratory of Biochemistry and Vascular Biology, FDA/CBER, 8800 Rockville Pike, NIH Bldg 29, Room 112, Bethesda, MD 20892, United States. Fax: +1 301 435 4034.

E-mail address: [abdu.alayash@fda.hhs.gov](mailto:abdu.alayash@fda.hhs.gov) (A.I. Alayash).

At the molecular level, the transcription factor HIF-1 $\alpha$  orchestrates the cellular response to low oxygen tension, as reviewed elsewhere [10]. HIF-1 $\alpha$ , in turn, links hypoxic events to the regulation of vascular tone by driving expression of ET-1, a potent vasoconstrictor secreted from endothelium [11]. In this report, we examine endothelial oxygen sensing by HIF-1 $\alpha$  and, for the first time, real-time mitochondrial oxygen consumption following CO delivery from CORM-2 and Hb-CO. CORM-2 reduced HIF-1 $\alpha$  and ET-1 expression while maintaining normal mitochondria oxygen consumption. At higher doses, CORM-2 disrupted mitochondrial function. When delivered by Hb, CO did not alter mitochondrial function, but failed to reduce HIF-1 $\alpha$  expression in hypoxic endothelial cells. These findings may directly relate to recent attempts by many to overcome the well-known vasculopathy associated with the infusion of Hb as oxygen therapeutics [12].

## 2. Materials and methods

### 2.1. Cell culture

Bovine aortic endothelial cells (BAECs) were cultured under normoxic and hypoxic conditions as described [13]. CORM-2 and RuCl<sub>3</sub> (control for CORM-2) were purchased from Sigma (St. Louis, MO) and dissolved in DMSO stocks. For hypoxia treatment, cells were gassed with 1% O<sub>2</sub> using a Modular Incubator Chamber from Billups-Rothenburg (Del Mar, CA).

To assess effects of O<sub>2</sub> and CO-ligated forms of Hb on endothelial cells, BAECs were grown to confluence on 12-well Transwell filter plates (Corning Life Sciences, Pittston, PA). Highly purified stroma free bovine Hb, used as a control in this study, was a kind gift from OPK Corporation, Cambridge, MA. Pegylated bovine Hb ligated to CO was a kind gift from Prolong Pharmaceuticals, South Plainfield, NJ. The isolation and manufacturing and the pegylation process of this Hb was described previously [14]. This PEG-Hb-CO is referred to throughout the manuscript for simplicity as Hb-CO. BAECs were serum-starved 2 h, and serum-free media was changed again immediately before beginning the experiment with 500  $\mu$ L in the top chamber and 700  $\mu$ L in the bottom chamber. Hb  $\pm$  CO (2 mM stock, 800  $\mu$ L) was added to the bottom chamber (final Hb  $\pm$  CO 1.07 mM in bottom chamber). Cells were incubated in 1% O<sub>2</sub> and at 37  $^{\circ}$ C for 2 h before harvesting for immunoblot analysis.

### 2.2. Immunoblot analysis

Cells were lysed in Novex Tricine SDS sample buffer (Invitrogen, Carlsbad, CA) supplemented with 2%  $\beta$ -ME (Sigma) for separation by SDS-PAGE. The HIF-1 $\alpha$  antibody was from R&D Systems (Minneapolis, MN), and the  $\beta$ -actin antibody was from Sigma. The secondary HRP-linked antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were visualized with Lumiglo (Cell Signaling Technology, Danvers, MA) and exposure to Amersham Hyperfilm MP (Buckinghamshire, UK). Images were scanned and analyzed with NIH Image J software (Bethesda, MD).

### 2.3. Endothelin-1 (ET-1) ELISA

The ET-1 ELISA kit obtained from Enzo Life Sciences (Ann Arbor, MI) was used according to the manufacturer's protocol to measure ET-1 secreted into cell culture media.

### 2.4. XF24 mitochondria oxygen consumption analysis

The OCR of BAECs was measured in real-time using XF24 technology from Seahorse Biosciences (Billerica, MA) essentially as de-

scribed for BAECs [15]. BAECs were grown to confluence on the 24-well XF cell culture plate before all experiments. One hour prior to experimentation, BAEC cell culture media (from above) was changed to serum-free and bicarbonate-free DMEM supplemented with 1000 mg/L glucose and L-glutamine (Sigma, D5523), and 1 mM pyruvate from Mediatech, Inc. (Herndon, VA), and pH was adjusted to 7.4 with NaOH. The oligomycin, FCCP, and rotenone used in XF24 experiments were all from Sigma, and dose-response experiments determined that the optimal amounts of compounds for our BAECs were 0.5  $\mu$ g/mL (oligomycin), 3  $\mu$ M (FCCP), and 1  $\mu$ M (rotenone). A typical evaluation of BAEC OCR in response to optimized inhibitors is described in Fig. 2. Data from multiple experiments are expressed in terms of percent change in OCR, and the OCR for area under the curve (AUC) measurements is expressed as pmol/min.

### 2.5. Statistics

Data are presented as mean  $\pm$  SEM. Statistical analyses were performed using ANOVA or Student's *t*-test as described in the figure legends. Differences were considered significant at  $p < 0.05$ .

## 3. Results

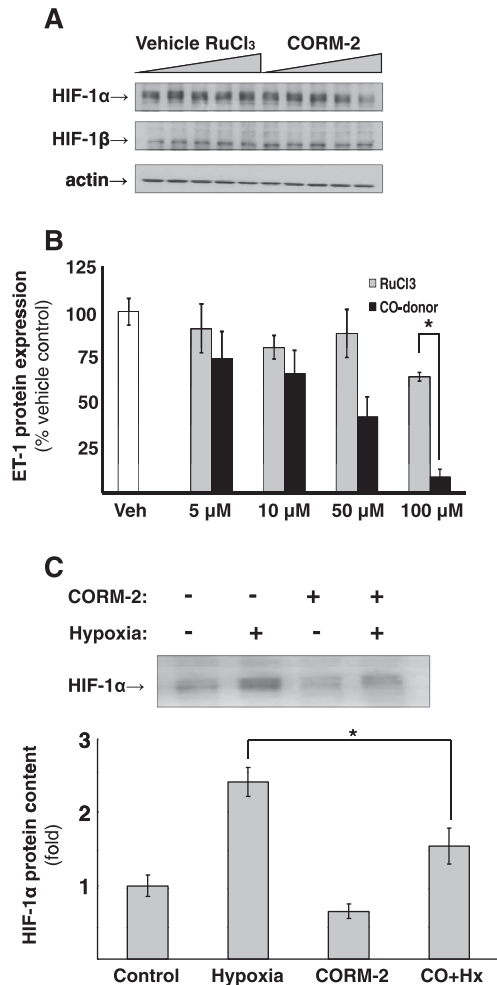
### 3.1. CORM-2 blocks HIF-1 $\alpha$ stabilization and ET-1 expression in endothelial cells

We first examined the effects of CORM-2 on HIF-1 $\alpha$  and ET-1 expression in endothelial cells. BAECs were treated with increasing doses of the control RuCl<sub>3</sub> or CORM-2. Fig. 1A depicts a representative immunoblot. HIF-1 $\alpha$  expression remained stable until cells were treated with 50 or 100  $\mu$ M CORM-2. Correspondingly, ET-1 secretion by BAECs was also reduced slightly after treatment with 50  $\mu$ M CORM-2, and was significantly lower after treatment with 100  $\mu$ M CORM-2 (Fig. 1B). Therefore, CO delivered by CORM-2 reduced basal HIF-1 $\alpha$  and its downstream target ET-1 in endothelial cells under normoxic conditions.

Next, we examined HIF-1 $\alpha$  expression in response to CORM-2 under hypoxic conditions. Confluent BAECs were treated for one hour with or without CORM-2 prior to hypoxia (1% O<sub>2</sub>) or continued normoxic conditions. A representative immunoblot and image analysis is shown in Fig. 1C. Hypoxia, as expected, increased HIF-1 $\alpha$  protein content of BAECs. CORM-2 (100  $\mu$ M), as Fig. 1A shows, reduced basal HIF-1 $\alpha$  expression in normoxic cells. BAECs that were treated first with CORM-2 had reduced HIF-1 $\alpha$  protein expression following the hypoxic challenge. Therefore, CORM-2 reduces basal HIF-1 $\alpha$  expression and attenuated upregulation of HIF-1 $\alpha$  expression in hypoxic endothelial cells.

### 3.2. Measurements of mitochondrial function in BAECs using XF24-extracellular flux analyzer

A Seahorse Bioscience XF24 extracellular analyzer was used to measure mitochondrial function in intact BAECs. The principle and operation of this technology has been recently reviewed [16]. Confluent BAECs were used in our model system, and this yielded a comparable OCR profile to that of previously published results [14] in BAECs (see Fig. 2). By using the indicated oxidative phosphorylation inhibitors, the OCR that corresponds to ATP production and maximal oxygen consumption by the mitochondria can be calculated. Therefore, we can exquisitely measure CO impacting mitochondrial function at cytochrome c oxidase [17] in real-time following CO delivery by CORM-2 or Hb.



**Fig. 1.** CORM-2 reduces HIF-1 $\alpha$  protein stabilization. (A) BAECs were treated with increasing doses of the CO-donor CORM-2 (0  $\mu$ M through 100  $\mu$ M) for 6 h and analyzed for HIF-1 $\alpha$  protein by immunoblot analysis. HIF-1 $\beta$  and actin serve as constitutive loading controls. Immunoblots are representative of three independent experiments. (B) BAECs were treated with the indicated doses as in A for 6 h and analyzed for ET-1 expression by ELISA. \* $p$  < 0.01 by Student  $t$ -test. (C) BAECs were treated without or with CORM-2 for 1 h before incubation under hypoxia (hx, 1% O<sub>2</sub>) or air for an additional 5 h. A representative immunoblot is shown, and densitometric image analysis demonstrates a reduction of HIF-1 $\alpha$  protein following CORM-2 treatment under normoxia and hypoxia ( $n$  = 3, \* $p$  < 0.05 by ANOVA and Tukey–Kramer post-test).

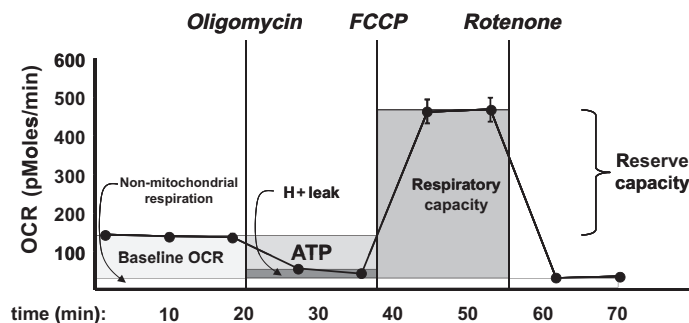
### 3.3. CORM-2 dose-dependently disrupted BAEC mitochondrial function

We examined mitochondrial OCR using XF24 technology in BAECs in response to a wide range of CORM-2 concentrations. As shown in Fig. 3A, BAECs were treated with 0–250  $\mu$ M (higher 500  $\mu$ M doses are not shown for clarity), and approximately 50 min later, basal OCR begins to steadily increase in BAECs treated with 100 or 250  $\mu$ M CORM-2. The effects of oligomycin and FCCP were also blunted in cells treated with 100 or 250  $\mu$ M CORM-2. Next, the area under the curve (AUC) that corresponds to basal OCR ( $t$  = 23–150 min in Fig. 3A), OCR due to ATP production ( $t$  = 150–170 min in Fig. 3A), and OCR that represents total respiratory capacity ( $t$  = 170–186 min in Fig. 3A) were calculated. As little as 50  $\mu$ M CORM-2 had no effect on any mitochondrial oxygen-consumption parameter, but 100  $\mu$ M or greater CORM-2 concentrations progressively disrupted mitochondrial function as reflected in increasing basal OCR or decreasing OCRs corresponding to ATP production and respiratory capacity. However, 500  $\mu$ M CORM-2 appeared highly toxic resulting in negative calculations of respiratory capacity. The 50  $\mu$ M CORM-2 dose that had little impact on cellular mitochondrial oxygen consumption is still greater than the 20  $\mu$ mol/kg CORM-2 dose that was reported to significantly reduce rat mean arterial pressure [18].

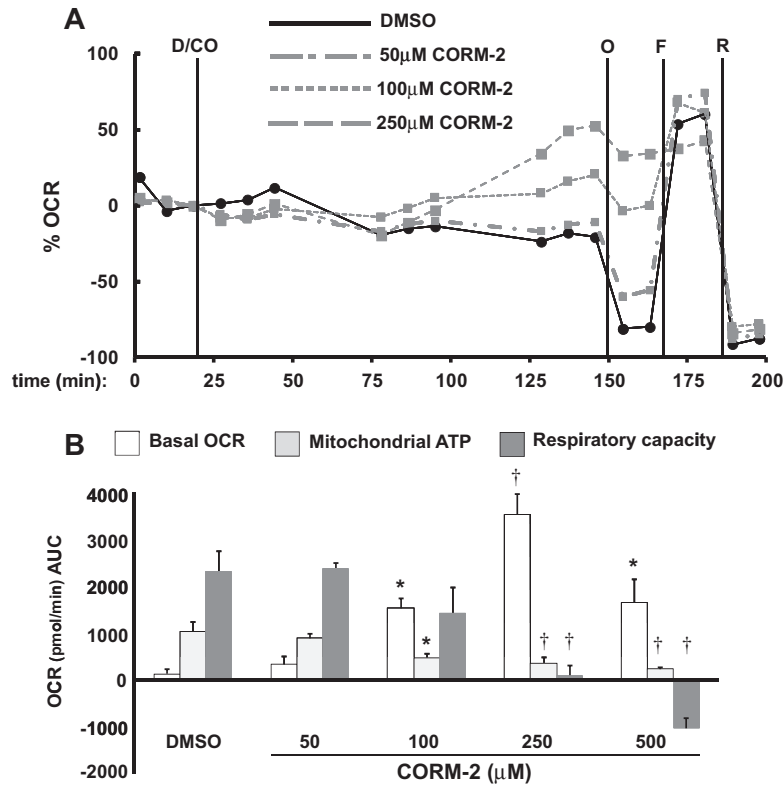
### 3.4. CO bound to Hb does not disrupt mitochondrial OCR nor inhibit HIF-1 $\alpha$ stabilization

We examined mitochondrial OCR and HIF-1 $\alpha$  expression in endothelial cells to evaluate Hb-CO potential toxicity and oxygen delivery, respectively. First, BAECs were examined for OCR while co-incubated with Hb or Hb-CO. The XF-24 Analyzer limits the amount of Hb that can be injected to co-culture with cells, therefore we examined 50  $\mu$ M heme, a maximum concentration that can be used without compromising the operation of the XF-24 Analyzer. As shown in Fig. 4A, the OCR of cells treated with control vehicle, Hb, or Hb-CO is nearly identical. In calculating AUC, only the basal rate following treatment with either Hb or Hb-CO induced a slight increase in OCR for  $t$  = 23–150 min (Fig. 4B). Treatment with the various mitochondrial inhibitors did not alter OCR compared with control. Therefore, Hb alone may induce alterations in OCR, which requires further investigation, but the addition of CO in the form of Hb-CO had no measurable effect on mitochondrial OCR.

Next, BAECs were exposed to hypoxia (1% O<sub>2</sub>) and co-cultured with Hb or Hb-CO, as described in Section 2, to ascertain oxygen delivery to cells (Fig. 4C). HIF-1 $\alpha$  expression was assessed as a bio-



**Fig. 2.** Typical real-time OCR data of BAECs using optimal electron transport chain inhibitors. Confluent BAECs were analyzed in the XF24 for oxygen consumption in response to treatment with ATP synthase inhibitor (oligomycin), uncoupler (FCCP), and complex I inhibitor (rotenone). The inhibitors allow measurement and quantification of the oxygen consumption rate (OCR) that correlates with baseline (unstimulated cells), ATP generation, proton (H<sup>+</sup>) leak, total respiratory capacity, reserve capacity, and any non-mitochondrial respiration.



**Fig. 3.** CORM-2 dose-dependently disrupts mitochondrial function in BAECs. (A) Representative OCR data of CORM-2 treatment of BAECs with XF24 technology (D/CO = DMSO or CORM-2, O = oligomycin, F = FCCP, R = rotenone). (B) The AUC measurements were calculated from mitochondrial OCR of BAECs treated with CORM-2 as in Fig. 3A. CO inhibits complex IV. Increasing CORM-2 progressively increased basal OCR resulting in decreased OCR for ATP production and reduced overall respiratory capacity and reserve capacity (\* $p < 0.05$  and † $p < 0.01$  by ANOVA and Dunnett's post-test compared to DMSO control of basal OCR, OCR due to ATP production, or OCR due to respiratory capacity, respectively). There was no statistical effect with 50  $\mu\text{M}$  CORM-2.

marker for hypoxia and oxygen delivery. As expected, BAECs co-cultured with Hb had reduced HIF-1 $\alpha$  expression in a hypoxic environment compared with BAECs co-cultured without Hb (Fig. 4C, compare lanes 1 and 2). However, BAECs that were co-cultured with Hb-CO had elevated HIF-1 $\alpha$  expression, suggesting that oxygen delivery to the cells was reduced, possibly due to CO ligation to the heme iron (compare lanes 1 and 3).

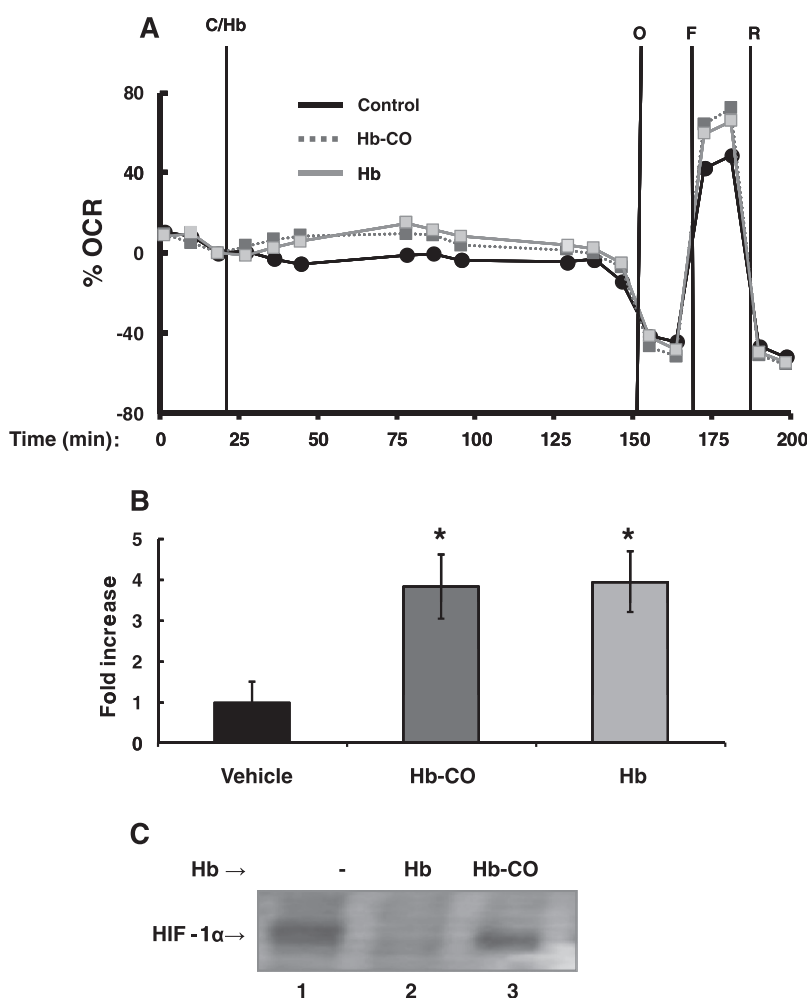
#### 4. Discussion

The possibility of CO binding to mitochondrial respiratory chain components and consequent impairments in oxygen consumption led us to investigate CO delivery by CORM-2 and by a CO ligated to chemically modified hemoglobin (Hb-CO). First, we determined the levels of HIF-1 $\alpha$  expressions in vascular endothelial cells to assess the impact of increasing levels of CORM-2 on cellular oxygen sensing mechanisms within these cells grown under normoxia and hypoxia. Our data clearly show that increasing doses of CO suppress the expression of HIF-1 $\alpha$  and its downstream target ET-1 (Fig. 1A and B), in support of observations made by others [19,20]. Under hypoxic (1% O<sub>2</sub>) stimulation of HIF-1 $\alpha$  stabilization, HIF-1 $\alpha$  expression was reduced with CORM-2 pretreatment (Fig. 1C), suggesting CORM-2 may enhance HIF-1 $\alpha$  degradation or interfere with HIF-1 $\alpha$  stabilization. Chin et al. [21] demonstrate that treatment of macrophages with CORM resulted in a rapid up-regulation of HIF-1 $\alpha$  that peaked at 30 min and returning to control levels within an hour; however, catalase (ROS scavenger) abrogated the stabilization of HIF-1 $\alpha$ . Astrocytes also upregulated HIF-1 $\alpha$  in response to CORM-2 [22], highlighting the varied cell- and tissue-specific responses to CO treatment. As a consequence of HIF-1 $\alpha$  inhibition in our experiments, ET-1 expression was

clearly inhibited as the concentration of CORM-2 increased (Fig. 1B). Since ET-1 secreted by the endothelium is known for its potent vasoconstrictor activity, the suppression of its expression may explain, in part, the vasodilatory activity reported on the use of CO.

We used the extracellular flux analyzer to measure mitochondrial function in BAECs in order to determine the effects of CORM-2 on the OCR, which correlates with electron transport chain function. We found that CORM-2 dose-dependently impacted mitochondrial function in these cells by increasing the basal resting-state OCR. This led to decreases of the OCR that correspond to ATP production and overall respiratory capacity of the mitochondria. Based on studies with CORM-3, this is likely due to a combination of cytochrome c oxidase inhibition and uncoupling of the electron transport chain leading to a loss of the proton gradient [23]. However, both Hb and Hb-CO showed little or no effects on overall mitochondrial function and biogenetics, except a small, but significant, rise in basal OCR (Fig. 4A and B). Probing mitochondrial function with inhibitors revealed no other alterations in OCR. Ferrous Hb however, suppressed the expression HIF-1 $\alpha$  whereas Hb-CO shows no effect on oxygen sensing elements due to blockage of Hb iron by CO. This suggests that time-in-circulation is required for CO off-loading and oxygen uptake and delivery by an HBOC utilizing this technology.

Gas messengers, such as CO and NO, and redox regulation by HIFs represent a different perspective in modulating potential therapeutic targets for normalizing cardiac, vascular, and respiratory disease states [24]. While it is unclear how CO reduces HIF-1 $\alpha$  protein levels following CORM-2 and to lesser extent with Hb-CO treatment, there may be at least two possible scenarios: (1) CO may directly attenuate HIF-1 $\alpha$  gene expression; or (2) CO



**Fig. 4.** CO delivered by Hb increases basal OCR but does not deliver oxygen. (A) Representative OCR data of CORM-2 treatment of BAECs with XF24 technology (C/Hb = media, Hb, or Hb-CO, O = oligomycin, F = FCCP, R = rotenone). (B) Hb and Hb-CO induced an increase in basal OCR between time of Hb or control treatment at  $t = 23$  min and oligomycin treatment at  $t = 151$  min in A ( $*p < 0.05$  by ANOVA and Tukey–Kramer post-test compared with vehicle). (C) BAECs were co-cultured in hypoxia either alone (–) or with Hb or Hb-CO to mimic an HBOC therapeutic to deliver oxygen. Only Hb, without CO (lane 2), delivered oxygen to reduce HIF-1 $\alpha$  expression in hypoxic BAECs. Immunoblot is representative of three independent experiments.

may indirectly enhance PHD2 hydroxylation activity. In the latter scenario, a disruption of the mitochondrial OCR by these compounds could transiently increase intracellular oxygen substrate availability for PHD2, leading to enhanced HIF-1 $\alpha$  degradation [24]. Chemically modified and stabilized Hb saturated with CO, in contrast to rapidly delivered CO by CORMs [25], represents another amenable option. This has recently been demonstrated in several transgenic mouse models of sickle cell disease in which vascular damage and even mortality was prevented under hypoxia [26]. Moreover, CO ligated to the ferric form of Hb was recently found to not only autoreduce its iron back to Fe $^{2+}$ , but it can also promote iron reduction in the respiratory chain hemoproteins under oxidative stress conditions and in the presence electron donating oxidants [27]. Together, the use of CO with chemically modified Hb may enhance the safe use of oxygen therapeutics and the development of new applications to treat sickle cell disease.

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