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# **A carbon monoxide-releasing molecule (CORM-3) uncouples mitochondrial respiration and modulates the production of reactive oxygen species**

**Luisa Lo Iacono<sup>1</sup>, Jorge Boczkowski<sup>2</sup>, Roland Zini<sup>3</sup>, Issam Salouage<sup>3</sup>, Alain Berdeaux<sup>3</sup>,  
Roberto Motterlini<sup>1,3#\*</sup> and Didier Morin<sup>3#</sup>**

<sup>1</sup>*Department of Drug Discovery and Development, Italian Institute of Technology, 16163 Genoa, Italy*

<sup>2</sup>*Inserm, U955 equipe 4 and <sup>3</sup>Inserm, U955 équipe 3, Université Paris Est, Faculté de Médecine, 94010  
Creteil, France*

<sup>#</sup>These authors equally contributed to the work

\* To whom correspondence should be addressed: Department of Drug Discovery and Development,  
Italian Institute of Technology, Via Morego 30, 16146 Genoa, Italy. Tel (+39) 010 71781547; Fax (+39)  
010 71781228; E-mail: [roberto.motterlini@iit.it](mailto:roberto.motterlini@iit.it).

Running title: Uncoupling activities by CO in mitochondria

## **Abstract**

Carbon monoxide (CO), produced during the degradation of heme by the enzyme heme oxygenase, is an important signaling mediator in mammalian cells. Here we show that precise delivery of CO to isolated heart mitochondria using a water-soluble CO-releasing molecule (CORM-3) uncouples respiration. Addition of low micromolar concentrations of CORM-3 (1-20  $\mu\text{M}$ ), but not an inactive compound that does not release CO (iCORM-3), significantly increased mitochondrial oxygen consumption rate (state 2 respiration) in a concentration-dependent manner. In contrast, higher concentrations of CORM-3 (100  $\mu\text{M}$ ) suppressed ADP-dependent respiration through inhibition of cytochrome c oxidase. The uncoupling effect mediated by CORM-3 was inhibited in the presence of the CO scavenger myoglobin. Moreover, this effect was associated with a gradual decrease in membrane potential over time ( $\Delta\psi/\text{min}$ ) and was partially reversed by malonate, an inhibitor of complex II activity. Similarly, inhibition of uncoupling proteins or blockade of adenine nucleotide transporters attenuated the effect of CORM-3 on both state 2 respiration and  $\Delta\psi$ . Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) produced by mitochondria respiring from complex I-linked substrates (pyruvate/malate) was increased by CORM-3. However, respiration initiated via complex II using succinate resulted in a 5-fold increase in  $\text{H}_2\text{O}_2$  production and this effect was significantly inhibited by CORM-3. These findings disclose a counterintuitive action of CORM-3 suggesting that CO at low levels acts as an important regulator of mitochondrial respiration.

**Keywords:** Carbon monoxide; CO-releasing molecules; mitochondrial respiration; uncoupling agents; reactive oxygen species

## Introduction

The toxic effects of carbon monoxide (CO) in eukaryotic organisms are manifested through its high affinity for the reduced iron-heme in hemoglobin, which is essential for O<sub>2</sub> delivery to tissues [1]. Typically, a significant and persistent increase in blood carbonmonoxy hemoglobin to concentrations of 30% and above following CO gas inhalation indicates a life-threatening condition [2]. Despite the lack of precise tissue biomarkers to assess CO poisoning in humans, it is known that CO can compete with O<sub>2</sub> for the binding to the heme of mitochondrial cytochrome c oxidase, thus becoming potentially hazardous for cellular respiration [3,4]. However, the perception that CO can exert only negative effects is challenged by reports showing strong cytoprotective and anti-oxidant activities by heme oxygenase-1 (HO-1), an inducible stress enzyme that endogenously degrades heme to CO in mammals [5-7]. The use of low doses of CO gas in pre-clinical experimental models of disease has also produced remarkable data highlighting its therapeutic properties in alleviating inflammatory processes and cardiovascular disorders [5,8]. It is apparent that the concentration of CO at the level of its targets is a key factor for producing its beneficial effects. Unfortunately, the delivery of an effective and precise dose of CO gas to the molecular target(s) in classical *in vitro* and *ex-vivo* experimental models is not easy to achieve. In this context an important progress was made by the development of CO-releasing molecules (CO-RMs), a class of compounds that deliver controlled amounts of CO to biological systems [8-12]. The use of CO-RMs has further corroborated the vasodilatory, anti-inflammatory and anti-ischemic effects of CO, opening promising perspectives for the therapeutic potentials of this gas. The notion that mitochondria may serve as important targets in transducing the beneficial signaling properties of CO has been proposed [4] and recent studies indicate that increased mitochondrial biogenesis is part of the mechanisms by which CO gas and CO-RMs exert protective effects against cardiomyopathy and cardiac dysfunction in sepsis [13,14]. However, the direct and specific effects of low concentrations of CO on mitochondrial bioenergetics and respiration have been so far poorly investigated.

Cellular respiration coupled with the production of ATP occurs in mitochondria when electrons from the oxidation of nutrients are sequentially transferred to molecular O<sub>2</sub> by a series of proteins known as the electron transport chain (ETC), thus leading to a trans-membrane difference of electrochemical potential harnessed by ATP synthase to generate ATP. It is known that mitochondrial oxidative metabolism in aerobic cells is accompanied by the generation of reactive oxygen species (ROS) which, if properly controlled, play an important role as ubiquitous mediators in post-translational signaling and regulation of gene expression [15]. However, persistent increased production of ROS is highly damaging to cellular components and represents the common denominator in the progression of several metabolic and aging related diseases [16]. Notably, mitochondria are the main source of cellular ROS during pathological conditions; this is because electrons accumulated in the ETC as result of disrupted metabolism can leak out primarily through a reverse electron flow from complex I and/or directly from complex III [15,17]. These electrons are then transferred directly to O<sub>2</sub> to generate superoxide and other potent oxidants such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical.

An inherent cellular mechanism to counteract the disrupted oxidative metabolism is mild mitochondrial uncoupling, which alters the efficiency of mitochondrial respiration and dampen ROS production by reducing reverse electron flow and superoxide generation [18]. Mitochondrial uncoupling essentially drives an energy-dissipating proton cycle across the mitochondrial inner membrane resulting in a compensatory increase in O<sub>2</sub> consumption that is not accompanied by production of ATP [19]. This can be achieved through various mechanisms including activation of adenine nucleotide transporters (ANT) and uncoupling proteins (UCPs), which have been shown to be crucial for cell survival and homeostasis in various disease states [20-24]. Here we report that low micromolar concentrations of CO, delivered to isolated heart mitochondria by a water-soluble CO-RM, uncouple mitochondrial respiration consequently modulating both ROS production and bioenergetic parameters. The importance of these counterintuitive data will be discussed in the context of the emerging findings on the cardioprotective actions exerted by CO.

## Material and Methods

*Reagents.* [Ru(CO)<sub>3</sub>Cl(glycinate)] (CORM-3) and its inactive counterpart [Ru(DMSO)<sub>4</sub>Cl<sub>2</sub>] (iCORM-3) were synthesized as previously described [11]. Depleted CORM-3, which does not release CO to myoglobin, was also prepared as previously described by placing CORM-3 in phosphate buffer solution for two days and then bubbling N<sub>2</sub> gas to remove the residual solubilised CO [10]. Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) was purchased from Invitrogen (Cergy-Pontoise, France). Guanosine 5' diphosphate (GDP), carboxyatractyloside (Catr), 5-hydroxydecanoate (5-OH-Dec), carbonyl cyanide p-trifluoro-methoxyphenyl-hydrazone (FCCP), oligomycin and all other reagents were purchased from Sigma unless otherwise specified.

*Detection of CO release from CO-RMs.* Rates of CO release from CO-RMs were assessed by a myoglobin assay and an amperometric CO electrode as previously described by our group [9,25].

*Isolation of mitochondria from rat heart.* Male Wistar rats (250-300 g) obtained from Janvier (Le Genest-St-Isle, France) were used for heart mitochondrial preparation. Briefly, hearts were removed and immediately immersed in ice cold 0.9% NaCl. The myocardial tissue was homogenized using a Polytron homogenizer in cold buffer (4 °C, pH 7.4) containing: mannitol (220 mM), sucrose (70 mM), Hepes (10 mM), EGTA (1 mM), and free fatty acid bovine serum albumin (BSA, 2 mg/ml). The sample was further homogenized for 5 consecutive times using a Potter homogenizer at 1500 rev/min. The homogenate was then centrifuged at 1000 g for 5 min at 4°C (Sorvall RC 5C Plus) to remove tissue debris and nuclei and the supernatant centrifuged for 10 min at 10000 g. The final mitochondrial pellet was resuspended in homogenization buffer without EGTA and BSA. The protein content was determined by the method of Lowry et al. [26]. Isolated mitochondria were kept on ice until assaying for mitochondrial respiration, change in membrane potential or production of reactive oxygen species (ROS).

*Measurement of mitochondrial respiration.* O<sub>2</sub> consumption was measured by a Clark-type oxygen microelectrode equipped with a water jacket thermostatically set at 30°C and fitted to an oxygen monitoring system (Hansatech). Prior to use, the electrode was calibrated with air-saturated water, assuming an O<sub>2</sub> concentration of 235 μM. Samples were placed in a gas-tight chamber and continuously stirred. Isolated mitochondria (0.4 mg/ml) were added to 500 μl respiration buffer [sucrose (50 mM), KCl (100 mM), Hepes (10 mM), KH<sub>2</sub>PO<sub>4</sub> (5 mM), pH 7.4] and mitochondrial respiration (State 2) was initiated by the addition of pyruvate/malate (5/5 mM). After 1 min, ATP synthesis (State 3) was induced by the addition of ADP to a final concentration of 0.3 mM. CORM-3 or iCORM-3 were incubated for 1 min with mitochondria in the stirring chamber before the addition of the substrate. In experiments using myoglobin as a scavenger of CO, deoxygenated myoglobin (stock solution 10 mM) was firstly prepared by adding sodium dithionite (5.7 mM) in deoxygenated water and then an aliquot of 100 μl (final concentration of myoglobin = 20 μM) was added to the mitochondrial respiration medium (0.4 ml) prior addition of CORM-3. O<sub>2</sub> consumption was also measured in the presence of CO gas by bubbling 100 % of CO gas (Air Products, Paris, France) at 25°C for 15 minutes in order to obtain a saturated respiration buffer (960 μM) before studying respiration. The concentration of CO in solution was determined spectrophotometrically by measuring the conversion of deoxymyoglobin to carbon monoxymyoglobin (MbCO), as previously described [9]. The following parameters were evaluated: substrate-dependent respiration rate (State 2 or State 4, i.e. oxygen consumption in the absence of exogenous ADP); ADP-stimulated respiration rate (State 3, i.e. oxygen consumption during ADP phosphorylation).

*Measurement of mitochondrial membrane potential.* Mitochondrial membrane potential ( $\psi$ ) was evaluated by the uptake of the fluorescent dye rhodamine 123, which accumulates electrophoretically into energized mitochondria in response to their negative-inner membrane potential [27]. The respiratory substrates pyruvate/malate (5/5 mM) and rhodamine 123 (0.2 μM), in the presence or absence of 5-OH-

Dec (1 mM), Catr (4  $\mu$ M) or GDP (1 mM), were added to 1.2 ml of respiration buffer into the cuvette maintained at 30°C. Oligomycin (1  $\mu$ M), an inhibitor of ATPase activity, was also used in these studies. Fluorescence was monitored over time using a Perkin-Elmer SA LS 50B fluorescence spectrometer (excitation wavelength = 503 nm, emission wavelength = 527 nm). After 100 s of initial monitoring, mitochondria (0.4 mg/ml protein) were added to initiate respiration while the effect of CORM-3 or iCORM-3 (1, 20 or 100  $\mu$ M) on membrane potential was examined following addition of the compounds at t = 350 s. FCCP (1  $\mu$ M) was added at the end of the experiment to depolarize the mitochondrial membrane potential thus acting as internal reference.  $\Delta\psi$  was calculated by measuring the change in membrane potential for 1 min after addition of CORM-3 or iCORM-3. The slope of control curve was calculated at same time points.

*Measurement of reactive oxygen species (ROS) production.* ROS generation was assessed by measuring the rate of hydrogen peroxide ( $H_2O_2$ ) production. This was determined fluorometrically by the oxidation of Amplex Red to fluorescent resorufin, coupled to the enzymatic reduction of  $H_2O_2$  by horseradish peroxidase (HRP). Essentially, the superoxide generated in mitochondria is converted endogenously to  $H_2O_2$  and then measured by the assay. Briefly, Amplex Red (10  $\mu$ M) and HRP (1 U/ml) were added to isolated mitochondria (0.2 mg/ml protein) in respiration buffer maintained at 30°C. The reaction was initiated by addition of the respiratory substrates (pyruvate/malate or succinate, 5 mM) in the presence or absence of 1  $\mu$ M rotenone (inhibitor of complex I). The subsequent increase in fluorescence was monitored over time using a fluorescence spectrometer (Perkin-Elmer SA LS 50B, excitation wavelength=563 nm; emission wavelength=587 nm). The effect of CORM-3 or iCORM-3 (1, 20 or 100  $\mu$ M) on  $H_2O_2$  production was assessed when the compounds were added to the reaction mixture at t = 200s.  $\Delta H_2O_2$  was calculated by measuring the slope of the curve for 1min after CORM-3 or iCORM-3 addition. Calibration was performed by sequential addition of known concentrations of  $H_2O_2$  and was used to calculate the rate of  $H_2O_2$  generation in nmol/min/mg mitochondrial protein.

*Assessment of mitochondrial respiratory complex activities.* Mitochondrial complex I activity (NADH decylubiquinone oxidoreductase, NQR) was measured by monitoring the decrease in absorbance ( $\lambda = 340$  nm) at 30°C resulting from the oxidation of NADH. The incubation medium contained  $\text{KH}_2\text{PO}_4$  (25 mM),  $\text{MgCl}_2$  (5 mM), decylubiquinone (100  $\mu\text{M}$ ), KCN (250  $\mu\text{M}$ ), BSA (1 mg/ml) and 0.1 mg/ml of freeze-thawed heart mitochondria. The reaction was initiated by the addition of NADH (200  $\mu\text{M}$ ). The rotenone-sensitive NQR activity was tested in the presence of rotenone (5  $\mu\text{M}$ ). Other respiratory chain enzyme activities were measured according to Barrientos et al. [28]. Briefly, mitochondrial succinate cytochrome c reductase (complex II + complex III activity) was measured by monitoring the change in absorbance ( $\lambda = 550$  nm) resulting from the reduction of cytochrome c. The assay mixture contained  $\text{KH}_2\text{PO}_4$  (10 mM), EDTA (2 mM), rotenone (2  $\mu\text{M}$ ), succinate (6 mM), BSA (1 mg/ml), KCN (250  $\mu\text{M}$ ) and freeze-thawed heart mitochondria (0.03 mg/ml). After an incubation period of 5 min, succinate cytochrome c reductase activity was measured in the presence of 50  $\mu\text{M}$  oxidized cytochrome c. Complex III (cytochrome c reductase) activity was measured as the rate of cytochrome c reduction at  $\lambda=550$  nm. The reaction mixture contained  $\text{KH}_2\text{PO}_4$  (10 mM), EDTA (2 mM), rotenone (2  $\mu\text{M}$ ), BSA (1 mg/ml), KCN (250  $\mu\text{M}$ ), freeze-thawed heart mitochondria (0.015 mg/ml) and reduced decylubiquinol (100  $\mu\text{M}$ ). The reaction was started by the addition of oxidized cytochrome c (50  $\mu\text{M}$ ). Mitochondrial complex IV activity (cytochrome c oxidase) was monitored following the decrease in absorbance ( $\lambda= 550$  nm) resulting from the oxidation of reduced cytochrome c (33  $\mu\text{M}$ ). The reaction medium contained  $\text{KH}_2\text{PO}_4$  (10 mM), EDTA (2 mM), rotenone (2  $\mu\text{M}$ ), BSA (1 mg/ml),  $\text{MgCl}_2$  (2 mM) and freeze-thawed heart mitochondria (0.015 mg/ml). CORM-3 was incubated for 1 min with mitochondria before the addition of the substrate required for the reaction. All absorbance measurements were performed using a Jasco V530 spectrophotometer. Complex activities were quantified by measuring the slope of the absorbance curves for the first 30 sec.

*Statistical analyses.* The data are reported as means  $\pm$  S.E.M. Differences between groups were analyzed using unpaired t-test or one-way analysis of variance followed by a Bonferroni's multiple comparison test when appropriate. The significance was accepted at  $p < 0.05$ .

## Results

***CORM-3 uncouples mitochondrial respiration at low micromolar concentrations.*** We first investigated the effect of different concentrations of CORM-3 (10 nM-100  $\mu$ M) on mitochondrial oxygen consumption rate in the absence (state 2 respiration) or in the presence (state 3 respiration) of exogenous ADP. Contrary to the common conception that CO is exclusively an inhibitor of respiration, we found that low concentrations of CORM-3 (2-50  $\mu$ M) elicit a concentration-dependent increase in oxygen consumption during state 2 respiration (Fig. 1A and 1B). This increase was maximal at approximately 20  $\mu$ M CORM-3 and was associated with a decrease in state 3 respiration rate (Fig. 1C and 1D). This is indicative of an uncoupling effect mediated by this compound. Higher concentrations of CORM-3 (50-100  $\mu$ M) exerted a concentration-dependent decrease in state 2 respiration (Fig. 1A and 1B). In contrast, 1-100  $\mu$ M CORM-3 caused a concentration-dependent inhibition of oxygen consumption in the presence of ADP (state 3 respiration), which is markedly pronounced at 100  $\mu$ M (Fig. 1C). Notably, the inactive form of CORM-3 (iCORM-3), which does not contain carbonyl groups and thus does not liberate CO, did not affect mitochondrial oxygen consumption neither during state 2 nor state 3 respiration (Fig. 1B and 1D). Identical results were obtained with depleted CORM-3 (data not shown), which is unable to release CO to myoglobin [10,29]. Moreover, we found that the increase in mitochondrial state 2 respiration by CORM-3 (20  $\mu$ M) is blocked by the presence of myoglobin, a potent CO scavenger (Figure 2A). Altogether these results, which were also repeated in purified mitochondria prepared as previously described [30] (data not shown), confirm that both the uncoupling effect and the inhibition of mitochondrial respiration observed at different concentrations of CORM-3 are mediated by CO. In order to explore whether CO gas itself could mimic the uncoupling effects of CORM-3 on mitochondrial respiration, we conducted experiments with CO gas in saturated solution. Interestingly, with this approach the uncoupling effects of CO on isolated mitochondria were noticeable only when high micromolar concentrations of the gas were used at the beginning of our experiment (Fig. 2B). These results highlight once more the technical limitations of using CO gas and confirm the unique ability of CO-RMs in transferring CO effectively at low

concentrations to biological targets. In fact, while CO gas diffuses homogeneously into the media, CORM-3 is able to release CO only in the presence of an appropriate biological acceptor. This is supported by the data presented in Figure S1 whereby the spontaneous release of CO from CORM-3 in aqueous solution was assessed using a sensitive CO electrode [25] and compared with the amount of MbCO measured after addition of CORM-3 to a solution containing deoxymyoglobin [10]. CORM-A1, a boron-based compound known to liberate CO spontaneously into solution with a half-life of 21 min, was used as a positive control as previously described [25]. As shown, no signal is detected by the CO electrode in the presence of CORM-3 indicating that this compound in water solution does not liberate CO (Supplementary Fig. S1A); in contrast, in the presence of deoxymyoglobin (Supplementary Fig. S1B) CORM-3 rapidly transfers CO to the heme leading to myoglobin carbonylation and confirming our previous data on the fast rate of CO release from this compound [10]. We then analyzed the effect of CORM-3 on basal (state 2) respiration over time in comparison with p-trifluoro-methoxyphenylhydrazine (FCCP), a well-known classical uncoupler. The data shown in Figure 3 indicate that 20  $\mu$ M CORM-3 but not iCORM-3 significantly accelerates O<sub>2</sub> consumption and this begins smoothly reaching a steady state approximately 1 min after addition of CORM-3 to the mitochondrial suspension. In contrast, the increase in O<sub>2</sub> consumption after addition of FCCP at low (20 nM) and high (1  $\mu$ M) concentrations is abrupt. It has also to be noted that the presence of oligomycin, an inhibitor of ATPase, did not affect the uncoupling effect mediated by CORM-3 (Supplementary Fig. S2A). We further investigated whether CORM-3 could influence the kinetics of electron transfer activities in individual complexes. Our results show that neither CORM-3 nor i-CORM-3 was able to modify the activity of complex I, II or III (Supplementary Fig. S3A, S3B and S3C). Predictably, inhibition of complex IV (cytochrome c oxidase) was observed at the highest concentration of CORM-3 used (100  $\mu$ M, Supplementary Fig. S3D) and the fact that iCORM-3 was ineffective clearly demonstrates that the inhibition of complex IV is mediated by the release of CO. While the inhibition of complex IV can explain the reduction in state 3 respiration observed in Fig. 1C and 1D, it cannot be associated with the increase in state 2 respiration rate. Thus,

complex IV is not a candidate target for CO-mediated uncoupling activity. On the other hand, it should be noted that malonate (10 mM), an inhibitor of succinate dehydrogenase (complex II), was able to reduce significantly the uncoupling effect mediated by 20  $\mu$ M CORM-3 (Fig. 4A and 4B) suggesting a possible contribution of complex II in the uncoupling process. The presence of oligomycin did not modify the effect of malonate (Supplementary Fig. S2A).

***CORM-3 decreases mitochondrial membrane potential in a concentration- and time-dependent manner.*** Since perturbations of the electron transfer activity in the respiratory chain are accompanied by a variation in mitochondrial membrane potential ( $\psi$ ), we then determined how this parameter is affected by CORM-3 during respiration. As shown in Fig. 5A and 5B, addition of CORM-3 (1-100  $\mu$ M) to respiring mitochondria resulted in a concentration-dependent decrease in membrane potential (i.e. increase in the fluorescence signal). While the change in membrane potential occurred gradually over time at concentrations of CORM-3 that mediate an uncoupling effect (1-20  $\mu$ M, Fig. 5A), a rapid and maximal discharge of the potential was obtained at the highest concentration used (100  $\mu$ M). In this case, the subsequent addition of the strong uncoupler carbonyl cyanide p-trifluoro-methoxyphenyl-hydrazone (FCCP, 1  $\mu$ M) induced no further decrease in membrane potential (data not shown). It is interesting to note that addition of 1  $\mu$ M FCCP alone to respiring mitochondria caused, an abrupt and marked decrease in membrane potential (Figure 5A). However, lower concentrations of FCCP (20 nM) exerted a sudden drop in membrane potential and then a progressive uncoupling effect similar to that produced by 20  $\mu$ M CORM-3. The negative control iCORM-3 (100  $\mu$ M) did not alter the respiratory membrane potential (Figure 5A and 5B), confirming once again the critical role of CO in this process. As in the case of respiration, the presence of oligomycin in the mitochondrial suspension did not affect the change in membrane potential mediated by CORM-3 (Supplementary Fig. S2B).

***Inhibition of UCPs and ANT reverses CORM-3-mediated uncoupling effect.*** To better understand the molecular mechanisms underlying the uncoupling effect of CO, we tested the possibility that CORM-3 could function as a protonophore, i.e. creating an intramembrane pore that enables protons to cross the lipid bilayers as in the case of the classical and strong uncoupling agent FCCP. We found that while the effect induced by FCCP (1  $\mu$ M) was almost fully reverted by 6-ketocholestanol (6-KTC, 400  $\mu$ M), CORM-3-mediated uncoupling activity was not affected by 6-KTC (Supplementary Fig. S4). Therefore, we next tested a set of known inhibitors of uncoupling respiration and assessed their effects in the presence of CORM-3. These compounds included: guanosine 5' diphosphate (GDP, 1 mM), an inhibitor of the uncoupling proteins family (UCPs); carboxyatractyloside (Catr, 4  $\mu$ M), an inhibitor of the adenine nucleotide transporter (ANT); and 5-hydroxydecanoate (5-OH-Dec, 1 mM), an inhibitor of mitochondrial ATP-dependent potassium channels. We found that both GDP and Catr, but not 5-OH-Dec, were able to reduce partially the increase in state 2 respiration mediated by 20  $\mu$ M CORM-3 (Fig. 6A). Interestingly, when the two drugs were combined (GDP+Catr), the reduction was more pronounced (Fig. 6A). To a similar extent, and in agreement with these data, we also found that GDP and Catr significantly attenuated the change in membrane potential ( $\psi$ ) exerted by CORM-3 (Fig. 6B and Fig. 6B *inset*). Also, the presence of the ATPase inhibitor oligomycin (1  $\mu$ M) in the incubation medium did not modify the effect of UCP and ANT inhibitors (data not shown). To exclude the possibility that CORM-3 could act by opening the mitochondrial permeability transition pore (MPTP), the well-known inhibitor cyclosporine A was used in the presence of CORM-3 but no effect on  $\psi$  was observed (data not shown). These results suggest that both UCPs and ANT partially contribute to the uncoupling properties of CORM-3.

***CORM-3-mediated uncoupling is associated with modulation of mitochondrial ROS production.***

Because CO has been shown to favor the generation of reactive oxygen species (ROS) from mitochondria [31] and mild uncoupling is assumed to prevent mitochondrial ROS formation [32], we next studied the effect of CORM-3 on the rate of ROS produced under different respiratory conditions. Mitochondrial

ROS can be produced by the ETC through electron leak at complexes I and III [19]. First, we analyzed the effect of CORM-3 on the rate of hydrogen peroxide production ( $\Delta\text{H}_2\text{O}_2$ ) when the electron transfer is physiologically initiated by addition of pyruvate/malate to isolated mitochondria. We found that CORM-3, but not iCORM-3, increases  $\Delta\text{H}_2\text{O}_2$  in a concentration-dependent manner (Fig. 7A). This result confirms that CO, via inhibition of complex IV, is able to promote electron leakage and ROS production at the level of complex III [31]. In a second experiment we tested the effect of CORM-3 on  $\Delta\text{H}_2\text{O}_2$  in conditions that stimulates ROS production mainly at the level of complex I. Such condition can be achieved by feeding the electron transfer chain via complex II with the addition of succinate. In contrast to the low basal rate of ROS production observed when the electron transfer is initiated by the addition of pyruvate through complex I ( $0.12\pm 0.01$  nmol  $\text{H}_2\text{O}_2$ /min/mg proteins), mitochondria produce a much higher amount of  $\text{H}_2\text{O}_2$  when electrons are donated to complex II via succinate oxidation ( $0.48\pm 0.03$  nmol  $\text{H}_2\text{O}_2$ /min/mg proteins). This dramatic increase in the basal rate of ROS is known to originate from complex I following reverse electron transfer at the level of coenzyme Q and is also known to be sensitive to a decrease in membrane potential [17,33]. We reasoned that CORM-3, thanks to its ability to uncouple mitochondria and decrease the proton electromotive force, could reduce reverse electron flow and thus ROS production at the level of complex I. Our results showed that CORM-3 is indeed capable of reducing  $\text{H}_2\text{O}_2$  in succinate-driven respiration (Fig. 7B), while iCORM-3 was again without effect. Such effect is already pronounced at low concentrations of CORM-3 (20  $\mu\text{M}$ ) being consistent with the uncoupling effects observed. To further demonstrate the ability of CO to reduce ROS production at the level of complex I, the effect of CORM-3 on succinate-driven electron transfer was tested in the presence of rotenone. As expected, this inhibitor of complex I decreased the rate of  $\text{H}_2\text{O}_2$  production to basal conditions and restored the effect of CORM-3 as a promoter of ROS generation similarly to the effect observed with pyruvate/malate-driven electron transfer (Fig. 7C). These important results suggest that CO acts both as a stimulus for the generation of physiologically relevant ROS in mitochondria and as an inhibitor in situations where excessive ROS are produced by these organelles. This dual activity opens

intriguing perspectives for the therapeutic exploitations of CO since its beneficial effects may strictly depend on the conditions and metabolic requirements of the cell.

## Discussion

The strong binding affinity of CO to mitochondrial cytochrome c oxidase, and the consequent impairment in O<sub>2</sub> consumption is substantiated experimentally only in the case of high millimolar levels of CO (i.e. CO poisoning) [2]. Very little is known about the effect of CO concentrations that might reflect more closely the amounts generated physiologically during the degradation of heme by heme oxygenase enzymes [1,2]. In this context the advent of CO-releasing molecules (CO-RMs) offers a useful tool to assess the precise effect of small quantities of CO on cellular respiration and mitochondrial activity [8,34,35]. In fact, contrary to CO gas that is distributed homogeneously in the mitochondrial suspension and can easily escape into the atmosphere, CO-RMs are able to shuttle with high efficiency molecules of CO to their targets, allowing a precise control of the concentration used, even at very low levels. In addition, the use of CO-RMs can easily overcome the problem related to atmospheric oxygen, which in *in vitro* and *ex-vivo* preparation becomes a serious obstacle for defining the effect of low concentrations of CO gas. Here we used a well characterized water-soluble CO releaser (CORM-3) to confirm that low micromolar concentrations of CO are able to uncouple mitochondrial respiration [34]. Three major findings originating from our experiments support this conclusion. First, CORM-3 in a concentration-dependent manner (1-20 μM) weakens the coupling between oxidation and ATP production as it accelerates substrate-dependent O<sub>2</sub> consumption (state 2) and concomitantly decreases ADP-dependent respiration (state 3). The direct contribution of CO liberated from CORM-3 on this effect appears to be plausible as uncoupling was not elicited by an inactive compound (iCORM-3) and the scavenger of CO myoglobin added to the mitochondrial suspension inhibited CORM-3-mediated increase in respiration. Second, CORM-3 decreases mitochondrial membrane potential at concentrations which do not inhibit cytochrome c oxidase. Third, the CO-mediated effects were attenuated by pharmacological agents known to inhibit mitochondrial uncoupling. The lack of effects on mitochondrial activities by iCORM-3, an inactive compound that does not liberate CO [11,34], substantiated the direct role of CO on the observed uncoupling mechanism. On the other hand CO gas alone was able to produce mitochondrial uncoupling

only when a saturated solution (960  $\mu\text{M}$ ) was used at the beginning of the experiment. At this concentration, CO gas should completely block respiration but evidently equilibration with atmospheric  $\text{O}_2$  occurs very rapidly so that the pharmacological effect of a given concentration of CO gas in open *in vitro* systems cannot be defined precisely. Thanks to the unique properties of CO-RMs to transfer CO to biological targets *in vitro* we were able to uncover an unprecedented uncoupling activity of low doses of CO even under conditions of high  $\text{O}_2$  levels (21%).

The uncoupling properties of CO with the consequent effects on mitochondrial respiration are counterintuitive but might have important implications for deciphering the exact pharmacological actions and the therapeutic potentials of this gas [8,34]. Interestingly, mitochondrial uncoupling has been proposed as an effective way to reduce body weight in humans [36]. Most compounds described so far as uncouplers act as protonophores either by inducing a cycling of protons across the inner mitochondrial membrane (2,4-dinitrophenol) or by binding to mitochondrial proteins involved in the uncoupling effect (FCCP or FCCP-derivatives) [37]. In the context of the present study, it is highly improbable that CO can act directly as carrier of protons although we cannot exclude a priori that CORM-3 may undergo a protonation/deprotonation cycling within mitochondria. It is also unlikely that CO or CORM-3 are protonophores since 6-KTC, which is known to abolish the uncoupling of respiration mediated by potent artificial protonophores [38], was without effect in mitochondria treated with CORM-3. However, we cannot exclude this possibility since the protonophoric uncoupler 2,4-dinitrophenol is not inhibited by 6-KTC [37]. What is interesting to point out is that CO behaves as a rather unusual uncoupler. In fact, unlike classic uncoupling agents which all elicit an abrupt decrease in membrane potential when applied to isolated mitochondria (see  $\psi$  in the presence of FCCP 20 nM and 1  $\mu\text{M}$ , Figure 5A, line f and g, respectively), we found that reductions in  $\psi$  caused by CORM-3 are not only concentration-dependent but occur gradually over time, indicating that prolonged mild uncoupling maybe achieved from this new class of molecules at low micromolar concentrations (see traces b, c and d, Figure 5A). Mild mitochondrial uncoupling is emerging as an intrinsic compensatory mechanism adopted by cells not only to restore

altered metabolism in diseases such as cancer and diabetes but also as a ubiquitous process to delay age-related disease and increase longevity [19]. Whether dynamic changes in mitochondrial membrane potential by sustained production of HO-1-derived CO in cells can result in mild uncoupling remain to be investigated.

One plausible mechanism by which CO, either directly or indirectly, would uncouple mitochondrial respiration is through activation of uncoupling proteins (UCPs) and/or the adenine nucleotide transporter (ANT). UCPs are inner mitochondrial membrane anion transporters that disrupt ATP synthesis, releasing heat and causing a compensatory increase in O<sub>2</sub> consumption [20]. Both UCP2 and UCP3 are expressed in cardiac mitochondria and their uncoupling activity is increased by free fatty acids and inhibited by purine nucleotides (such as GDP) [39]. Similarly, ANT, which exchange ADP for ATP during oxidative phosphorylation, is known to catalyze the basal proton conductance of mitochondria [21] and its activation mediates the uncoupling process induced by several agents such as 2,4-dinitrophenol or free fatty acids [40]. Our data demonstrating that GDP partially attenuates the uncoupling effect of CORM-3 suggest the involvement of UCPs activity in the observed uncoupling mechanism mediated by CO. In addition, one of the most effective and widely used inhibitors of ANT (Catr) also limited the increase in respiration rate and reduced the drop in  $\Psi$  level induced by CORM-3 in isolated mitochondria. Notably, inhibition of the two proteins using a combination of GDP and Catr almost completely suppressed CORM-3-mediated increase in mitochondrial state 2 respiration suggesting that both ANT and UCP operate concomitantly in the uncoupling process exerted by CO. In support of these findings are recent reports showing that ANT activity is enhanced by CO in non-synaptic mitochondria and that increased HO-1 expression by gene delivery in aortas results in enhanced expression of ADP/ATP and deoxynucleotide carrier proteins [41,42].

Another interesting result emerging from our study is that malonate, a well-known inhibitor of complex II activity (succinate dehydrogenase or SDH), also significantly reverses the increase in state 2 respiration by CORM-3 indicating a possible contribution of this complex in the concerted mechanism(s)

of CO-mediated mitochondrial uncoupling. Due to its unique redox properties, and in partnership with ubiquinone, SDH activity maintains a high reduction state of the ubiquinone pool which in turns improves the anti-oxidant capacity of mitochondria, and thus the resistance to oxidative stress [43]. Although we did not find any change in the activity of isolated complex II following treatment with CORM-3, further work should be undertaken to verify whether a direct effect of CO on this complex uncouples mitochondria in respiring cells. In this context, circumstantial evidence on how mitochondrial function is affected by smoking in human circulating lymphocytes is rather instructive. In fact, compared to non-smokers, lymphocytes isolated from cigarette smokers displayed a significant decrease in complex IV activity, while oxidative rate with succinate and thus SDH activity were significantly enhanced. In addition, intact cell respiration was higher in smokers compared to non-smokers [44]. It is also intriguing that structural and kinetics studies performed on activators of purified SDH identified, among other compounds, the prototypic uncoupling agent 2,4-dinitrophenol [45].

The observed uncoupling effects mediated by CORM-3 have obvious consequences on the increased production of mitochondrial ROS [17]. It is known that complex I-driven ROS generation is sensitive to variation of membrane potential and is therefore susceptible to a decrease in the presence of uncoupling agents such as 2,4-dinitrophenol or ADP [17,19,46]. In the case of CORM-3 we found that 20  $\mu$ M induces a slight increase in H<sub>2</sub>O<sub>2</sub> production in isolated mitochondria respiring from complex I-linked substrates (pyruvate/malate) and this effect was more pronounced at 100  $\mu$ M, the concentration needed for cytochrome C oxidase inhibition. However, a totally opposite outcome can be observed when succinate is used as substrate for mitochondrial respiration. It is known that mitochondria respiring on succinate, the substrate for complex II, produce much more superoxide than mitochondria fed with pyruvate due to reverse electron transport into complex I; thus, superoxide production during reverse electron transport is greater than during forward electron transport. Although the mechanisms and physiological relevance of this phenomenon is unknown, evidence indicates that the level of succinate increases sharply in pathological conditions associated with cardiac ischemic injury and oxidative stress

[47,48]. Our results confirm first of all that succinate-driven mitochondrial respiration generates approximately 5 times more  $H_2O_2$  than in the presence of pyruvate/malate and that under these conditions CORM-3 is capable of markedly reducing the excessive production of  $H_2O_2$ . Notably, the effect was already significant at 20  $\mu M$  CORM-3, consistent with the low concentrations of compound required for exerting uncoupling activities. Inhibition of complex I by rotenone decreased the rate of  $H_2O_2$  production to basal conditions and restored the ability of CORM-3 to generate ROS in a similar fashion to that observed during pyruvate/malate-driven respiration. This demonstrates the specific effect of CORM-3 in reducing complex I-derived ROS. Recent data reveal that both CO-RMs and CO gas in different cell types promote a transient and subtle increase in mitochondrial ROS production [31,49,50], a signaling process that might well justify the emerging cardioprotective properties of CO. In fact, CO and CO-RMs effectively mitigate myocardial ischemia-reperfusion injury, cardiac allograft rejection, cardiomyopathy and heart failure through mechanisms that appear to involve the preservation of mitochondrial function, bioenergetics and cardiac metabolism [10,13,14,51].

Taken together, our data demonstrate that CORM-3, through the liberation of CO, represents a novel regulator of mitochondrial respiration which in addition to fatty acids, thyroid and steroid hormones could play a crucial role in those pathological conditions where strategies aimed at targeting mitochondrial uncoupling and metabolism are developed for therapeutic interventions.

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**List of abbreviations:**

ANT = adenine nucleotide transporter

BSA = bovine serum albumin

Catr = carboxyatractyloside

CO-RMs = CO-releasing molecules

CORM-3 = Ru(CO)<sub>3</sub>Cl(glycinate)

$\psi$  = mitochondrial membrane potential

ETC = electron transport chain

FCCP = carbonyl cyanide p-trifluoro-methoxyphenyl-hydrazone

GDP = guanosine 5' diphosphate

H<sub>2</sub>O<sub>2</sub> = hydrogen peroxide

HRP = horseradish peroxidase

iCORM-3 = [Ru(DMSO)<sub>4</sub>Cl<sub>2</sub>]

6-KTC = 6-ketocholestanol

NQR = NADH decylubiquinone oxidoreductase

5-OH-Dec = 5-hydroxydecanoate

ROS = reactive oxygen species

SDH = succinate dehydrogenase

UCPs = uncoupling proteins

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## Figure Legends

**Figure 1. CORM-3 is an uncoupling agent at low micromolar concentrations.** Oxygen consumption rate in the absence of ADP (state 2 respiration) was recorded **(A)** and quantified **(B)** in isolated heart mitochondria pre-incubated (1 min) with increasing concentrations of CORM-3 (1, 20 and 100  $\mu\text{M}$ ) or its inactive form iCORM-3 used as negative control. Similarly, oxygen consumption rate in the presence of ADP (state 3 respiration) was also recorded **(C)** and quantified **(D)** as reported in the *Experimental Procedures*. CORM-3 increases state 2 respiration, which is maximal at 20  $\mu\text{M}$ , while decreasing state 3 in a concentration-dependent manner. Data are expressed as percentage of control (untreated mitochondria). Each bar represents the mean $\pm$ S.E.M. of 6 independent experiments. Data were analyzed with one-way ANOVA followed by Bonferroni's multiple comparison post-hoc adjustment (\*\* $p < 0.001$ ).

**Figure 2. CO liberated from CORM-3 and CO gas in solution uncouple mitochondrial respiration.** **(A)** Basal respiration rate (state 2 respiration) was measured in isolated heart mitochondria in the presence of CORM-3 (20  $\mu\text{M}$ ), deoxy-myoglobin (Mb, 20  $\mu\text{M}$ ) or a combination of CORM-3 plus Mb (see Materials and Methods for details). Data are expressed as percentage of control. Each bar represents the mean $\pm$ S.E.M. of 5 independent experiments. Symbols indicate: \* $p < 0.05$  with unpaired t-test. **(B)** Basal respiration rate (state 2 respiration, *left panel*) as well as ADP-dependent respiration (state 3 respiration, *right panel*) were measured in isolated heart mitochondria in a solution saturated with CO gas (960  $\mu\text{M}$ ) at the beginning of the experiment. As shown, CO is able to significantly increase state 2 and decrease state 3 respiration compared to control (untreated mitochondria). Data are expressed as percentage of control. Each bar represents the mean $\pm$ S.E.M. of 6 independent experiments. Symbols indicate: \* $p < 0.05$  with unpaired t-test.

**Figure 3. Time-dependent effect of uncoupling concentrations of CORM-3 on basal respiration rate: comparison with the uncoupling agent FCCP.** Basal respiration rate (state 2) was recorded in isolated heart mitochondria. After 1 min of incubation, different compounds were added in independent experiments as indicated by the arrow: line a, 20  $\mu$ M CORM-3; line b, 20  $\mu$ M iCORM-3; line c, 20 nM FCCP; line d, 1  $\mu$ M FCCP. Numbers next to the traces indicate the rate of O<sub>2</sub> consumption (nmol/min/mg proteins) calculated from the slope of the curve.

**Figure 4. Malonate, an inhibitor of complex II activity, partially inhibits the uncoupling effect of CORM-3.** Mitochondrial basal respiration rate (state 2 respiration, **A**) as well as ADP-dependent respiration (state 3 respiration, **B**) were measured in isolated heart mitochondria in the presence of malonate (10 mM), CORM-3 (20  $\mu$ M) or CORM-3 + malonate. As shown, CORM-3-dependent increase in state 2 respiration and decrease in state 3 respiration are significantly reverted in the presence of malonate. Data are expressed as percentage of control (untreated mitochondria). Each bar represents the mean $\pm$ S.E.M. of 6 independent experiments. Symbols indicate: \* $p$ <0.05 compared to CORM-3 with unpaired t-test.

**Figure 5. Effect of different concentrations of CORM-3 on mitochondrial membrane potential. (A)** Membrane potential ( $\psi$ ) in isolated heart mitochondria treated with CORM-3 were recorded over time by measuring the uptake/release of the fluorescent dye rhodamine 123 (see *Experimental Procedures* for details). The following compounds and concentrations were tested: 100  $\mu$ M iCORM-3 (negative control, *line a*); 1  $\mu$ M CORM-3 (*line b*); 20  $\mu$ M CORM-3 (*line c*); 30  $\mu$ M CORM-3 (*line d*); 100  $\mu$ M CORM-3 (*line e*). Carbonyl cyanide p-trifluoro-methoxyphenyl-hydrazone (FCCP), a well-known uncoupling agent, was used for comparison (*line f*, 20 nM; *line g*, 1  $\mu$ M). **(B)** Changes in membrane potential over time ( $\Delta\psi$ /min) were quantified in isolated mitochondria treated with different concentrations of CORM-3 and compared to control (untreated mitochondria). Each bar represents the mean $\pm$ S.E.M. of 5 independent

experiments. Data were analyzed with one-way ANOVA followed by Bonferroni's multiple comparison post-hoc adjustment. Symbols indicate: \* $p < 0.05$  and \*\*\* $p < 0.001$  compared to control.

**Figure 6. Inhibitory effects of GDP and Catr on the uncoupling activity of CORM-3.** **(A)** The effect of CORM-3 (20  $\mu\text{M}$ ) on ADP-independent oxygen consumption rate (state 2 respiration) was measured in isolated heart mitochondria in the presence of the following inhibitors of respiratory uncoupling: guanosine 5' diphosphate (GDP, 1 mM), carboxyatractyloside (Catr, 4  $\mu\text{M}$ ), and 5-hydroxydecanoate (5-OH-Dec, 1 mM). GDP and Catr, but not 5-OH-Dec partially inhibit CORM-3-dependent increase in state 2. Data are expressed as percentage of control (untreated mitochondria). Each bar represents the mean  $\pm$  S.E.M. of at least 5 independent experiments. **(B)** Membrane potential ( $\psi$ ) in isolated mitochondria treated with CORM-3 (20  $\mu\text{M}$ ) were recorded over time in the presence of different uncoupling inhibitors. The following conditions were analyzed: untreated mitochondria (control, *line a*); CORM-3 + 1 mM GDP (*line b*); CORM-3 + 4  $\mu\text{M}$  Catr (*line c*); CORM-3 (*line d*); CORM-3 + 1 mM 5-OH-Dec (*line e*); CORM-3 + 10 mM malonate (not shown, see the inset). Changes in membrane potential over time ( $\Delta\psi/\text{min}$ ) were then quantified for the correspondent experiments (see the inset). Each bar represents the mean  $\pm$  S.E.M. of at least 3 independent experiments. Symbols indicate: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to CORM-3-treated mitochondria with unpaired t-test.

**Figure 7. CORM-3 modulates hydrogen peroxide production.** The rate of hydrogen peroxide production over time ( $\Delta\text{H}_2\text{O}_2$ ) was quantified in isolated heart mitochondria treated with different concentrations of CORM-3 or iCORM-3 (negative control) by measuring the oxidation of Amplex Red to the fluorescent dye resorufin (see *Experimental Procedures* for details).  $\text{H}_2\text{O}_2$  production was initiated by feeding the electron transfer chain via complex I with the addition of pyruvate/malate (**panel A**), via complex II with the addition of succinate (**panel B**), or via complex II with the addition of succinate in the presence of rotenone, a strong inhibitor of complex I (**panel C**). A schematic diagram for each

condition is shown. Data are presented as percentage of control (untreated mitochondria). Control values correspond to  $0.12 \pm 0.01$ ,  $0.48 \pm 0.03$  and  $0.19 \pm 0.04$  nmol  $H_2O_2$ /min/mg proteins in Figure 7A, 7B and 7C, respectively. Each bar represents the mean  $\pm$  S.E.M. of 3 independent experiments. Data were analyzed with one-way ANOVA followed by Bonferroni's multiple comparison post-hoc adjustment. Symbols indicate: \* $p < 0.05$  and \*\* $p < 0.01$ .

**Figure S1. Detection of CO release from CORM-3 and CORM-A1: comparison between CO electrode and myoglobin assay. (A)** Typical tracings showing the current measured by an amperometric CO electrode immersed in an aqueous solution at  $37^\circ C$  upon addition of  $100 \mu M$  CORM-3 (solid line) or CORM-A1 (dotted line). Note that while CO is gradually detected over time from CORM-A1, no CO is measured after addition of CORM-3. **(B)** The amount of carbonmonoxy myoglobin (MbCO) formed over time at  $37^\circ C$  was measured after addition of  $50 \mu M$  CORM-A1 (open bars) or CORM-3 (closed bars) to a solution containing  $50 \mu M$  deoxymyoglobin. Note that the release of CORM-3 is much faster (half-life  $< 1$  min) compared to CORM-A1 (half-life  $\cong 21$  min). Bars represents the mean  $\pm$  S.E.M. of three experiments.

**Figure S2. Blockade of F1F0ATPase does not affect the uncoupling activity of CORM-3 alone or in the presence of malonate. (A)** Mitochondrial basal respiration rate (state 2) was measured in isolated heart mitochondria in the absence or in the presence of  $1 \mu M$  oligomycin, an inhibitor of F1F0ATPase. The following conditions were analyzed: untreated mitochondria (pyruvate/malate  $2.5$  mM); iCORM-3 ( $20 \mu M$ ); CORM-3 ( $20 \mu M$ ); CORM-3 + malonate ( $10$  mM). **(B)** Changes in membrane potential were recorded over time ( $\Delta\psi$ /min) in isolated mitochondria and quantified in the following conditions: control (pyruvate/malate  $2.5$  mM); iCORM-3 ( $20 \mu M$ ); CORM-3 ( $20 \mu M$ ); CORM-3 + oligomycin ( $1 \mu M$ ). Each bar represents the mean  $\pm$  S.E.M. of at least 4 independent experiments. Symbols indicate: \*\* $p < 0.01$  and \*\*\* $p < 0.001$  with unpaired t-test.

**Figure S3. Effect of CORM-3 on the electron transfer activity of mitochondrial complexes.** The activity of respiratory complex I (A), complex II+III (B), complex III (C) and complex IV (D) was measured spectrophotometrically in isolated heart mitochondria in the absence or presence of different concentrations of CORM-3 (20 and 100  $\mu$ M) or its inactive form iCORM-3 (100  $\mu$ M). Data are calculated as the slope of the absorbance curve and are expressed as percentage of control. Each bar represents the mean $\pm$ S.E.M. of 6 independent experiments. Symbols indicate: \*\*\*  $p < 0.001$  compared to iCORM-3 with unpaired t-test.

**Figure S4. 6-ketocholestanol (6-KTC) does not affect the uncoupling activity of CORM-3.** Mitochondrial basal respiratory rate (state 2) was measured in isolated heart mitochondria in the presence of respectively: FCCP (1  $\mu$ M), FCCP + 6-KTC (400  $\mu$ M), CORM-3 (20  $\mu$ M), and CORM-3 + 6-KTC (400  $\mu$ M). While 6-KTC blocks FCCP uncoupling activity, it does not affect CORM-3-mediated increase in state 2. Data are expressed as percentage of control (untreated mitochondria). Each bar represents the mean $\pm$ S.E.M. of 4 independent experiments. Symbols indicate: \* $p < 0.01$  with unpaired t-test.

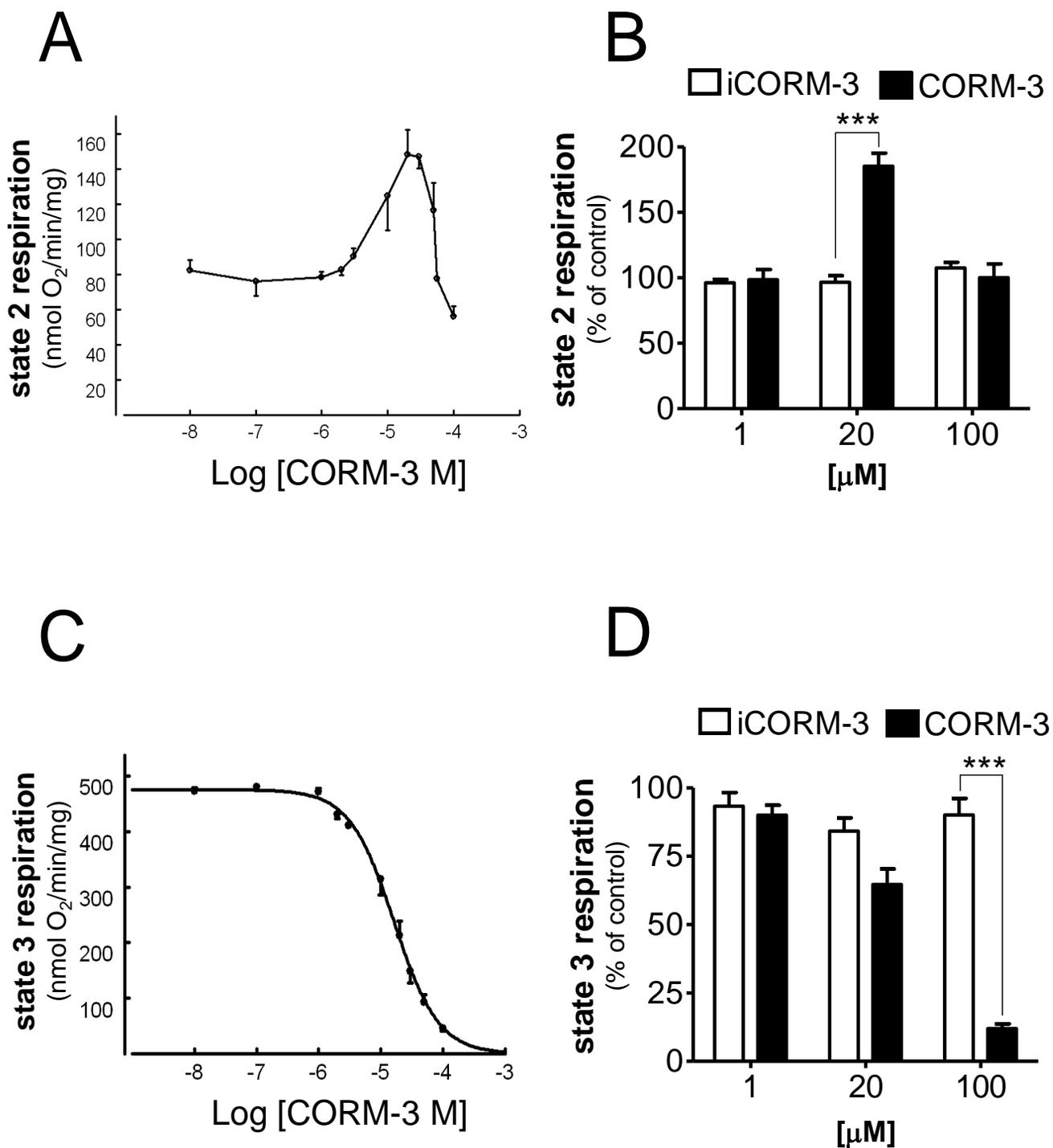
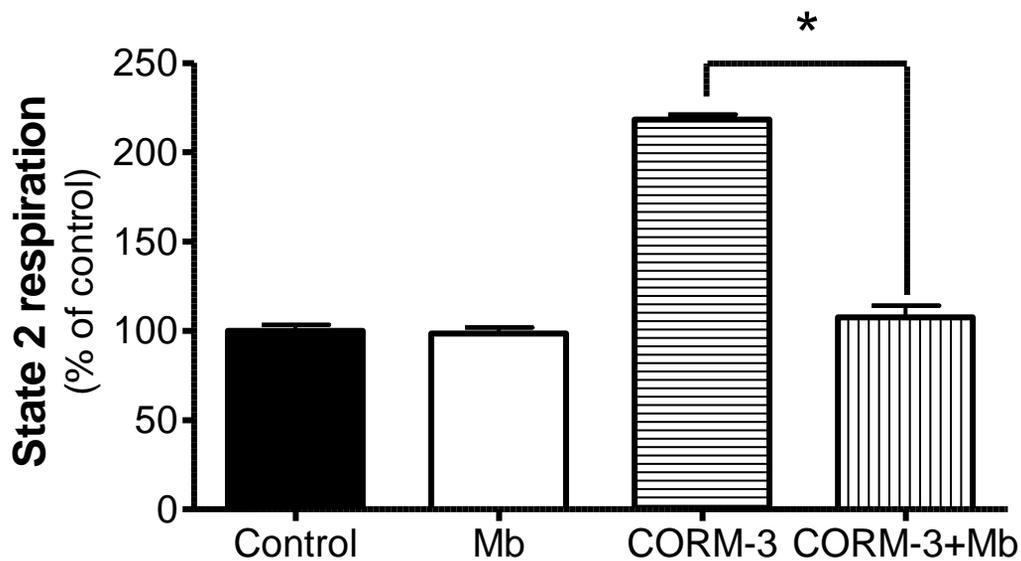


Figure 2

A



B

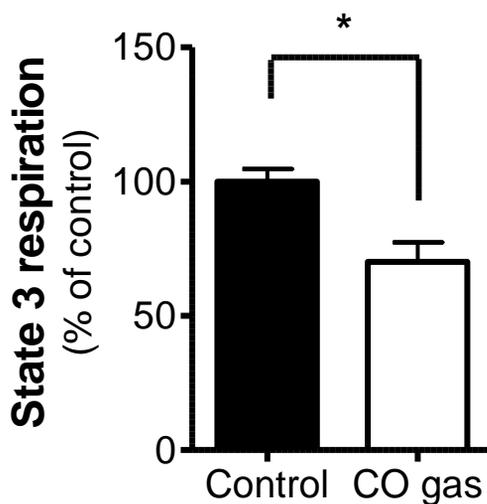
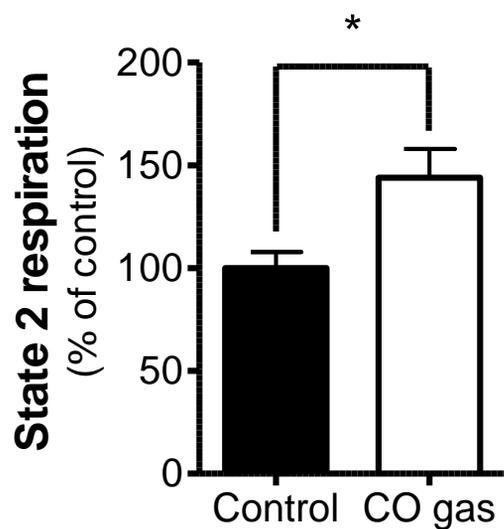


Figure 3

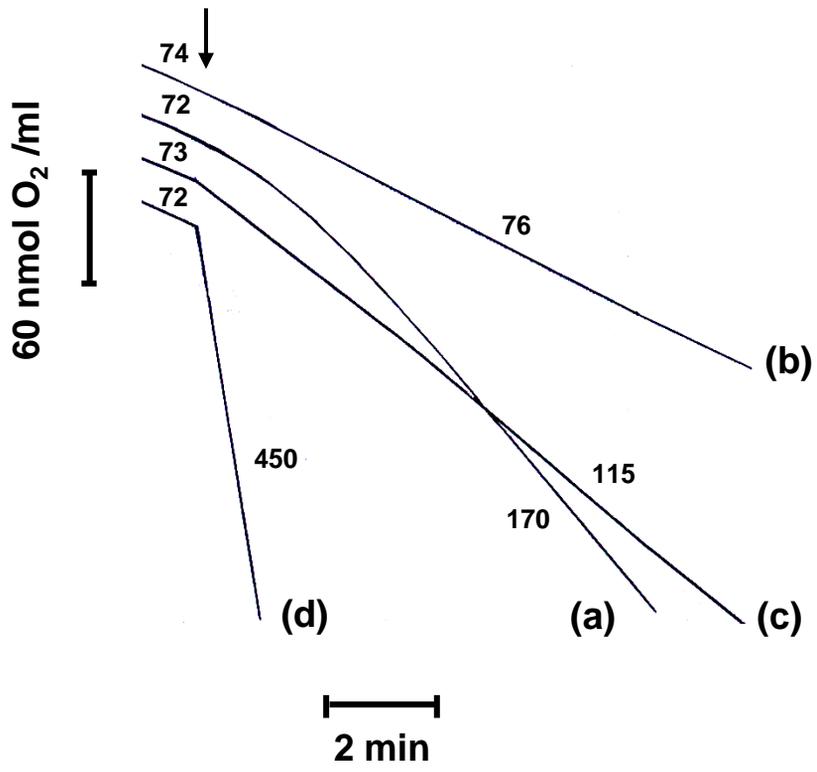
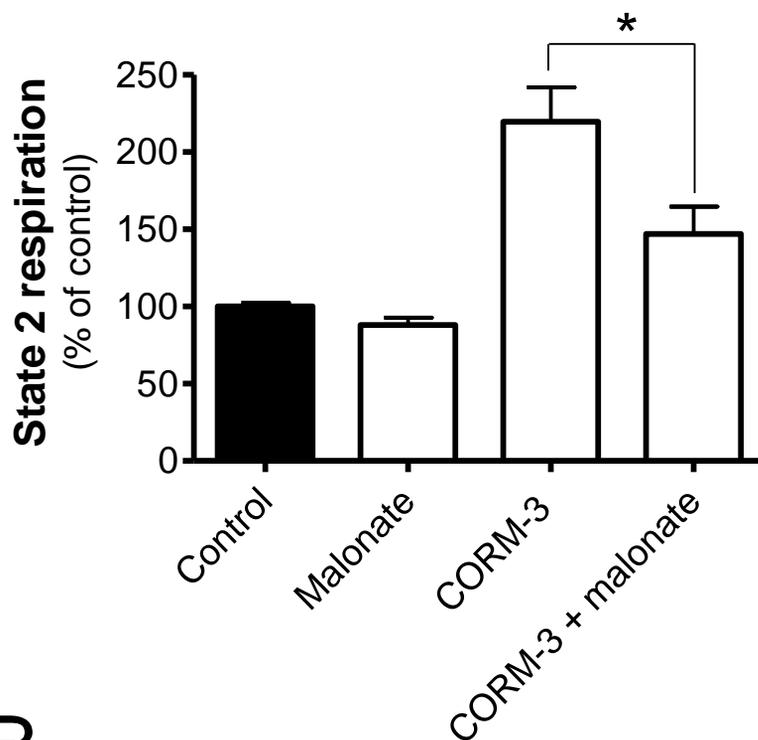


Figure 4

**A**



**B**

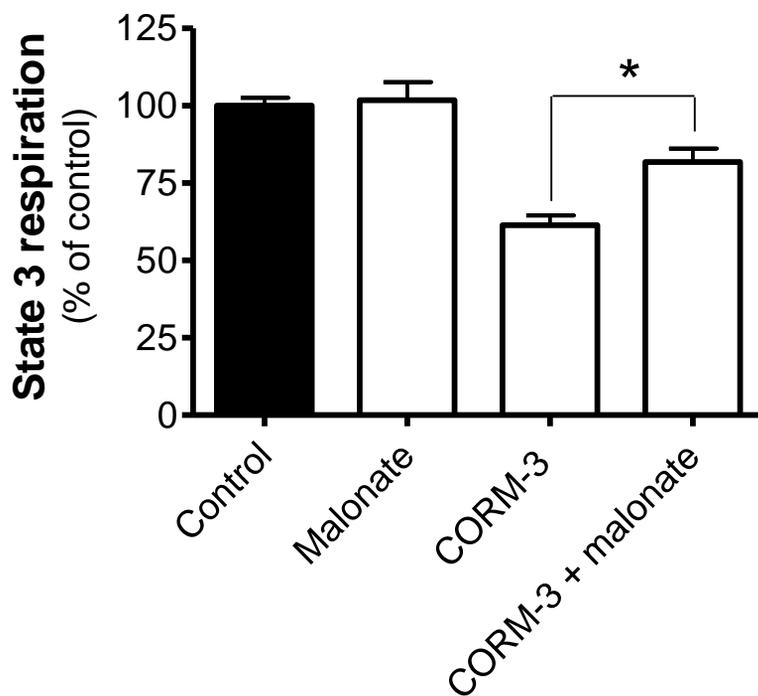
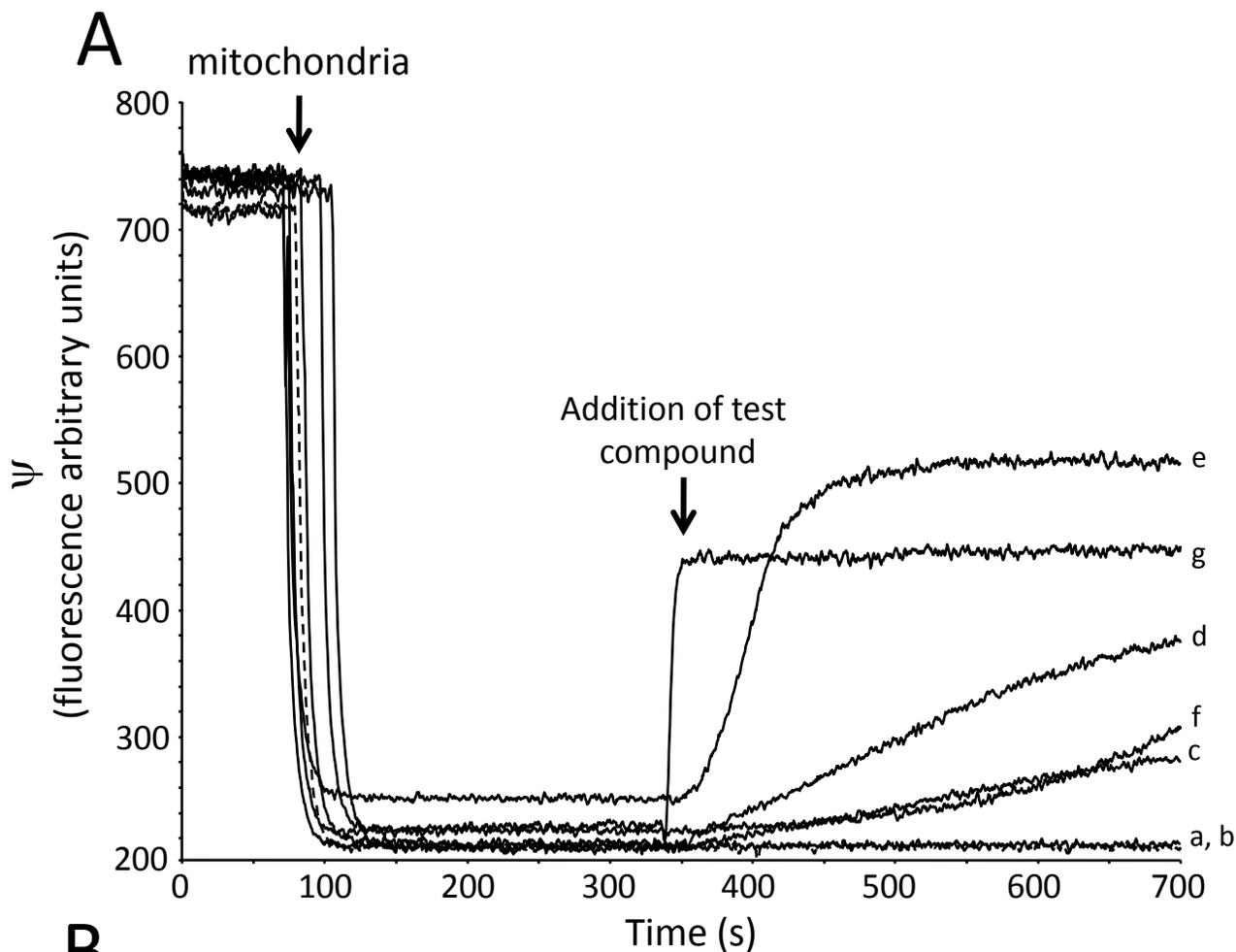


Figure 5



**B**

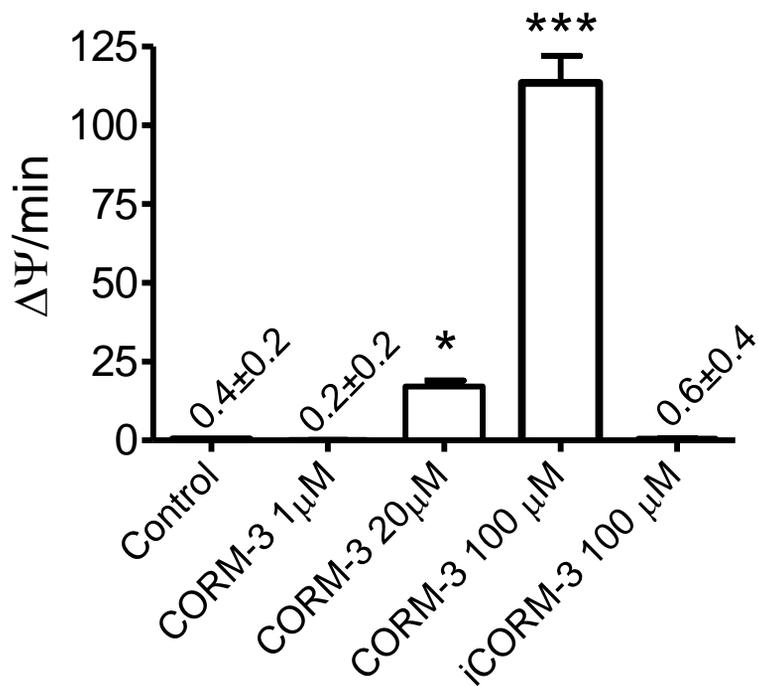
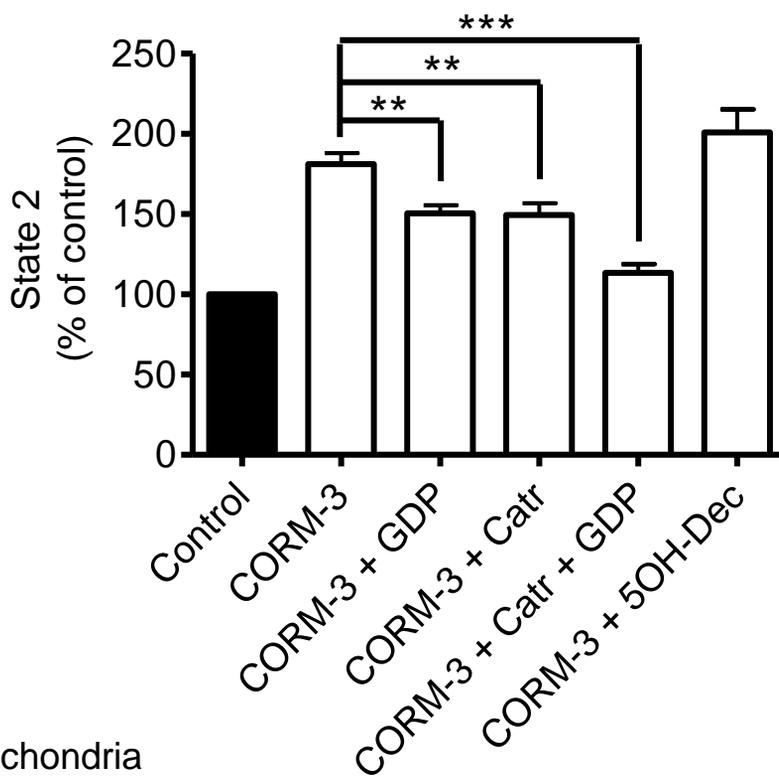


Figure 6

**A**



**B**

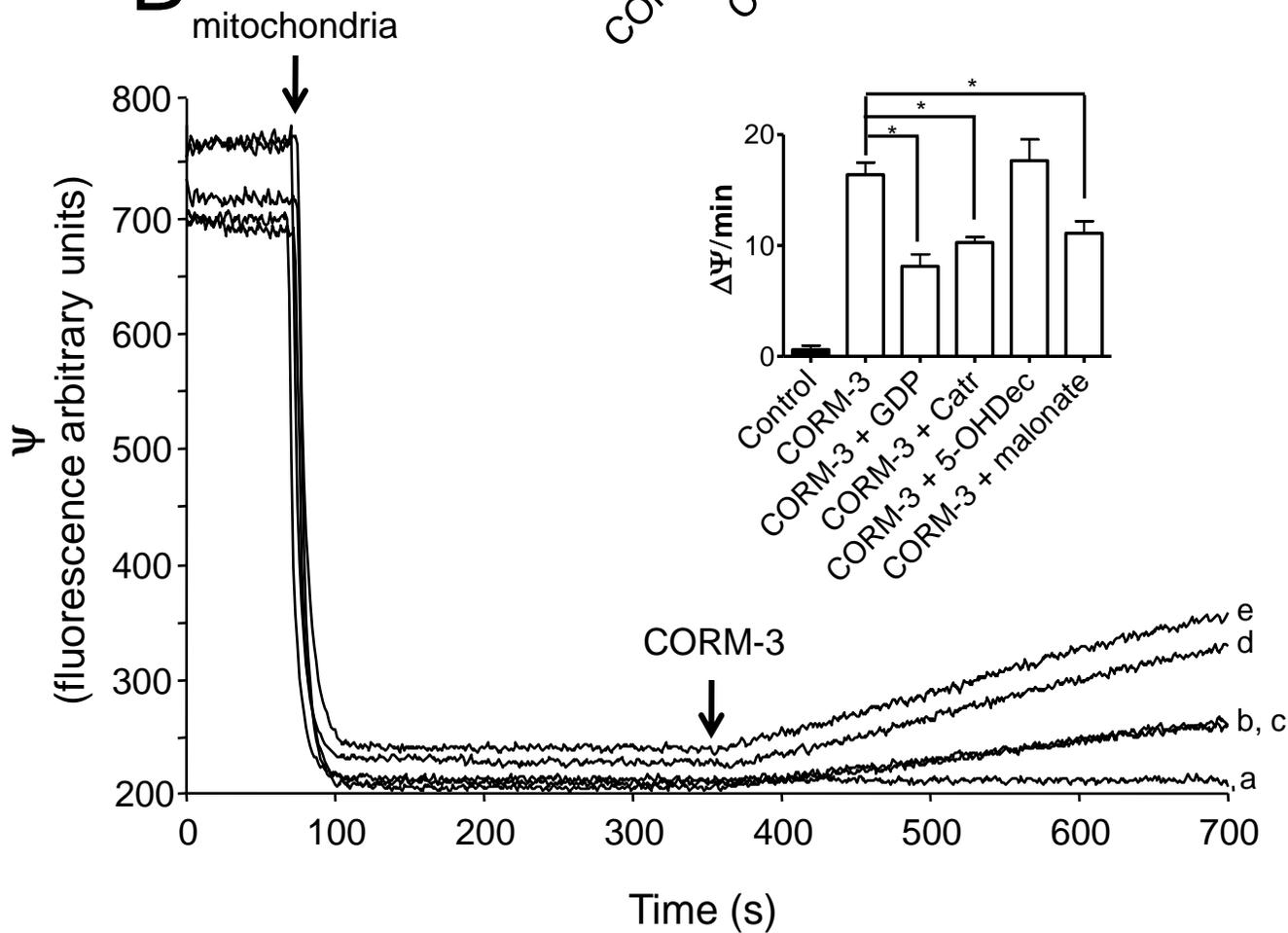
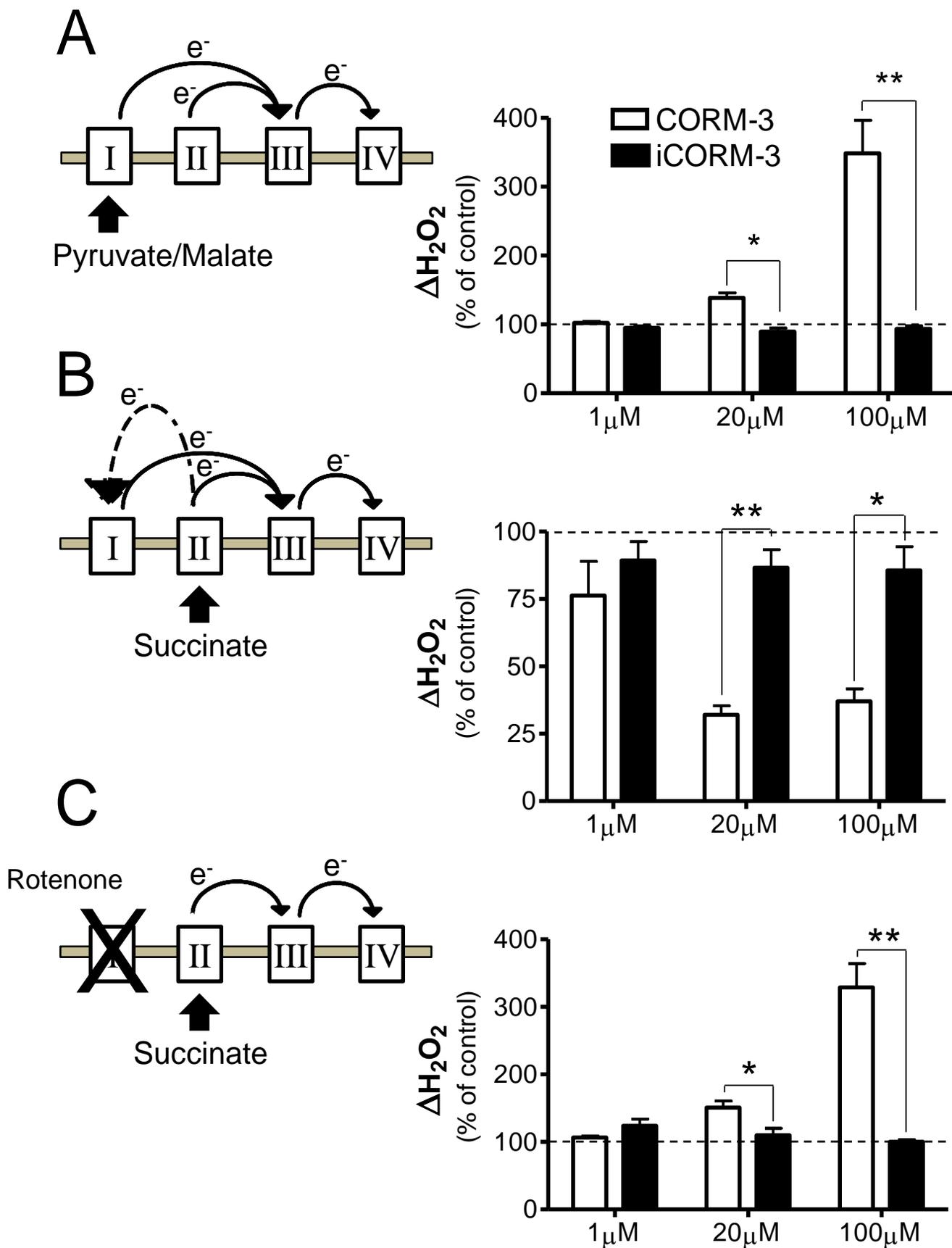
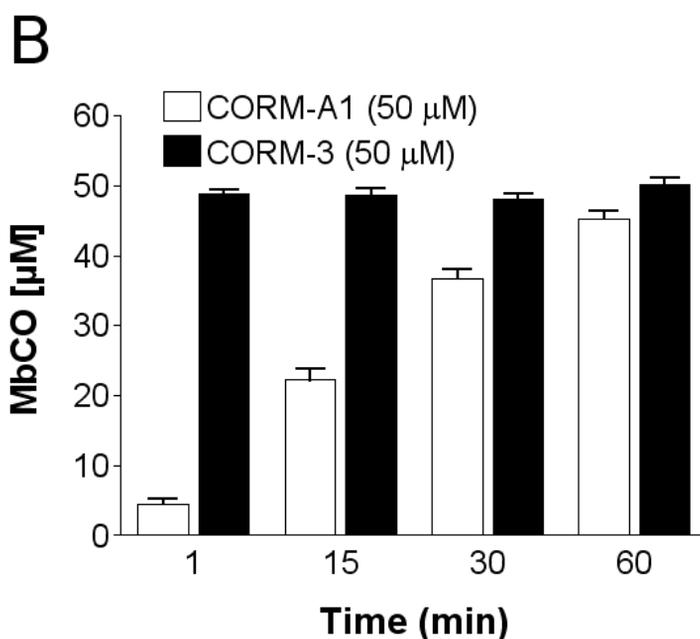
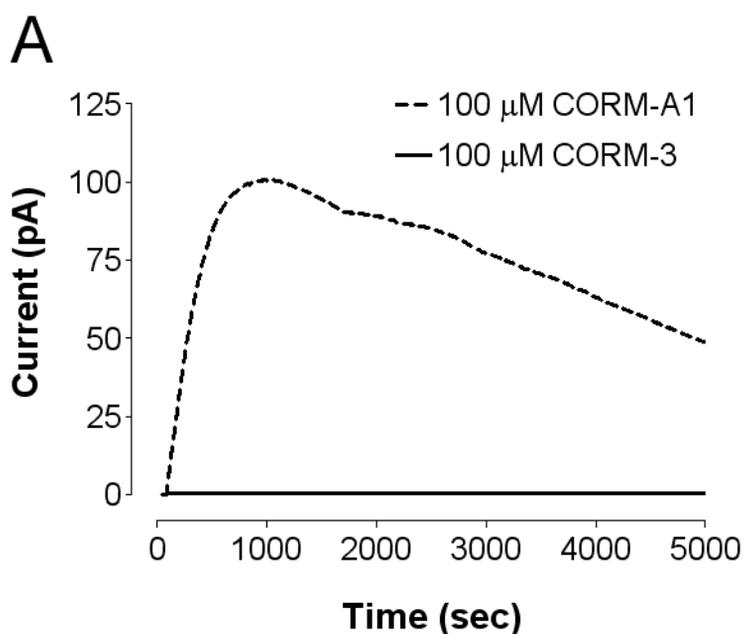
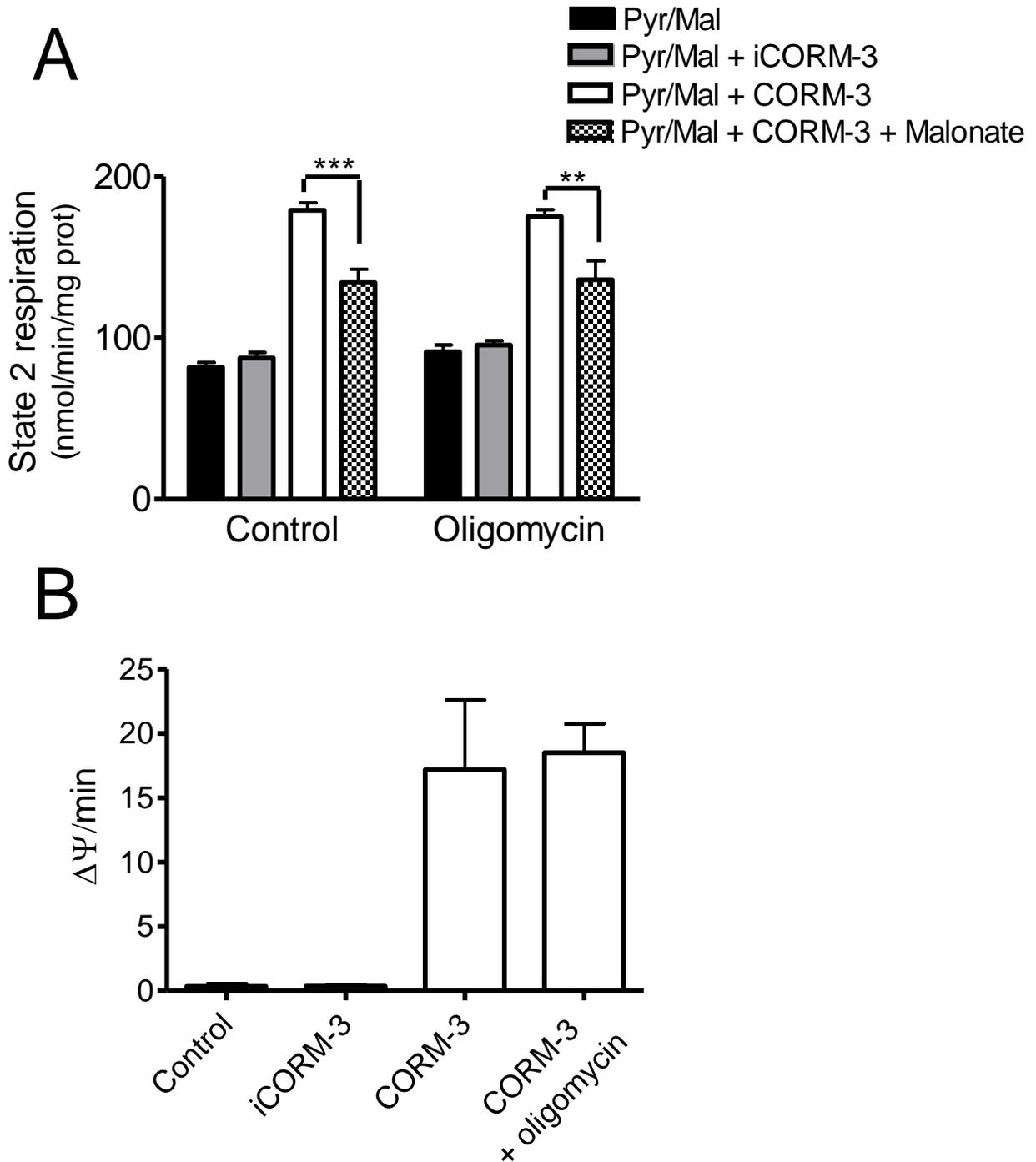


Figure 7

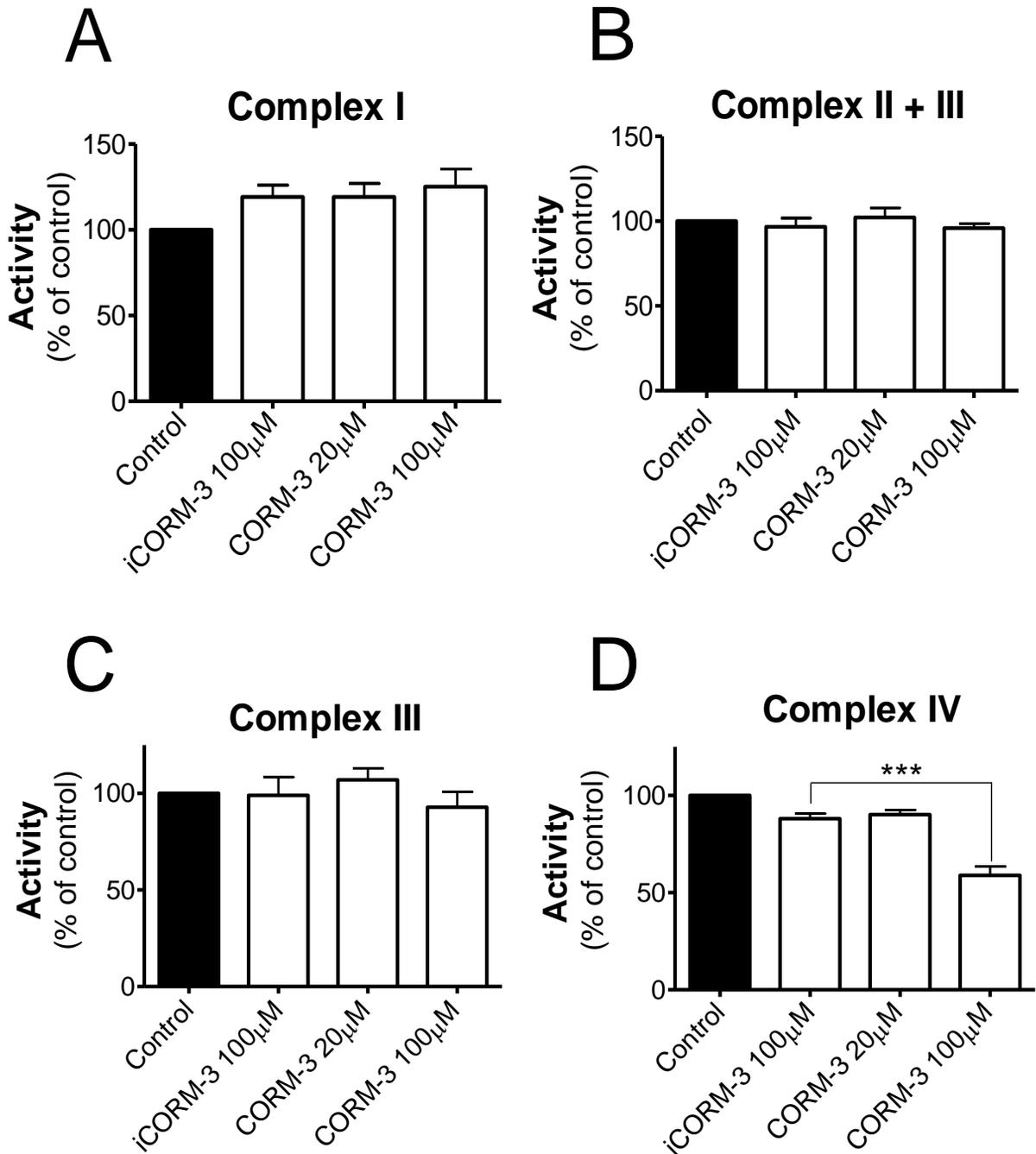




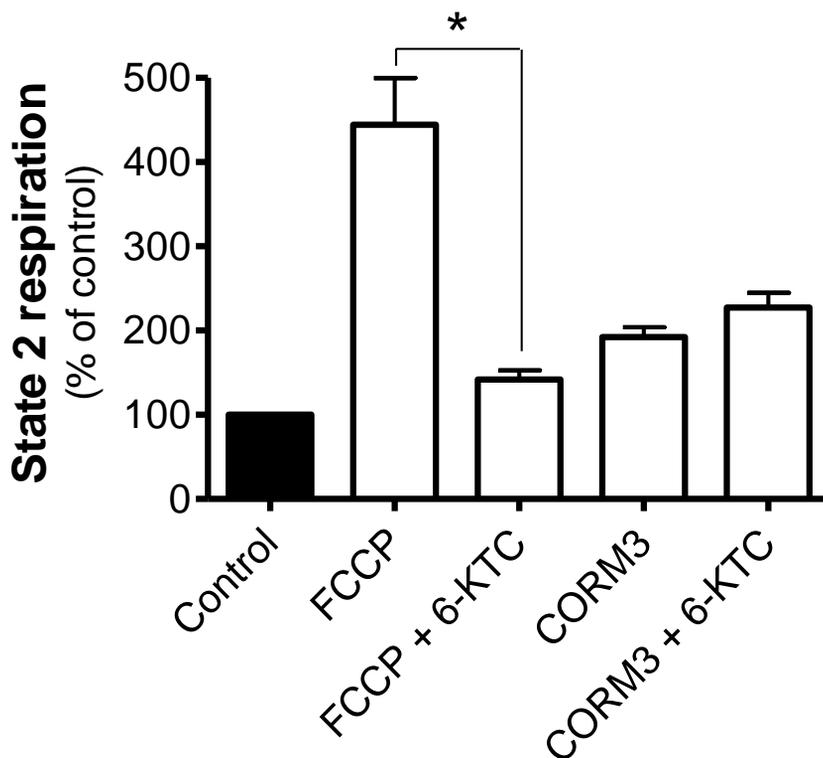
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