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Carbon monoxide released by CORM-401 uncouples mitochondrial respiration and inhibits glycolysis in endothelial cells: A role for mitoBK$_{Ca}$ channels

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ABSTRACT

Carbon monoxide (CO), a product of heme degradation by heme oxygenases, plays an important role in vascular homeostasis. Recent evidence indicates that mitochondria are among a number of molecular targets that mediate the cellular actions of CO. In the present study we characterized the effects of CO released from CORM-401 on mitochondrial respiration and glycolysis in intact human endothelial cells using electron paramagnetic resonance (EPR) oximetry and the Seahorse XF technology. We found that CORM-401 (10–100 μM) induced a persistent increase in the oxygen consumption rate (OCR) that was accompanied by inhibition of glycolysis (extracellular acidification rate, ECAR) and a decrease in ATP-turnover. Furthermore, CORM-401 increased proton leak, diminished mitochondrial reserve capacity and enhanced non-mitochondrial respiration. Inactive CORM-401 (iCORM-401) neither induced mitochondrial uncoupling nor inhibited glycolysis, supporting a direct role of CO in the endothelial metabolic response induced by CORM-401. Interestingly, blockade of mitochondrial large-conductance calcium-regulated potassium ion channels (mitoBK$_{Ca}$) with paxilline abolished the increase in OCR promoted by CORM-401 without affecting ECAR; patch-clamp experiments confirmed that CO derived from CORM-401 activated mitoBK$_{Ca}$ channels present in mitochondria. Conversely, stabilization of glycolysis by MG132 prevented CORM-401-mediated decrease in ECAR but did not modify the OCR response. In summary, we demonstrated in intact endothelial cells that CO induces a two-component metabolic response: uncoupling of mitochondrial respiration dependent on the activation of mitoBK$_{Ca}$ channels and inhibition of glycolysis independent of mitoBK$_{Ca}$ channels.

1. Introduction

Carbon monoxide (CO) is an endogenous gasotransmitter [1] produced during heme degradation by heme oxygenase [2]. For a long time CO has been considered merely as a 'silent killer' due to its strong affinity to hemoglobin and high toxicity when delivered to organisms *via* inhalation. More recently, CO produced in low concentrations has been recognized as an important endogenous mediator involved in vascular homeostasis (for review see [3–5]). Being a gaseous molecule, CO can freely enter the cell and influence diverse cellular processes without the involvement of receptors or endocytosis. Importantly, and unlike other gases (oxygen and nitric oxide), CO is not metabolized by mammals but eliminated through exhalation in the lungs [6]. Many reports support beneficial, cytoprotective and antioxidant effects of heme oxygenase-1 (HO-1)-derived CO [3,4,7–9] and the development of CO-

Abbreviations: CO, carbon monoxide; CO-RMs, CO-releasing molecules; CORM-401, Mn(CO)4{S2CNMe(CH2CO2H)}; iCORM-401, inactive CORM-401; BK$_{Ca}$, large-conductance calcium-regulated potassium ion channels; mitoBK$_{Ca}$, mitochondrial large-conductance calcium-regulated potassium ion channels; HO-1, heme oxygenase-1; EPR, electron paramagnetic resonance; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; mHCTPO, 4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrrolin-1-yloxy; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; MG132, Z-Leu-Leu-Leu-al

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releasing molecules (CO-RMs) has facilitated studies on the role of CO in diverse cellular processes in *in vitro* and *in vivo* models. CO-RMs are a class of compounds formed by complexation of a transition metal with CO, which allows the delivery of controlled amounts of CO to biological systems, mimicking in many instances the effect of endogenous CO [3,7]. A number of CO-mediated vasoprotective activities induced by CO-RMs have been described, including inhibition of platelet aggregation, anti-thrombotic and anti-inflammatory effects [6,10,11]. The HO-1 pathway was also shown to display pronounced anti-atherosclerotic action that may be partially due to specific effects of CO on the endothelium [12]. Indeed, HO-1 deficiency in humans resulted in extensive endothelial injury [13] and a number of reports demonstrate beneficial roles of CO on endothelial function. For example, CORM-2 exhibited anti-inflammatory actions in lipopolysaccharide (LPS)-stimulated human umbilical vein endothelial cells (HUVECs) by decreasing LPS-induced production of reactive oxygen species (ROS) and nitric oxide [14]. Furthermore, it was shown that CORM-2 suppressed prothrombotic (tissue factor, TF) and anti-fibrynolytic (PAI-1) activities of the endothelium stimulated by inflammatory cytokines (TNF-α) and regulated activation of MAPKs and NF-kB signaling pathways [15]. However, the target responsible for the beneficial action of CO in the endothelium remains elusive. Recent studies conducted mainly in isolated mitochondria from various types of cells or tissues suggest that these organelles participate in the regulation of cellular activity by CO [16–18], but limited evidence is available in intact cells. Reiter et al. [19] demonstrated CO-dependent increase in oxygen consumption rate (OCR) in endothelial cells, which was accompanied by a disruption of mitochondrial function. In addition, Wegiel and colleagues [20] showed that CO inhibits respiration in normal cells, but CO accelerates oxidative metabolism and ROS generation and decreases glucose metabolism in cancer cells. Nevertheless, the detailed mechanism(s) explaining the action of CO on mitochondria remain to be fully investigated.

It is known that plasma membrane large-conductance calcium-regulated potassium channels (BK_{Ca}) contain a heme-binding domain [21]. Reduced heme can in turn be a functional CO receptor, providing a mechanism for the regulatory activity of the channel by CO [22]. For example, it was shown that CO activates BK_{Ca} channels in endothelial cells directly and indirectly *via* involvement of nitric oxide and cGMP-dependent pathways [23] and that BK_{Ca} channels activity in the endothelium is heme oxygenase-dependent, suggesting that CO represents an endogenous regulator of BK_{Ca} activity [24]. It is interesting to note that large-conductance calcium-regulated potassium channels ($mitoBK_{Ca}$) in the mitochondrial inner membrane were described recently in the endothelium [25]. Based on these findings, we hypothesized that the modulation of mitochondrial function exerted by CO is associated with regulation of $mitoBK_{Ca}$ channels activity. Therefore, we determined the effect of CO released by CORM-401 on mitochondrial respiration and glycolytic flux in intact endothelial cells and whether $mitoBK_{Ca}$ channels mediate this response. Our results demonstrate that CO induces a two-component metabolic response: uncoupling of mitochondrial respiration dependent on the activation of mitochondrial BK_{Ca} channels and inhibition of glycolysis.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), GlutaMAX, HAT supplement, penicillin/streptomycin, sodium pyruvate, and trypsin were obtained from Gibco. Oligomycin was obtained from Calbiochem. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), rotenone and antimycin A used for the EPR oximetry experiments, DMSO, methyl cellulose, paxilline and MG132 (Z-Leu-Leu-Leu-al) were obtained from Sigma. Base XF medium, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin, rotenone, antimycin A and 2-deoxy-glucose were obtained from Seahorse Bioscience. All other reagents used in patch-clamp experiments and not listed above were obtained from Sigma.

CORM-401, synthesized as described previously [26], was dissolved in PBS and protected from light in all experiments. PBS was used as vehicle in control experiments conducted with CORM-401. mHCTPO (4-protio-3-carbamoyl-2,2,5,5-tetraperdeuteromethyl-3-pyrrolin-1-yloxy) was synthesized as described previously [27]. As paxilline and MG132 were dissolved in DMSO, in control experiments DMSO was used as a vehicle.

2.2. Cell culture

The hybridoma endothelial EA.hy926 cell line, formed by fusion of human umbilical vein endothelial cells (HUVEC) with the A549 human lung carcinoma cell line, was kindly provided by Dr. C-J Edgell (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) (Edgel 1983). Cells were propagated using three weekly feedings of DMEM containing 10% FBS, 1 g/l glucose, 110 mg/l sodium pyruvate, 2 mM GlutaMAX™, antibiotics (100 IU penicillin, 100 μg/ml streptomycin) and 2% HAT Supplement. Cultures were maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air.

2.3. EPR oximetry

Oxygen consumption by EA.hy926 cells in suspension was measured by the spin label method in a closed chamber system (glass capillary tubes) using an EMX Bruker spectrometer. EA.hy926 cells were cultured to 95% confluence, harvested by trypsinization, washed with PBS buffer (centrifugation 300 ×*g*, 5 min, RT), re-suspended in DMEM without FBS and stored on ice until the start of measurement to preserve their functional responses for a period of couple of hours. Five min before the experiment cells were transferred to room temperature. Measurements were performed at 37 °C on cells suspended at a density of 5 million per ml in DMEM with 0.2% methylcellulose and 100 μM mHCTPO as a spin probe (spectral parameters of mHCTPO were calibrated for dissolved oxygen concentration in DMEM at 37 °C, data not shown). The total volume of sample was 50 μl. Samples were run using the following instrumental settings: microvawe power, 1 mW; modulation amplitude, 0.03 mT; scan width, 0.3 mT; and scan time, 23 s. All reagents investigated (CORM-401 or mitochondrial modulators) were added just before the start of measurements. The mitochondrial function assay employed in the study relied on separate measurements of oxygen consumption by untreated cells (control) or cells treated with oligomycin (1 μg/ml), CCCP (1 μM) or rotenone (1 μM) together with antimycin A (1 μM). Slopes of linear functions fitted to the data points reflect the oxygen consumption rate (OCR) of cells. The mitochondrial function parameters determined were: 1) basal respiration (difference between OCR of untreated cells and cells incubated with rotenone/antimycin A), 2) ATP-linked respiration (difference between OCR of untreated cells and cells treated with oligomycin), 3) proton leak (difference between OCR of cells incubated with oligomycin and cells treated with rotenone/antimycin A), 4) maximal respiration (difference between OCR of cells treated with CCCP and cells additioned with rotenone/antimycin A), 5) reserve respiratory capacity (difference between OCR of cells treated with CCCP and untreated cells) and 6) non-mitochondrial respiration (OCR value of cells after incubation with rotenone/antimycin A).

Spin labeled EPR oximetry has been described and widely used for measurements of oxygen consumption by cells in suspension [28,29]. In this method OCR is measured in cells placed in a closed chamber system (glass capillary tube), which does not allow the diffusion of gases from the atmosphere. Thus, the system is especially useful in experiments aimed at investigating the effect of gaseous mediators, such as CO, because it ensures that the entire amount of gas remains within the vial containing the sample. Interestingly, EPR oximetry is normally used for measurements of basal OCR by cells, but here we show that a complete profile of mitochondrial respiration can be obtained with

this method by measuring the response to oligomycin, the uncoupler CCCP, and rotenone with antimycin A.

2.4. Extracellular flux technology

A Seahorse Bioscience XF24 Analyzer was used to measure mitochondrial function and extracellular acidification in adherent endothelial EA.hy926 cells. Cells were seeded into Seahorse XF24-well plates approximately 24 h before the experiment according to the Seahorse protocol. A density of 30,000 cells per well was selected as ideal after preliminary experiments. One day before the experiment sensor cartridges were hydrated in XF calibrant and maintained at 37 °C in air without CO_2. On the day of the experiment cells were washed once and incubated with bicarbonate-free low-buffered assay medium (glucose 1 g/l, GlutaMAX 2 mM, sodium pyruvate 1 mM, pH adjusted with NaOH) for one hour at 37 °C in the absence of CO_2 prior to the beginning of the assay. Changes in cellular respiration were assessed over time after addition of CORM-401 or in a mitochondrial functional assay where sequential injections of vehicle, CORM-401 or other reagent in port A followed by addition of 1 μg/ml oligomycin in port B, 0.7 μM FCCP in port C and 1 μM rotenone/antimycin A in port D were performed.

From the mitochondrial assay we determined the following parameters: stimulated respiration (the last value of OCR after exposure to PBS or CORM-401 and preceding oligomycin injection), ATP-linked respiration (the difference between OCR before and after oligomycin injection), proton leak (the difference between OCR after oligomycin and rotenone/antimycin A injection), maximal respiration (the difference between OCR after FCCP and rotenone/antimycin A injection), reserve capacity (the difference between OCR after FCCP and stimulated respiration before oligomycin injection), non-mitochondrial respiration (the OCR value after rotenone/antimycin A injection).

The glycolysis stress test was performed according to the Seahorse Bioscience protocol. Briefly, on the day of the experiment cells were washed once and incubated with bicarbonate-free low-buffered glycolysis assay medium (glucose-free, GlutaMAX 2 mM, pH adjusted with NaOH) for one hour at 37 °C in the absence of CO_2 prior to the beginning of the assay. The glycolysis stress test employed in the present study used sequential injections of glucose (10 mM, port A), PBS or CORM-401 (port B), oligomycin (1 ug/ml, port C) and 2-deoxy-glucose (100 mM, port D). In certain experiments cells were incubated for 6 h with 10 μM MG132, a protease inhibitor that stabilizes glycolysis, prior to performing the glycolysis stress test.

2.5. Mitoplast preparation and patch-clamp experiments

Patch-clamp experiments in mitoplasts (mitochondria without outer membranes) were performed as described previously [25]. Firstly, EA.hy926 cells were scraped, collected in ice-cold PBS, centrifuged (800 ×*g* for 10 min) and resuspended in sucrose solution (250 mM sucrose, 5 mM HEPES, pH 7.2). Next, cells were homogenized with a glass-glass homogenizer and centrifuged again (9200 ×*g* for 10 min). The resulting pellet was resuspended in sucrose solution and centrifuged (790 ×*g* for 10 min). The mitochondria-enriched supernatant was collected and centrifuged again (9200 ×*g*, 10 min). The resulting pellet was resuspended in storage solution (150 mM KCl,10 mM HEPES, pH 7.2) and used in patch clamp experiments (Fig. 6A). All procedures were performed at 4 °C.

Mitoplasts were prepared from the mitochondrial preparation by changing the isotonic solution to a hypotonic one (5 mM HEPES, 100 μM $CaCl_2$, pH 7.2) to induce swelling and disruption of the mitochondrial outer membrane. To restore the sample to an isotonic condition (150 mM KCl, 10 mM HEPES, 100 μM $CaCl_2$, pH 7.2) a hypertonic solution (750 mM KCl, 30 mM HEPES, 100 μM $CaCl_2$, pH 7.2) was added (Fig. 6A). The purity of the mitoplasts preparation has been confirmed in our previous publication [25] and by a novel PCR approach (unpublished results).

The experiments to assess the mitoBK$_{Ca}$ channel activity were carried out in patch-clamp inside-out mode. Reported voltages are those applied to the interior of the patch-clamp pipette. Hence, positive potentials represent the physiological polarization of the inner mitochondrial membrane (outside positive). CORM-401 or iCORM-401 were added as dilutions in isotonic solution containing 1 μM Ca^{2+}. The mitoplast attached to the tip of the measuring pipette was put into a glass perfusion tube in which its outer face was washed by the test solutions (Fig. 6A). The electrical connection was made using Ag/AgCl electrode and an agar salt bridge as the ground electrode. The current was recorded using a patch-clamp amplifier (Axopatch 200B). The pipettes made of borosilicate glass had a resistance of about 15 MΩ. The currents were low-pass filtered at 1 kHz and sampled at a frequency of 100 kHz. The illustrated single-channel recordings are representative for the most frequently observed conductance in given condition and the conductance of the channel was calculated from the current-voltage relationship. The probability of channel opening was determined using the single-channel search mode of the Clampfit software.

2.6. Statistics

The EPR results are presented as means ± SD from four independent experiments. The Seahorse XF results are presented as means ± SEM from four independent experiments (4–5 replicates in each experiment) or as means ± SD (4–5 replicates) in representative experiments. Details are included under the figures. For statistical analysis Student's t-test or One-way ANOVA was performed and statistical significance was considered at $p < 0.05$. Data from the patch-clamp experiments are reported as means ± SD. Student's t-test was used for statistical analysis.

3. Results

3.1. Effect of CORM-401 on endothelial mitochondrial function as measured by EPR oximetry

As shown in Fig. 1A, endothelial cells in suspension (5 million/ml) in a glass capillary tube consumed oxygen with a linear kinetic. To confirm the validity of our experimental methodology, we measured oxygen consumption following treatment of cells with different modulators of mitochondrial function. We observed that inhibition of ATP synthase with oligomycin decreased OCR, the uncoupler CCCP strongly accelerated oxygen consumption and rotenone/antimycin A virtually abolished cell respiration (Fig. 1A). Differences in OCR values in control cells or cells treated with oligomycin, CCCP, or rotenone/antimycin A were used to calculate basal respiration, ATP-linked respiration, proton leak, maximal respiration and reserve capacity as illustrated in Fig. 1B. Interestingly, incubation with CORM-401 induced a concentration-dependent acceleration of OCR in comparison to untreated cells (Fig. 2A) and an increase in basal respiration, proton leak and non-mitochondrial respiration with concomitant decrease in ATP-linked respiration was observed (Fig. 2B). Maximal respiration and reserve capacity were slightly decreased, but the difference did not reach statistical significance. Importantly, oxygen concentration in the chamber remained unchanged in the presence of CORM-401 (1 mM) but without cells.

3.2. The effect of CORM-401 on mitochondrial respiration and glycolytic flux is concentration-dependent

In parallel sets of the experiments using EPR oximetry we assessed the effect of CORM-401 on cellular respiration and glycolysis using the Seahorse XF analyzer. As shown in Fig. 3, CORM-401 (10, 30 or 100 μM) added to endothelial cells induced a concentration-

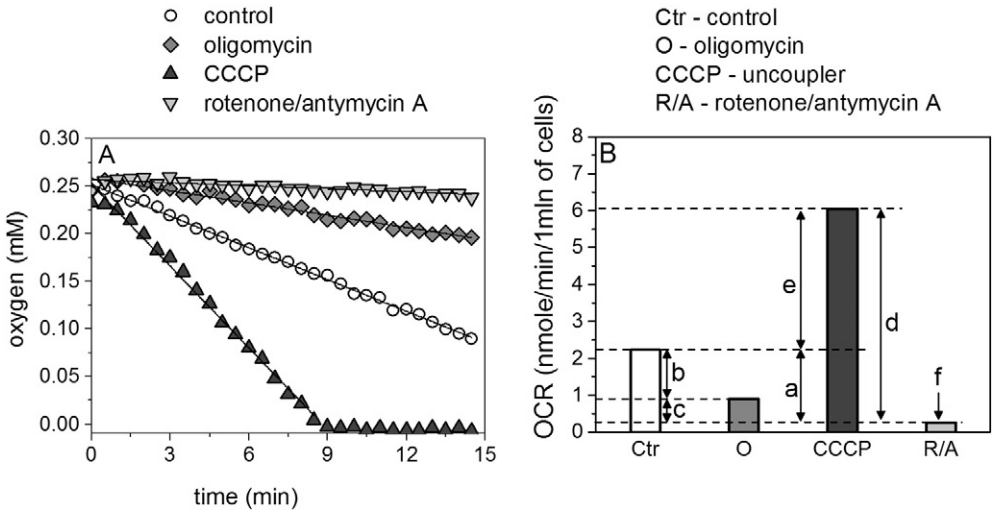

Fig. 1. *Assesment of oxygen consumption in endothelial EA.hy926 cells using EPR oximetry.* (A) The kinetic of oxygen consumption by EA.hy926 cells in suspension (5 mln/ml) was measured using 100 μM of the mHCTPO spin probe in a closed chamber system. Cells were untreated (control) or treated independently with the following mitochondrial modulators: 1 μg/ml oligomycin (O), 1 μM CCCP (uncoupler), 1 μM rotenone in combination with 1 μM antimycin A (R/A). Data are from a representative experiment. (B) Oxygen consumption rate (OCR) calculated for different conditions: (a) basal respiration is the difference between OCR of untreated cells and cells incubated with R/A; (b) ATP-linked respiration is the difference between OCR of untreated cells and cells treated with O; (c) proton (H^+) leak is the difference between OCR of cells incubated with O and cells treated with R/A; (d) maximal respiration is the difference between OCR of cells treated with CCCP and cells additioned with R/A; (e) reserved respiratory capacity is the difference between OCR of cells treated with CCCP and untreated cells; (f) non-mitochondrial respiration is the OCR value of cells incubated with R/A.

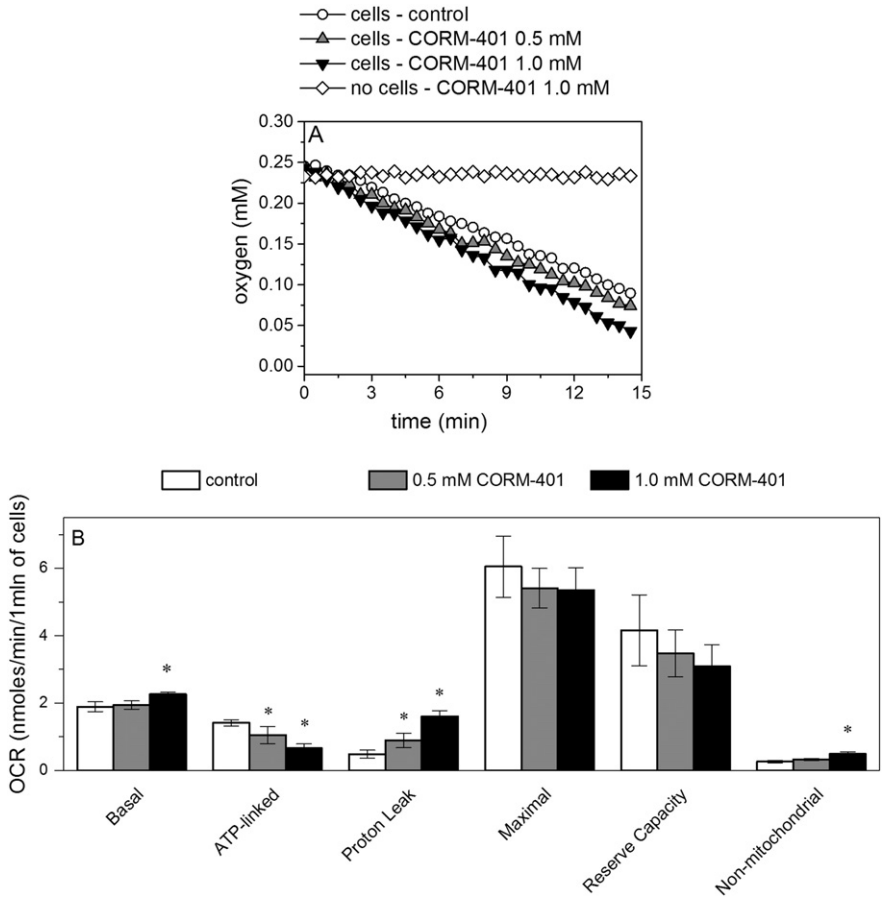

Fig. 2. *Effect of CORM-401 on mitochondrial function of endothelial EA.hy926 cells monitored by EPR oximetry.* (A) Oxygen consumption by endothelial cells (5 mln/ml) in control conditions or in the presence of CORM-401 (0.5 mM or 1.0 mM); 1 mM CORM-401 did not change oxygen levels in the absence of cells (data from a representative experiment). (B) Mitochondrial function assay performed in control conditions or in the presence of CORM-401 (0.5 mM or 1.0 mM) as described in Materials and Methods. In these experiments 1 mM CORM-401 equals to 200 nmoles of CORM-401 per 1 mln of cells. Results are calculated as described in the legend of Fig. 1. Data are means ± SD of four independent experiments, * $p < 0.05$ compared to control.

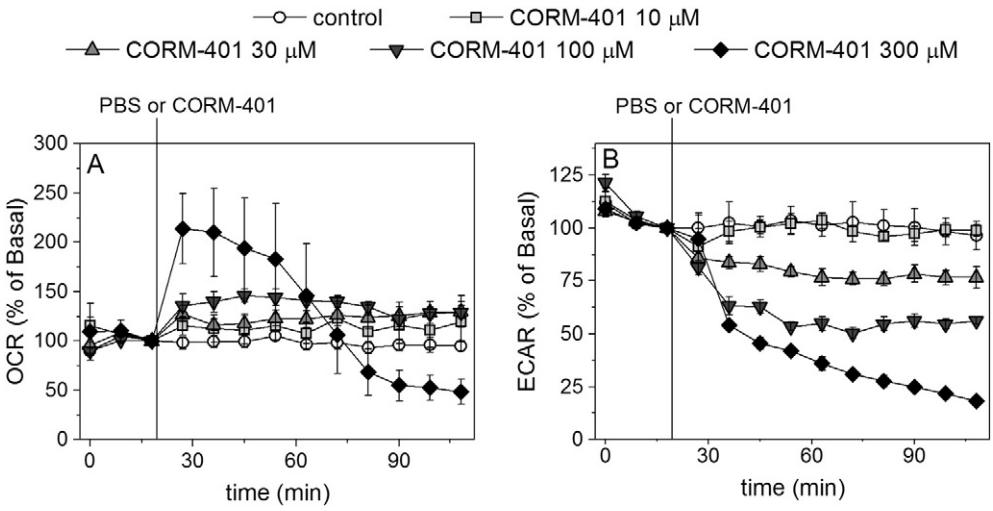

Fig. 3. *Effect of CORM-401 on basal mitochondrial respiration and glycolytic flux in endothelial EA.hy926 cells as monitored by the Seahorse XF Analyzer.* Basal oxygen consumption rate (OCR) (A) and extracellular acidification rate (ECAR) (B) were measured in cells after addition of PBS (control) or CORM-401 (10, 30, 100, 300 μM). Data are expressed as a percentage of the basal OCR or ECAR. Data represent the means ± SD of n = 3–4 replicates from a representative experiment.

dependent increase in OCR and a simultaneous decrease in ECAR. By contrast, 300 μM CORM-401 caused a rapid initial increase in OCR followed by a profound decrease after ~45 min of incubation. A persistent decrease in ECAR was observed with CORM-401 at 300 μM. No evident cytotoxicity was observed at all the concentrations of CORM-401 tested in these experiments (Supplementary Fig. S1). Therefore, we selected 10, 30 and 100 μM concentrations of CORM-401 for further analysis of mitochondrial function.

We next performed a mitochondrial function assay to understand whether the OCR response elicited by CORM-401 is linked to ATP production or whether the compound induces a shift from glycolysis to oxidative phosphorylation. In these experiments, CORM-401 (10, 30 and 100 μM) or 100 μM inactive CORM-401 (iCORM-401, containing $MnSO_4$ and the CORM-401 ligand), which does not release CO, were used. We confirmed that addition of CORM-401 increased cell respiration (Fig. 4A) and that the compound reduced the response of cells to oligomycin, FCCP and rotenone/antimycin A. Although iCORM-401 induced an initial increase in OCR, its effect on mitochondrial function was very different from the same concentration of active compound since the responses to oligomycin, FCCP and rotenone/antimycin were similar to control (Fig. 4B). Thus, it appears that CO released from CORM-401 is a major player in modulating mitochondrial respiration. These findings were supported by additional experiments in which OCR was followed over time after prolonged exposure of cells to either CORM-401 or iCORM-401 (100 μM). We observed that the increase in OCR induced by iCORM-401 was transient and was accompanied by an initial increase in ECAR, while CORM-401 exerted a higher and sustained increase in OCR with a substantial decline in ECAR (Supplementary Fig. S2). Since 30 μM CORM-401 induced clear-cut effects in OCR as well as in ECAR (Fig. 4C and D), we selected this concentration for further analysis of mitochondrial functional parameters and for mechanistic studies. CORM-401 (30 μM) consistently increased OCR, proton leak and non-mitochondrial respiration. In contrast, ATP-linked respiration, maximal respiration and reserve capacity were substantially decreased (Fig. 4E, for concentration-dependent effects see also Fig. S3 in Supplementary data).

To further investigate the effect of CORM-401 on glycolysis we performed a glycolysis stress test using CORM-401 (from 10 to 100 μM). It should be noted here that cells in the glycolysis stress test were starved for 90 min (in the absence of glucose) prior to addition of glucose (10 mM), while cells in the mitochondrial function assay had continuous access to glucose (5.5 mM) in the medium. Therefore, the different experimental conditions between the glycolysis stress test and the mitochondrial functional assay may give rise to different responses to CORM-401. Interestingly, CORM-401 added during the glycolysis stress test caused a 'hormesis-like' effect since the lowest concentration (10 μM) induced an increase in ECAR. However, CORM-401 (30 μM) did not elicit significant effects while this compound at 100 μM substantially diminished ECAR (Fig. S4A in Supplementary data).

3.3. Inhibition of mitoBK$_{Ca}$ channels with paxilline abolishes the increase in respiration induced by CORM-401

We hypothesized that activation of mitoBK$_{Ca}$ channels is involved in the increase in mitochondrial respiration induced by CORM-401. Therefore, we performed experiments in which the effect of paxilline, a selective inhibitor of BK$_{Ca}$ type channels, was evaluated. Injection of paxilline (10 μM) alone did not influence OCR values compared to control, however injection of paxilline with CORM-401 (30 μM) abolished the effect of CORM-401 (Fig. 5A). Interestingly, the presence of paxilline did not influence glycolytic flux and the decrease in ECAR observed with CORM-401 (Fig. 5B). The calculation of parameters from the mitochondrial function assay revealed that paxilline blocked the increase in basal OCR and partially inhibited the increase in proton leak caused by CORM-401 (Fig. 5C).

3.4. Direct activation of endothelial mitoBK$_{Ca}$ channels by CO released from CORM-401

It was previously shown that mitoBK$_{Ca}$ channels are present in the mitochondrial inner membrane of human endothelial EA.hy926 cells. Moreover, it seems that these channels share similar pharmacology properties and kinetics with their plasma membrane counterparts [25]. To corroborate our findings showing that mitoBK$_{Ca}$ channels mediate the increased respiration by CORM-401, we performed patch-clamp experiments to assess the capacity of CORM-401 to directly activate mitoBK$_{Ca}$ channels. The presence of mitoBK$_{Ca}$ channels in EA.hy926 cells was confirmed by single-channel recordings at different voltages in symmetrical isotonic solution. As expected, the channel activity was inhibited in low-calcium solution (Fig. 6B). Interestingly, despite the lack of calcium stimulus, the mitoBK$_{Ca}$ channel regained its activity upon addition of 30 μM CORM-401 (Fig. 6B). When currents were measured as a function of applied potentials (from 60 mV to −60 mV) the

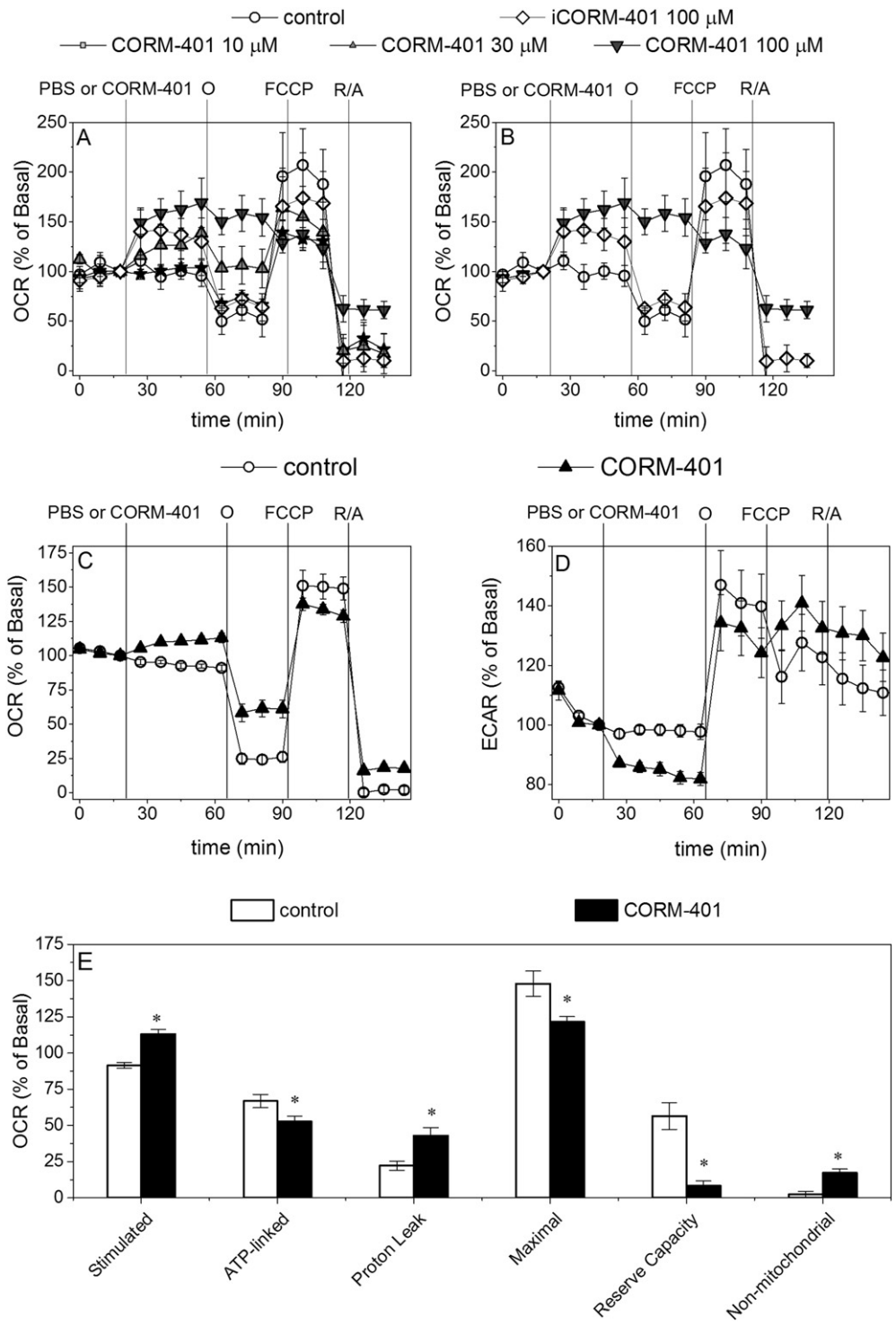

Fig. 4. *Effect of CORM-401 on mitochondrial bioenergetic parameters in endothelial EA.hy926 cells as monitored by the Seahorse XF Analyzer.* OCR measurements of cells treated with PBS (control), CORM-401 (10, 30, 100 μM, A) or iCORM-401 (100 μM, B) followed by sequential addition of oligomycin (1 μg/ml), FCCP (0.7 μM) and rotenone/antimycin A (1 μM/1 μM) (data represent the means ± SD of n = 3–4 replicates from a representative experiment). OCR (C) or ECAR (D) measurements of cells treated with PBS (control) or 30 μM CORM-401 followed by sequential addition of oligomycin (1 μg/ml), FCCP (0.7 μM) and rotenone/antimycin A (1 μM/1 μM), (data represent the means ± SEM of nine independent experiments). Bioenergetic parameters (E) were calculated from the results presented in Fig. 4C: stimulated respiration as the last value of OCR after exposure to CORM-401 and before oligomycin injection; ATP-linked respiration as the difference between OCR before and after oligomycin injection; proton leak as the difference between OCR after oligomycin and rotenone/antimycin A injection; maximal respiration as the difference between OCR after FCCP and rotenone/antimycin A injection; reserve capacity as the difference between OCR after FCCP injection and before oligomycin injection; non-mitochondrial respiration as the OCR value after rotenone/antimycin A injection. Data represent the means ± SEM of nine independent experiments, n = 4–5 replicates in each experiment, * $p < 0.05$ compared to control.

channel showed a linear current–voltage relationship in the presence of 100 μM Ca^{2+} or CORM-401 (Fig. 7A). However, CORM-401 slightly shifted this relation towards higher values compared with the positive control (100 μM of Ca^{2+}). Therefore, the conductance of mitoBK$_{Ca}$ channels calculated for control recordings oscillated around 280 pS ± 10 pS and rose insignificantly to 300 pS ± 10 pS after CORM-401. Both values

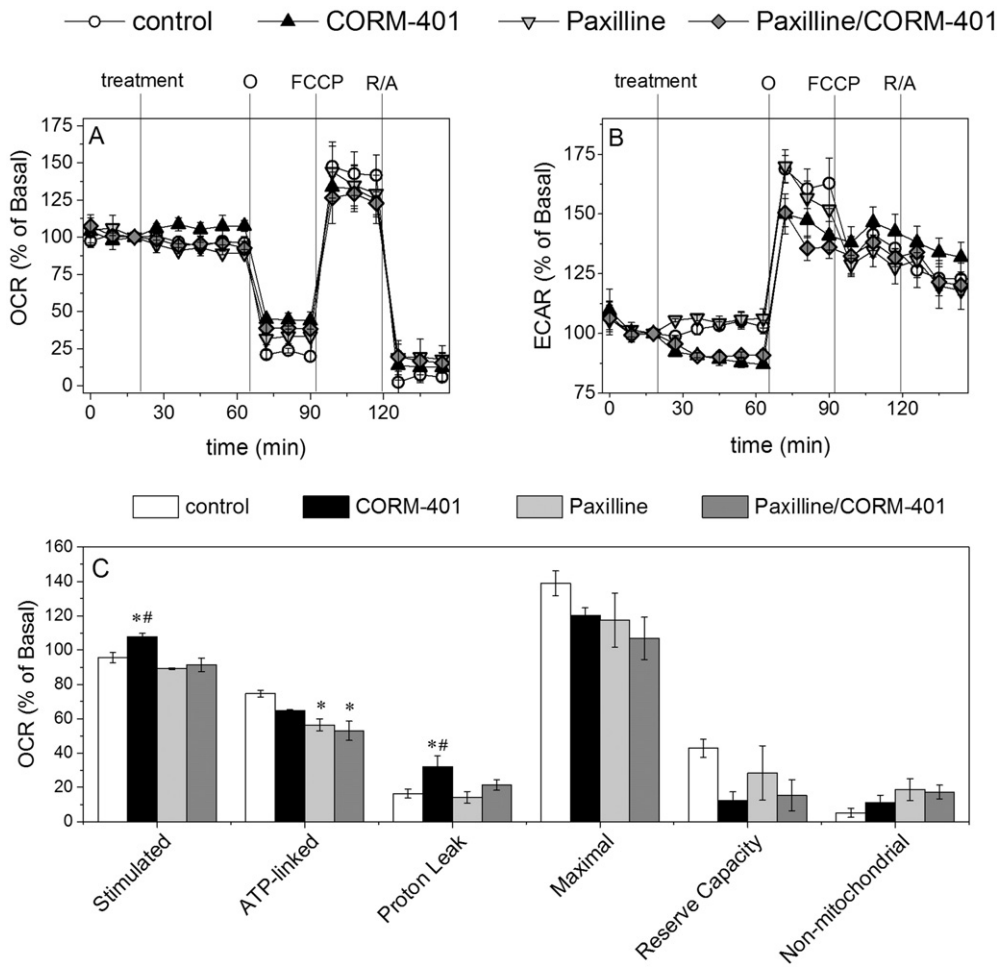

Fig. 5. *Inhibition of mitoBK$_{Ca}$ channels prevents CORM-401-dependent changes in mitochondrial function in EA.hy926 cells.* Three basal OCR (A) and ECAR (B) measurements were performed prior to injection of PBS (control), 30 μM CORM-401, 10 μM paxilline or a combination of CORM-401 and paxilline. C) Bioenergetic parameters were calculated after sequential addition of the following modulators of mitochondrial function: oligomycin (O, 1 μg/ml), FCCP (0.7 μM) and rotenone/antimycin A (R/A, 1 μM). Data are expressed as a percentage of the initial OCR or ECAR before injection of compounds. Data represent the means ± SEM of four independent experiments (4–5 replicates in each experiment), * $p < 0.05$ compared to control, # $p < 0.05$ compared to paxilline.

indicate large-conductance channel activity. Rectification of the channel was not observed (Fig. 7A).

The probability of channel opening (P_o) was distinctive for a mitoBK$_{Ca}$ channel and ranged from ~4% at −60 mV to ~90% at 60 mV as calculated from recordings in a symmetrical isotonic solution with Ca^{2+} (100 μM). After channel inactivation with low Ca^{2+} (1 μM) and reactivation by CORM-401 (30 μM), the probability of opening of the channel appeared to be somewhat higher than in the control, especially when calculated from recordings carried out at −20 mV and 20 mV. In fact, the P_o in the presence of CORM-401 rose from ~14% to ~35% and from ~61% to ~75%, respectively (Fig. 7B).

The distribution of mean lifetime of channel closure and opening at different voltages was also altered by CORM-401 as compared to the positive control. Thus, the maximal mean lifetime of the closed state calculated from recordings at −60 mV reached ~120 ms in the presence of 100 μM Ca^{2+} while after channel inactivation in 1 μM Ca^{2+} and reactivation by 30 μM CORM-401 it dropped to only ~40 ms (Fig. 7C). Similarly, the maximal mean lifetime of the open state at 60 mV reached ~70 ms in 100 μM Ca^{2+} but was shortened to ~15 ms in the CORM-401 group (Fig. 7D). These results indicate that the kinetics of the channel has changed and the dramatic shortening of mean lifetime of both closed and open state without corresponding changes in P_o suggest that CORM-401 significantly increases the frequency of closure/opening events. The effect of CORM-401 was reversed by paxilline (1 μM, Fig. 7E). iCORM-401 (30 μM) did not reactivate the mitoBK$_{Ca}$ channel in low-calcium solution (Fig. S5 in Supplementary data).

3.5. Effect of glycolysis stabilization by MG132 on CORM-401-induced changes in glycolytic flux

As the increase in OCR induced by CORM-401 was accompanied by a decrease in ECAR, we examined whether these two events were metabolically related, that is, the reduction in ECAR was a direct consequence of the increase of OCR, or they were two effects of CORM-401 acting independently on mitochondrial and glycolysis targets. Therefore, we treated cells with the protease inhibitor MG132, which stabilizes the methylated form of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and increases glycolysis ([30], see Scheme in Fig. 9), followed by addition of CORM-401. As expected, pre-incubation with MG132 lowered the basal OCR and increased basal ECAR compared to control (Fig. 8A and B). However, CORM-401 still induced an acceleration of OCR, and a mitochondrial function assay (Fig. 8C) confirmed that CORM-401 caused an increase in basal respiration and proton leak, as well as a decrease in ATP-linked respiration even in the presence of MG132. In contrast, the glycolysis stress test showed that the decrease in ECAR induced by CORM-401 was lost in the presence of MG132, suggesting that the potential target responsible for inhibition of

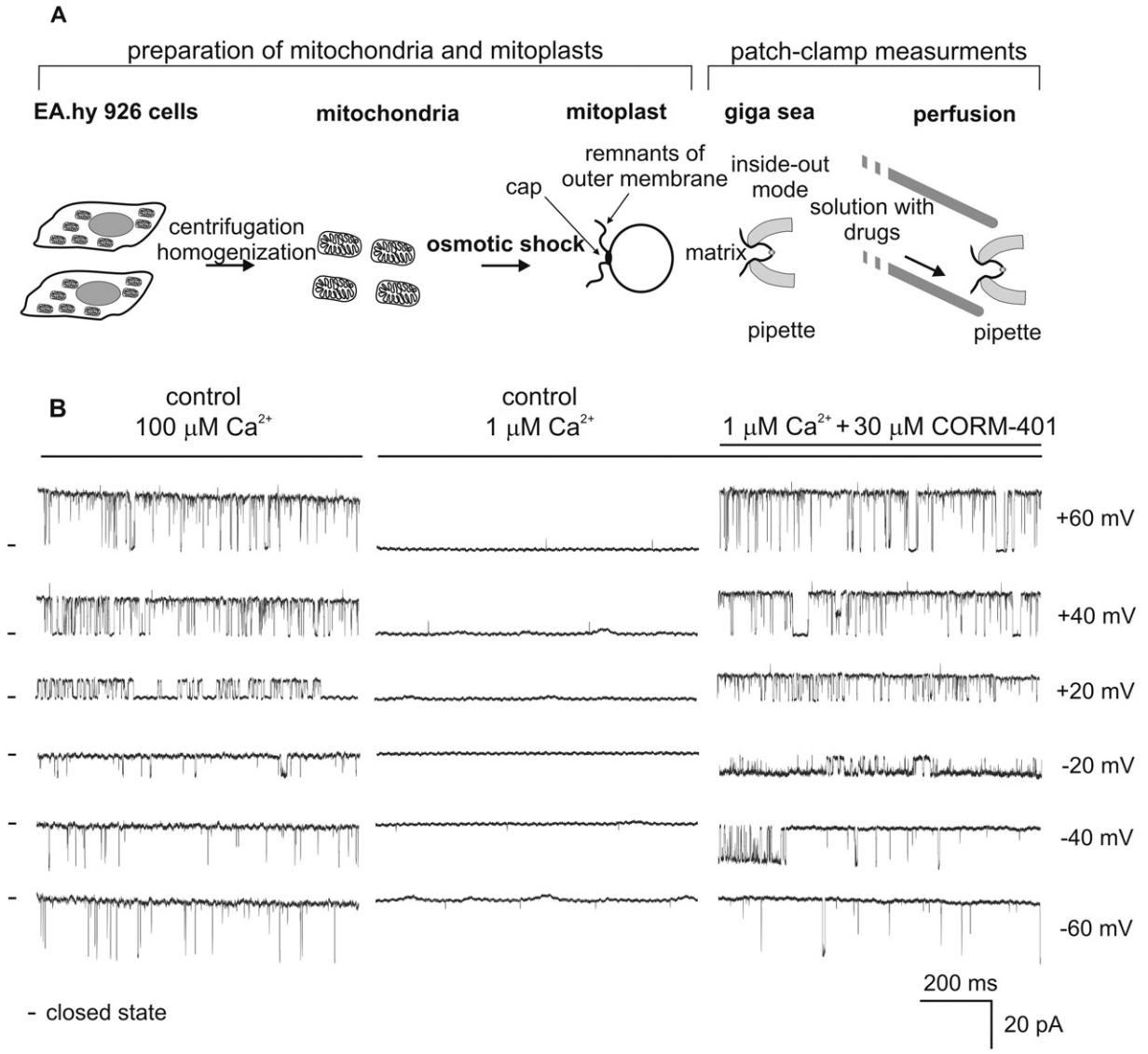

Fig. 6. *Effect of CORM-401 on the activity of the mitoBK$_{Ca}$ channel derived from mitochondria of endothelial EA.hy926 cells.* (A) Scheme summarizing the preparation of mitoplasts and the patch-clamp experiment. (B) Representative traces of single-channel recordings of the mitoBK$_{Ca}$ channel at different voltages in the rage of −60 mV to +60 mV performed in a symmetric 150/150 mM KCl control solution (100 μM Ca^{2+}), low-calcium solution (1 μM Ca^{2+}) or low-calcium solution containing 30 μM CORM-401.

glycolysis by CO was either PFKFB3 or an enzyme upstream of PFKFB3 (Fig. 9 and Fig. S4B in Supplementary data).

4. Discussion

Recent evidence suggest that CO targets mitochondria [9,16,17,31–34], though the mechanisms involved are not clear. Herein, we demonstrate in intact endothelial cells that CO induces uncoupling of mitochondrial respiration and inhibition of glycolysis. We provide evidence supporting the effect of CO released by CORM-401 on OCR, index of mitochondrial respiration, in intact endothelial cells using two complementary techniques: EPR-based oximetry and the Seahorse XF-based methodology. The main advantage of using both techniques is that mitochondrial function can be investigated in whole cells, as opposed to isolated mitochondria. Moreover, two different systems – opened and closed chambers – are especially useful in studies on gaseous moieties such as CO. Most importantly, we showed that the increase in respiration induced by CO is mediated by activation of mitoBK$_{Ca}$ channels, while the effect on glycolysis was mitoBK$_{Ca}$-independent.

In the present work we used a recently synthesized CO-releasing molecule, CORM-401, a Mn-based metal carbonyl soluble in PBS that releases at least three moles of CO per mole of compound with a half-life of 13–14 min (Crook et al. 2011 [26] and unpublished data). Therefore, CORM-401 differs from previously characterized CO-RMs, such as CORM-3 and CORM-A1, which release only one mole of CO per mole of compound [35,36]. Here we demonstrate that CORM-401 consistently accelerates OCR in intact endothelial EA.hy926 cells using both EPR oximetry (closed chamber) and the Seahorse XF analyzer (open system). It is known that CO at high concentrations is a potent inhibitor of cytochrome oxidase, as it directly competes with oxygen binding and its inhibition is strictly competitive with respect to oxygen [37,38]. In our experiments we observed a rapid acceleration of respiration followed by a decrease in oxygen consumption rate only after prolonged exposure to the highest concentration of CORM-401 (300 μM). This later effect is likely due to inhibition of cytochrom oxidase as greater amounts of CO are liberated by the compound over time. Alternatively, depletion of substrates for respiration may also explain this result. However, the aim of the study was to investigate subtle effects of non-toxic concentration of CORM-401 and at 30 μM CORM-401 we only observed

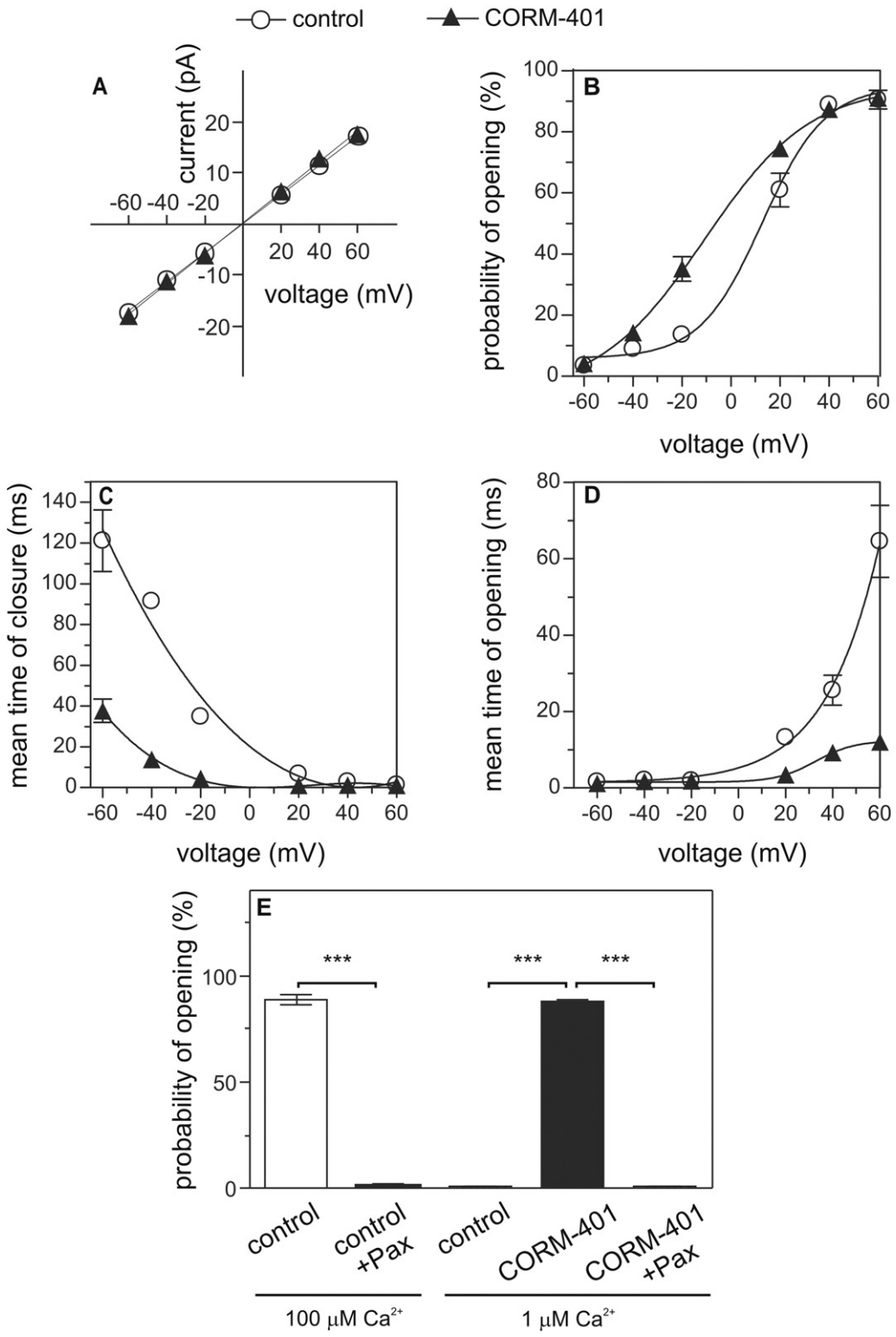

Fig. 7. *Influence of CORM-401 on the biophysical properties of the mitoBK$_{Ca}$ channel present in mitochondria of endothelial EA.hy926 cells.* Measurements were performed in a symmetric 150/150 mM KCl solutions: control solution (100 μM Ca^{2+}) or low-calcium solution (1 μM Ca^{2+}) containing 30 μM CORM-401. (A) Current–voltage characteristics of single-channel events at different voltages. (B) Distribution of the probability of channel opening. The mean lifetime of the closure (C) and opening (D) of the mitoBK$_{Ca}$ channel at different voltages. (E) Mean probability of the channel opening at 40 mV in control solution, control solution containing 1 μM paxilline, low calcium solution, low calcium solution containing 30 μM CORM-401 or a combination of 30 μM CORM-401 and 1 μM paxilline. Data represent the means ± SD of four independent experiments (7–13 replicates in each experiment), Student's t-test, *** $p < 0.001$.

acceleration and not a slowdown of oxygen consumption rate. We also show that the increase in basal respiration by CORM-401 is accompanied by an increase in proton leak and non-mitochondrial respiration and a decrease in ATP-linked respiration and reserve capacity. It is important to note that the concentration of CORM-401 used in EPR oximetry (0.5-1 mM) corresponds to 100-200 nmoles of compound per

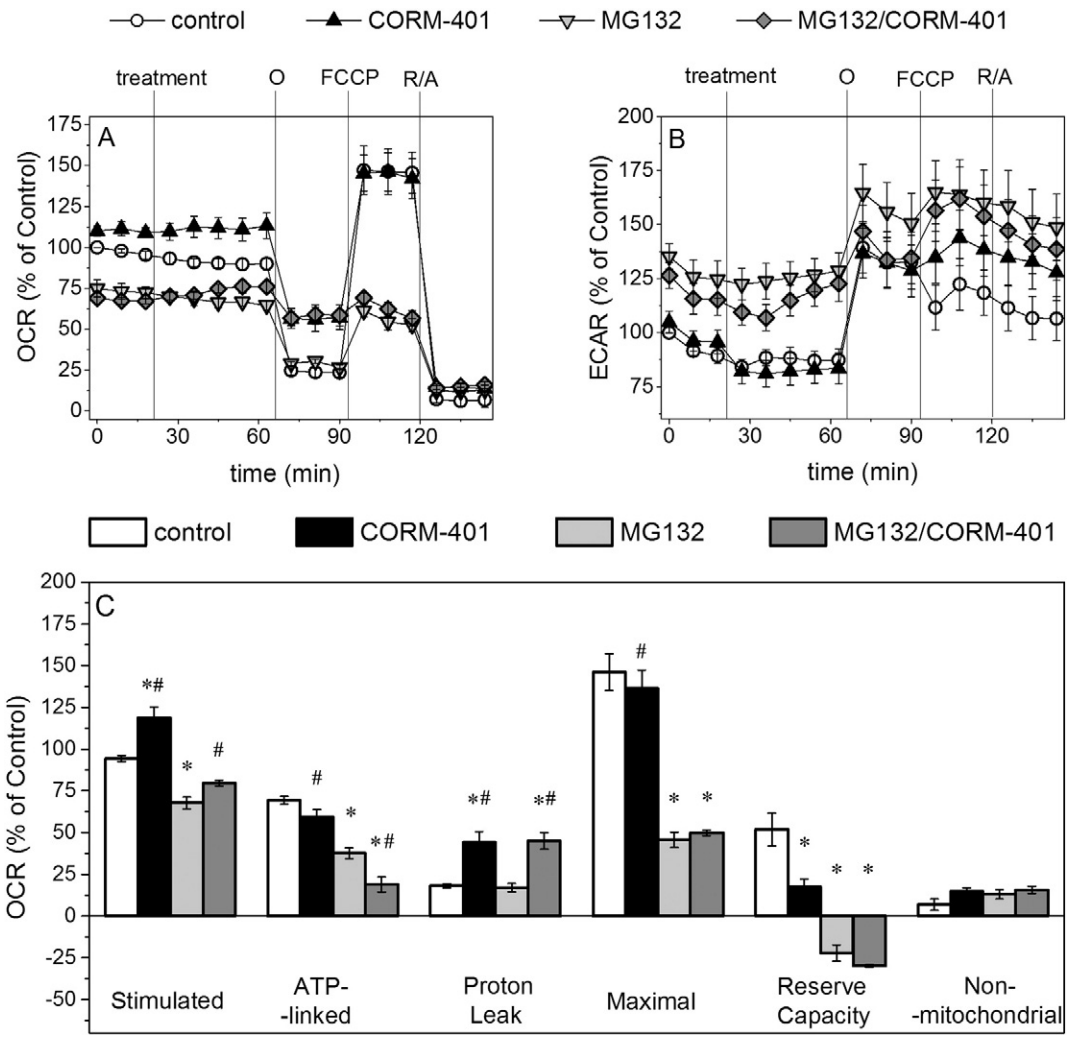

Fig. 8. *Effect of glycolysis stabilization on CORM-401-mediated changes in mitochondrial function and glycolytic flux in endothelial EA.hy926 cells.* Endothelial cells were pre-incubated with DMSO or 10 μM MG132 for 6 h. Three basal OCR (A) and ECAR (B) measurements were performed prior to injection of PBS (control) or 30 μM CORM-401. (C) Bioenergetic parameters were calculated after sequential addition of the following modulators of mitochondrial function: oligomycin (O, 1 μg/ml), FCCP (0.7 μM) and rotenone/antimycin A (R/A, 1 μM). Data are expressed as a percentage of the first OCR or ECAR value in control group. Data represent the means ± SEM of four independent experiments (4 – 5 replicates in each experiment), (One Way ANOVA test, * $p < 0.05$ compared to control, # $p < 0.05$ compared to MG132).

1 mln of cells, while the reference concentration of CORM-401 used for the majority of the Seahorse experiments (30 μM) corresponds to 600 nmoles per 1 mln of cells. It is possible that the slightly higher ratio of nmoles of CORM-401 per 1 mln of cells needed to observe an acceleration of oxygen consumption in the Seahorse is due to the facts that the Seahorse apparatus is a closed system only transiently, during the measurement phase, and that in between each measurement of oxygen consumption the medium is mixed several times to re-equilibrate oxygen levels. Consequently, a loss of CO from the medium is expected to occur over the course of the experiment in the Seahorse apparatus. Importantly, in both systems we observed a concentration-dependent effect of CO on respiration of endothelial cells. In the Seahorse experiments, where CORM-401 was tested at 10, 30, 100 and 300 μM, the highest concentration appeared to be toxic as mitochondrial function and glycolytic flux were considerably inhibited over time.

The increase in OCR by endothelial EA.hy926 cells treated with CORM-401 combined with a decline in ATP-linked respiration and a rise in proton leak suggest mitochondrial uncoupling by CO. This is in line with our recent findings in mitochondria isolated from cardiomyocytes, in which CORM-3 uncoupled oxidative phosphorylation from ATP synthesis through a mechanism that involves activation of uncoupling proteins (UCPs), adenine nucleotide transporter (ANT) and phosphate carriers (PiC, DIC) [16,17]. Interestingly, in the present work we provide evidence that mitoBK$_{Ca}$ channels also contribute to the uncoupling activity of CO in endothelial cells since paxilline, an inhibitor of the BK$_{Ca}$ type channel, abolished the increase in OCR caused by CO and lowered the effect on proton leak. Importantly, we further show a direct action of CORM-401 in the activation of mitoBK$_{Ca}$ channels in mitoplasts derived from mitochondria of EA.hy926 cells. CORM-401 induced re-activation of mitoBK$_{Ca}$ channels in low-calcium solution, increasing the frequency and decreasing the time span of closure/opening events in comparison to control high calcium solution. The effect was directly related to CO, as inactive iCORM-401 was not able to reactivate mitoBK$_{Ca}$ channels in low-calcium solution. Activation of mitoBK$_{Ca}$ channel-dependent K^+ influx increases the matrix volume of mitochondria, which can alter electron flow through the respiratory chain [39,40] and increase OCR. The influx of K^+ is balanced by the K^+/H^+ antiporter, which expels K^+ from the matrix and induces an influx of H^+ [41], seen as an increased proton leak. Therefore, the effect of CO on mitoBK$_{Ca}$ channels seems to underscore the role of endogenous CO in the regulation of coupling tightness between respiration and ATP synthesis in mitochondria, contributing to the maintenance of a balance between energy supply and demand in cells. On the other hand, activation of mitoBK$_{Ca}$ channels elicited by CO may function as a

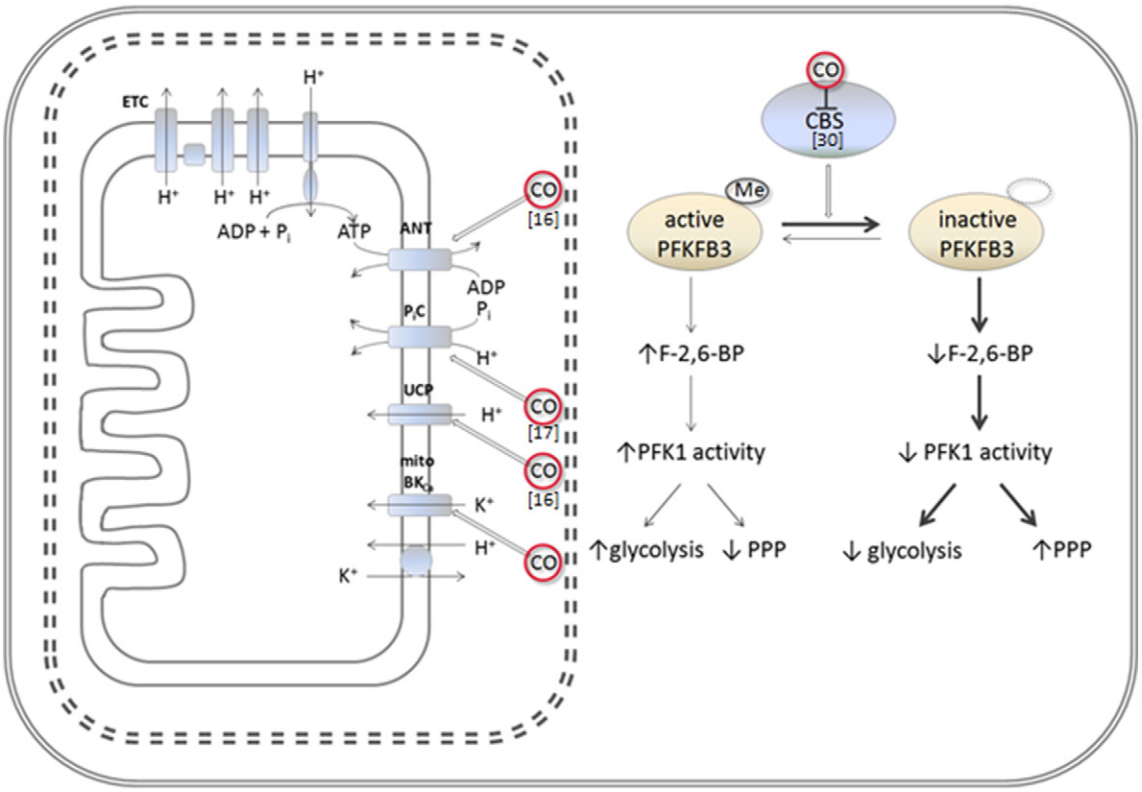

Fig. 9. *A schematic model summarizing the proposed molecular processes by which CO influences mitochondrial respiration and glycolytic flux.* Findings from the literature and this report suggest that CO can uncouple mitochondrial respiration through: (1) activation of UCP and/or ANT, the inner mitochondrial membrane anion transporters that disrupt ATP synthesis, releasing heat and causing a compensatory increase in oxygen consumption [16]; (2) stimulation of P_iC activity thus increasing the matrix concentration of phosphate and protons [17] and (3) activation of the mitochondrial large-conductance calcium-regulated potassium channel (mitoBK$_{Ca}$). Activation of mitoBK$_{Ca}$ channel-dependent K^+ influx increases matrix volume of mitochondria, but the effect is balanced by the K^+/H^+ antiporter, which expels K^+ from the matrix and induces an influx of H^+ [41]. The active, methylated form of phosphofructokinase/fructose biphosphatase 3 (PFKFB3) increases the level of fructose 2,6-bisphosphate (F-2,6-BP), an allosteric activator of phosphofructokinase-1 (PFK1), resulting in activation of PFK1 and an increase in glycolytic flux, together with a decrease in pentose phosphate pathway (PPP). CO can suppress the activity of cystathionine β-synthase (CBS) leading to a decrease in the level of the methylated form of PFKFB3. As a result, the glycolytic flux is decreased and glucose is shifted towards the PPP. The scheme depicting the role of CO in inhibiting CBS and regulating glycolysis and the PPP is based on findings by Yamamoto et al. [30].

signaling pathway inducing a change in mitochondrial membrane potential [25] and reactive oxygen species production [42] as it was shown with using NS1619, an activator of the mitoBK$_{Ca}$ channel. We also demonstrated that CO induced an increase in non-mitochondrial respiration indicating additional cellular pathways that consume oxygen that may be targeted by CO. However, the identity of these pathways remains at present unknown.

It was interesting to observe in our experiments that the increase in respiration exerted by CO was associated with inhibition of glycolysis. Similar findings previously reported by Wegiel and colleagues [20] demonstrated that CO accelerated oxidative metabolism and ROS generation together with a decrease in glucose metabolism in cancer cells. At first glance these results could suggest a shift in energy metabolism from glycolysis to oxidative phosphorylation, but this hypothesis is not supported by our data showing a decrease in ATP-linked respiration induced by CO during the mitochondrial function assay. Moreover, paxilline prevented the increase in oxygen consumption by CO but did not change the slowdown of glycolic flux induced by CORM-401, suggesting that the inhibition of glycolysis by CO is independent of its effect on oxygen consumption and the activation of mitoBK$_{Ca}$ channels. This idea was further corroborated in additional experiments in which we stabilized glycolysis using the protease inhibitor MG132, which maintains PFKFB3 in the methylated form. Methylation of PFKFB3 increases glycolysis, as shown recently by Yamamoto et al. [30] and confirmed in our study by a decrease in OCR and an increase in ECAR during a mitochondrial functional assay and a glycolysis stress test after incubation with MG132. Interestingly, under these conditions we observed that the effect of CO on ECAR was abolished, while the increase in respiration and proton leak induced by CO was not affected. These data obtained using two separate pharmacological approaches strongly support the idea that the activation of mitochondrial respiration and the inhibition of glycolysis by CORM-401 are two independent cellular events, most likely occurring *via* the participation of different pathways and mechanisms.

Our results may have implications in the context of various physiological and pathological changes in which alterations in endothelial metabolism are linked to endothelial phenotype (reviewed by Goveia et al. [43]). For example, repression of glycolysis induced by laminar shear-stress at the level of PFKFB3 *via* KLF2 seems to be a determinant of quiescent endothelial phenotype [44]. In addition, pulmonary arterial hypertension was associated with a 3-folds increase in glycolysis in the pulmonary endothelium [45]. Interestingly, a robust activation of PFKFB3-glycolysis by migrating vascular tip cells was required for angiogenic sprouting, whereas PFKFB3 silencing impairs tip cells activity [46]. In view of our findings, it would be interesting to determine which of the above mentioned phenomena might be modulated by CO due to its effect on glycolysis.

In conclusion, we demonstrated that CO released from CORM-401 induced a mitoBK$_{Ca}$ channel-dependent uncoupling of mitochondrial respiration and a mitoBK$_{Ca}$ channel-independent repression of glycolysis. To our knowledge, we also showed for the first time that CO can reactivate the mitoBK$_{Ca}$ channel in low-calcium conditions. Our results suggest a signaling significance of CO in the metabolic reprogramming of the endothelium, which may have implications for the activation of

a vasoprotective phenotype of the endothelium or the inhibition of pathological angiogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2015.07.004.

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