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CORM-3, a water soluble CO-releasing molecule, uncouples mitochondrial respiration via interaction with the phosphate carrier

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Abbreviations: CO, carbon monoxide; CO-RMs, CO-releasing molecules; CORM-3, Ru(CO)3Cl(glycinate); iCORM-3, inactive CORM-3; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; pHi, intramitochondrial pH; mPTP, mitochondrial permeability transition pore; PiC, phosphate carrier; DIC, dicarboxylate carrier; NEM, N-ethylmaleimide.

Abstract

Carbon monoxide is continuously produced in small quantities in tissues and is an important signaling mediator in mammalian cells. We previously demonstrated that CO delivered to isolated rat heart mitochondria using a water-soluble CO-releasing molecule (CORM-3) is able to uncouple mitochondrial respiration. The aim of this study was to explore more in depth the mechanism(s) of this uncoupling effect. We found that acceleration of mitochondrial O_2 consumption and decrease in membrane potential induced by CORM-3 were associated with an increase in mitochondrial swelling. This effect was independent of the opening of the mitochondrial transition pore as cyclosporine A was unable to prevent it. Interestingly, removal of phosphate from the incubation medium suppressed the effects mediated by CORM-3. Blockade of the dicarboxylate carrier, which exchange dicarboxylate for phosphate, decreased the effects induced by CORM-3 while direct inhibition of the phosphate carrier with N-ethylmaleimide completely abolished the effects of CORM-3. In addition, CORM-3 was able to enhance the transport of phosphate into mitochondria as evidenced by changes in mitochondrial phosphate concentration and mitochondrial swelling that evaluates the activity of the phosphate carrier in de-energized conditions. These results indicate that CORM-3 activates the phosphate carrier leading to an increase in phosphate and proton transport inside mitochondria, both of which could contribute to the non-classical uncoupling effect mediated by CORM-3. The dicarboxylate carrier amplifies this effect by increasing intra-mitochondrial phosphate concentration.

1. Introduction

Carbon monoxide (CO) is well-known for its poisonous effects when inhaled at high concentrations and for prolonged times in eukaryotic organisms. Its high affinity for heme makes it a powerful competitor of molecular oxygen (O_2) at the level of both haemoglobin and mitochondrial cytochrome c oxidase, which are essential for O_2 supply and cellular respiration, respectively. The toxic properties of CO are counterbalanced by its intrinsic cell signalling function since CO is continuously produced in small quantities in tissues, regulates several physiological processes and exerts beneficial effects against pathophysiological conditions [1,2,3]. Corroborating data obtained using preclinical models of diseases showed that administration of low doses of CO in its gaseous form mitigates the progression of vascular and inflammatory pathologies affecting the cardiovascular and nervous systems [3,4]. Because the delivery of accurate doses of CO to the selected molecular target by direct gas inhalation remains difficult to achieve, CO-releasing molecules (CO-RMs) have been developed as a promising alternative technology. This class of compounds are primarily based on transition metal carbonyls that are able to store the gas in a stable chemical form and thus deliver controlled amounts of CO to biological systems [3]. Different CO-RMs have been tested in a number of *in vitro* and *in vivo* models demonstrating the vasodilatory, anti-inflammatory and anti-ischemic effects of CO [3,5,6,7].

Recent data revealed that mitochondria might be important targets in transducing the beneficial effects of CO. For instance, it has been reported that the protective effects of CO gas and CO-RMs against cardiomyopathy and cardiac dysfunction in sepsis, respectively, are mediated by increased mitochondrial biogenesis [8,9]. These data have been reinforced by our recent observation showing that low-micromolar concentrations of CO delivered to isolated rat heart mitochondria by CORM-3 promote a mild uncoupling effect resulting in increased basal respiration and decreased mitochondrial membrane potential in the absence of ADP. These effects were inhibited by malonate suggesting a partial role for complex II of the respiratory chain in the uncoupling effect mediated by CO [10]. The purpose of the present study was to further investigate the mechanism(s) by which low concentrations of CORM-3 uncouple mitochondrial respiration.

2. Material and Methods

2.1. Animals

Animal procedures used in this study were conducted in accordance with the Directives of the European Parliament (2010/63/EU-848 EEC) and recommendations of the French Ministère

de l'Agriculture. Male Wistar rats, weighting 250 to 300 g (7-8 weeks), were purchased from Janvier (Le Genest-St-Isle, France). They were housed in a room maintained under constant environmental conditions (temperature 22-25°C and a constant cycle of 12-h light/dark). They were acclimatized to the animal room before being used and received standard rat diet and water ad libitum.

2.2. Reagents

Ru(CO)3Cl(glycinate) (CORM-3) was synthesized as previously described [6]. Depleted inactive CORM-3 (iCORM-3), which does not release CO to myoglobin, was also prepared as previously described by placing CORM-3 in phosphate buffer solution for 2 days and then bubbling the solution with N_2 gas to remove the residual solubilized CO [6]. Deoxygenated myoglobin (20 µM) prepared as previously described [10] was used as CO scavenger. 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) was obtained from Invitrogen (Cergy-Pontoise, France). All other reagents were purchased from Sigma (Saint-Quentin Fallavier, France).

2.3. Isolation of mitochondria from rat heart

Male wistar rats (250-300 g, Janvier, Le Genest-St-Isle, France) were used for the preparation of heart mitochondria as described before [11]. Briefly, hearts were initially homogenized for 5 s in a cold buffer solution (220 mM mannitol, 70 mM sucrose, 10mM HEPES, 2 mM EGTA, pH 7.4 at 4°C), using a Polytron homogenizer. Samples were then further homogenized for five consecutive times in a Potter-Elvehjem glass homogenizer at 1500 rpm. The homogenate was centrifuged at 1000 g for 5 min at 4°C. The supernatant was centrifuged at 10000 g for 10 min at 4°C. The mitochondrial pellet was resuspended in homogenization buffer without EGTA and protein concentration was determined using the "Advanced protein assay reagent" (Sigma, catalogue number 57697).

2.4. Measurement of mitochondrial respiration

O_2 consumption was measured by a Clark-type oxygen microelectrode fitted to a water-jacketed reaction chamber (Hansatech, Cergy, France) as described before [12]. Mitochondria (0.4 mg/mL protein) were incubated in a respiration buffer containing 100 mM KCl, 50 mM sucrose, 10 mM HEPES, 5 mM KH_2PO_4, pH 7.4 at 30°C. Mitochondrial respiration (State 2) was initiated by addition of pyruvate/malate (5 mM each) and after 1 min CORM-3 (20 µM) or iCORM-3 was added to the medium.

2.5. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (Ψ) 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. Rhodamine 123 (0.2 μM) and the respiratory substrates pyruvate/malate were added to respiration buffer contained in a cuvette maintained at 30°C. Fluorescence was monitored over time using a PerkinElmer SA LS 50B (excitation wavelength 503 nm; emission wavelength 527 nm). After 20 s of recording, mitochondria (0.4 mg/mL) were added in the cuvette and the effect of CORM-3 or iCORM-3 on the membrane potential was examined after addition of the compounds at 120 s. tyrphostine A9 (1 μM) was added at the end of the experiment to depolarize mitochondrial membrane potential.

2.6. Mitochondrial swelling assays

The effect of CORM-3 on mitochondrial swelling was assessed by measuring the change in absorbance at 540 nm (A_{540}) using a Jasco V-530 spectrophotometer. Experiments were carried out at 30°C in the respiration buffer. Mitochondria (0.4 mg/mL) were incubated in the presence of various substrates (5 mM each; pyruvate, malate, glutamate, succinate or a combination of these substrates) for 50 s and swelling was induced by addition of increasing concentrations of CORM-3. When inhibitors were used, they were introduced at the beginning of the incubation period.

2.7. Phosphate transport assays

Two complementary methods were used for the measurement of phosphate transport in mitochondria. First, phosphate transport was evaluated by a swelling assay in de-energized conditions as previously described [13], by measuring the change in absorbance at 540 nm (A_{540}) using a Jasco V-530 spectrophotometer. Mitochondria (0.4 mg/mL) were incubated at 30°C in KCl buffer (125 mM KCl, 20 mM MOPS, 10 mM Tris, 0.5 mM EGTA, 4 μM nigericin, 0.5 μM rotenone, 0.5 μM antimycin A, pH 7.2) in the presence of 40 mM KH_2PO_4 to initiate phosphate transport and 1 μM cyclosporine A to prevent mPTP opening. CORM-3 was added after 50 s.

Secondly, phosphate transport was assessed by direct measurements of mitochondrial phosphate concentration. Mitochondria (0.4 mg/mL protein) were incubated in the respiration buffer in the presence of pyruvate/malate (5 mM each). After 2 min, CORM-3 (10, 20 or 40

μM) or iCORM-3 were added to the medium and the incubation was followed for 0.25, 0.5 1, 2, 5 or 10 min. At each time point, mitochondria were put on ice and immediately centrifuged at 13000g for 10 min at 4°C. The pellet containing mitochondria was resuspended in 1 ml of water, was subjected to three cycles of freezing/thawing (10 min at -80°C/ 5 min at 30°C) and inorganic phosphate concentration was determined according to the green malachite method [14]. Results are reported in nmol/mg proteins.

2.8. Measurement of intra-mitochondrial pH

Intra-mitochondrial pH (pHi) was evaluated using the non fluorescent probe BCECF-AM, which is converted to fluorescent BCECF via the action of esterases. BCECF fluorescence is pH-dependent. Isolated mitochondria were loaded with BCECF-AM (30 µM) for 1 h at 4 °C. Mitochondria were then washed in 15 mL homogenization buffer and after centrifugation (10 min, 10000 *g*) the pellet was resuspended in 200 µL homogenization buffer. Loaded mitochondria (0.4 mg/mL) were incubated in the respiration buffer (1 mL) maintained at 30 °C under constant agitation and fluorescence was followed using a temperature-controlled spectrofluorometer (Jasco FP-6300, excitation wavelength 488 nm; emission wavelength 525 nm). After 30 s, various substrates were introduced and then iCORM-3, tyrphostine A9 (1 µM) or increasing concentrations of CORM-3 were added. At the end of the experiment, tyrphostine A9 was added. When inhibitors were used, they were introduced at the beginning of the incubation period. The effect of CO on pHi was also evaluated using CO gas. Respiration buffer was bubbled with 100% CO gas at 30 °C to obtain a saturated respiration buffer. Deoxygenated myoglobin (20 µM) was used as CO scavenger with both CORM-3 and CO gas. To investigate the interaction between phosphate and BCECF, loaded mitochondria (0.4 mg/ml) were broken by three cycles of freezing/thawing (10 min at -80°C/ 5 min at 30°C) to release BCECF in the medium. A suspension of broken mitochondria was then introduced in the respiration buffer containing various concentration of phosphate and BCECF fluorescence was followed. When inhibitors were used, they were introduced at the beginning of the incubation period.

2.9. Statistical analysis

The data are reported as mean ± S.E.M. Statistical significance was determined using either Student's two-tailed unpaired t-test or one-way analysis of variance (ANOVA) followed by Scheffe's post test. Significance was accepted when $p < 0.05$.

3. Results

3.1. CORM-3-mediated uncoupling effect needs phosphate

We previously showed that CORM-3 increased the basal respiration rate and decreased the mitochondrial membrane potential in isolated cardiac mitochondria respiring on pyruvate/malate and that these effects were mediated by CO. These features are typical of an uncoupling effect but contrary to classical uncoupling agents we demonstrated that the effects of CORM-3 were not sudden but occurred slowly over time [10]. To further investigate the mechanism of this uncoupling effect, we studied the effect of CORM-3 on pHi using the BCECF method. This represents an interesting approach to study uncoupling agents as most of them recycle protons across mitochondrial inner membranes, disrupting proton gradient and decreasing pHi. As shown in Figure 1A, feeding the electron transport chain via complex I with pyruvate/malate increased pHi as the electron transport chain worked and extruded protons. The uncoupling agent, tyrphostine A9, abolished the gradient of protons and thus strongly decreased pHi confirming the feasibility of this method. Surprisingly, CORM-3 did not decrease but rather increased intra-mitochondrial pHi in a concentration-dependent manner which *a priori* appears counterintuitive for an uncoupling agent. This effect was also inhibited by deoxy-myoglobin, a scavenger of CO, and was not reproduced by the depleted or inactive CORM-3 (iCORM-3), which does not release CO (Figure 1A). Although to a lesser extent, an increased pHi was also observed after addition of CO gas confirming the critical role of CO (Figure 1B). We observed, however, that the variation of pHi induced by 20 µM CORM-3 was inhibited in the absence of phosphate (Figure 2A). The same effect was observed in the presence of 1 mM ADP which induces ATP synthesis, leading to phosphate consumption and matrix acidification and, confirming the role of phosphate in the effect mediated by CORM-3 (Figure 2A). The data of Figure 2 confirm that phosphate greatly contributes to the effect of CORM-3 as the acceleration of O_2 consumption and the decrease in membrane potential were almost completely inhibited when respiration was measured in a buffer without phosphate ions (Figure 2B, 2C and 2D). The next question we tried to address was: how can phosphate contribute to the increased O_2 consumption induced by CORM-3? As phosphate is a well-documented activator of mitochondrial permeability transition pore (mPTP) opening [15,16] that induces swelling and increases respiration, we investigated whether cyclosporine A, a well-known de-sensitizer of mPTP opening, might inhibit the effects of CORM-3. Cyclosporine A did not affect the effect of CORM-3 either on O_2 consumption, membrane potential or pHi, excluding the possibility that CORM-3 could act through mPTP opening. The absence of effect of drugs acting on Ca^{2+}, which is a crititical

factor for mPTP opening, reinforced this hypothesis. Indeed, neither the calcium chelator EGTA (Figure 2) nor the specific inhibitor of the mitochondrial Ca^{2+} uniport ruthenium red modified the effect of CORM-3 (data not shown).

3.2. CORM-3 induces phosphate-dependent mitochondrial swelling

Mitochondrial swelling can be induced independently of mPTP opening. Hence, we examined whether CORM-3 could induce mitochondrial swelling per se. As depicted in Figure 3A, CORM-3 induced swelling in energized mitochondria in a concentration-dependent manner as attested by the decrease in absorbance. Once again, iCORM-3 was without effect confirming the direct involvement of CO. In the same way, cyclosporine A did not inhibit CORM-3-induced swelling confirming that mPTP opening was not involved in the mechanism. When phosphate was omitted from the respiration buffer no swelling could be observed (Figure 3B). The selective addition of ion to a minimal sucrose-HEPES buffer validated the crucial role of phosphate. Indeed, the addition of 5 mM of phosphate in the form of potassium or of sodium phosphate induced swelling. This was not observed when 5 mM KCl or 5 mM NaCl were added to the buffer, excluding a role for potassium, chloride and sodium when they were alone in the medium (Figure 3C). Addition of 1 mM ADP, which as previously mentioned consumes phosphate, reduced swelling and this was reversed in the presence of oligomycin (Figure 3D). In addition, both the rate and the extent of swelling induced by 20 μM CORM-3 were dependent on the amount of phosphate added (Figure 3B). Taken together, these data demonstrate that the entry of phosphate into mitochondria is absolutely necessary for the effects of CORM-3 but a role for potassium and/or sodium could not be excluded as phosphate was added under the form of potassium or of sodium phosphate. Indeed, one can imagine a mechanism in which phosphate would enter through a phosphate carrier and either potassium and/or sodium would enter through a channel induced by the CORM-3.

Thus, we used a Tris phosphate buffer (phosphoric acid neutralized with Tris base) that did not contain any of these cations. In this medium CORM-3 was without effect but this experiment did not allow us to draw any conclusion as we observed that Tris alone inhibited CORM-3-induced swelling by an unknown mechanism (data not shown).

3.3. CORM-3 induces phosphate transport

In order to monitor phosphate transport in energized cardiac mitochondria we developed a technique to measure mitochondrial phosphate concentration (see Material and Methods). Figure 4A shows that CORM-3, but not iCORM-3, induces a rapid entry of phosphate into

mitochondria. The maximal effect was observed 1 min after addition of CORM-3 followed by a decrease and then a plateau corresponding probably to a re-equilibrium of the phosphate concentration. This effect was concentration-dependent and was observed at concentrations of CORM-3 that induced the uncoupling and swelling effects

3.4. Mitochondrial phosphate carriers are involved in the effects mediated by CORM-3

Phosphate is transported across mitochondrial membranes by two carriers: 1) the mitochondrial phosphate carrier (PiC), which facilitates the transport of phosphate together with an equivalent amount of protons or in exchange for hydroxyl ions and 2) the dicarboxylate carrier (DIC), which catalyses the electroneutral exchange of phosphate with dicarboxylates (malate and succinate). Thus, we investigated whether these carriers could be involved in the effect mediated by CORM-3. First, we used high concentrations of the substrate of DIC malonate which can inhibit malate-phosphate exchange by saturating the external binding sites of DIC [17]. In these conditions, malonate reduced both mitochondrial swelling and the increase in intra-mitochondrial pH induced by CORM-3 (Figure 5A and 5B). This change in pH value was also limited by butylmalonate that is considered as a very effective inhibitor of DIC. Unfortunately, we could not use butylmalonate in swelling experiments because butylmalonate per se caused swelling in our experimental conditions. The omission of pyruvate in the medium, which is necessary for malate entry into mitochondria via the citrate carrier and for DIC activation [18], or replacement of malate by glutamate, limited both CORM-3-induced swelling and increase in intra-mitochondrial pH (Figure 5A and 5C). The effect of CORM-3 persisted when malate was substituted by succinate. It should be noted that all the experimental conditions that reduced DIC activity (malonate, changes of substrates) also altered the effect of CORM-3 on O_2 consumption (Figure 5D). From these results we can conclude that DIC activity is involved in CORM-3-induced both phosphate entry into mitochondria and swelling.

CORM-3-induced swelling was also inhibited by the thiol reagent N-ethylmaleimide which is a PiC inhibitor at concentrations that are ineffective at inhibiting DIC [19] (Figure 5A). This inhibition was total, indicating that PiC could be the main target of CORM-3 for phosphate entry. Importantly, N-ethylmaleimide strongly inhibited phosphate transport even when a high concentration of CORM-3 was used (Figure 4B) and totally suppressed the increase in mitochondrial O_2 consumption (Figure 5D). However, the fact that N-ethylmaleimide also fully prevented mitochondrial matrix alkalinisation (Figure 5B) was contradictory as PiC

activation leads to co-transport of phosphates and protons inside mitochondria, and therefore an acidification might be expected. Thus, we suspected a non-specific effect independent of pH variations and we speculate that it could result from a competition between BCECF and phosphates for protons. This was relevant as the pKa value for phosphate is slightly higher than that of BCECF at physiological pH, 7.2 and 6.97 for phosphate and BCECF, respectively. In order to investigate this possibility, mitochondria were loaded with BCECF-AM to generate the fluorescent drug BCECF, were broken by three cycles of freezing/thawing and BCECF fluorescence was followed in the presence of increasing concentrations of phosphate. As shown in figure 6, the presence of pyruvate/malate did not modify BCECF fluorescence (confirming the loss of mitochondrial integrity) whereas phosphate induced a concentration-dependent increase in fluorescence. These data demonstrate that in our experimental conditions BCECF and phosphate compete for protons and that the matrix alkalinisation described in Figure 1 reflected in fact phosphate entry into mitochondria and was not the result of proton exit. Taken together, these data provide further evidence that the increase in mitochondrial phosphate is involved in the uncoupling effect of CORM-3 and that PiC could be responsible for this effect.

3.5. CORM-3 stimulates PiC activity

If PiC is the main target for the induction of swelling by CORM-3, this effect must be obtained in a model monitoring specifically the transport of phosphate by PiC. Figure 7 shows that this is indeed the case. To do this, we used a swelling assay for phosphate transport in de-energized mitochondria as previously described [13]. In these experiments, DIC is inactive and mitochondrial swelling is caused by the entry of phosphate into mitochondria via PiC. Note that cyclosporine A (1 μM) was present in the medium to ensure that no mPTP-dependent swelling occurred following the addition of phosphate. In these conditions, CORM-3, but not iCORM-3, caused a concentration-dependent activation of mitochondrial swelling. This effect was inhibited in the presence of several potent PiC inhibitors. In fact, both N-ethylmaleimide and the sulfhydryl reagent mersalyl [20] suppressed mitochondrial swelling whereas the more recently described inhibitor ubiquinone 0 [13] decreased it. This effect was abolished by 1) malate, which is known to stimulate DIC activity and thus phosphate exit from mitochondria and 2) glutamate, which is able to co-transports protons into the mitochondrial matrix. These data show that PiC is activated by CORM-3.

4. Discussion

CO is produced during the degradation of heme by heme oxygenase enzymes in mammals and has been suggested to act as an endogenous signaling molecule in many tissues [21,22]. Although the poisoning effects of high concentrations of CO that lead to impairment of both O_2 transport by haemoglobin and mitochondrial respiration have been well described, most of the effects of CO at concentrations that might reflect the amount generated physiologically remain unknown.

Recently, we have shown that low micromolar concentrations of CO are able to regulate oxidative phosphorylation by uncoupling mitochondrial respiration [10]. In the present paper we demonstrated that this effect is caused by activation of PiC that leads to the increase in intra-mitochondrial phosphate and proton concentrations, which are both known to play an important role in the uncoupling effect. As in the previous study, we used the well-characterized water soluble CO releaser CORM-3 to perform these experiments. The advantage of this carbonyl complex is its ability to release low and precise concentrations of CO within the biological milieu. This is not the case of CO gas that distributes homogeneously in the medium and can escape into the atmosphere. Moreover, recent studies using fluorescent probes specific for CO confirmed that CO-RMs such as CORM-2 and CORM-3 are more efficient to deliver CO inside cells than applying solutions saturated with CO gas [23,24].

We have previously highlighted that malonate, a well-known inhibitor of complex II, significantly reversed CO-mediated mitochondrial uncoupling suggesting a possible contribution of complex II in the uncoupling effect. However, the fact that we did not find any change in the activity of complex II in the presence of increasing concentrations of CORM-3 [10] leaves open the possibility that additional mechanisms may take place. Besides to its complex II-inhibiting properties, malonate is also a substrate of DIC and is able to reduce phosphate transport. Phosphate being known to be able to induce an increase in mitochondrial membrane permeability [25,26], a subsequent swelling and thus an increase in O_2 consumption and a decrease in membrane potential, we focused on the role of phosphate transport on the effects mediated by CORM-3. Our results demonstrate that phosphate is indeed required to enable CO to exert its uncoupling effects. Using a method that directly assesses mitochondrial phosphate concentration, we showed that CO enhanced the transport of phosphate into mitochondria in a concentration-dependent manner. This effect was sudden and reached a maximal effect after 1 min, which is concomitant to the fast release of CO by CORM-3 as previously shown [6]. We also demonstrated that CO induces a concentration-

dependent swelling and this effect also needs phosphate. Although phosphate is a critical factor for mPTP opening, the CO-dependent uncoupling of cardiac mitochondria is not caused by mPTP opening because the effects of CO persist even in the presence of cyclosporine A, a potent de-sensitizer of mPTP opening.

In mitochondria, phosphate transport is mediated by 2 carriers: DIC, which exchanges phosphate with dicarboxylate, and PiC, which co-transports phosphate and protons or exchange phosphate and hydroxyl ions [18]. Our results show that PiC might be the target of CO and DIC may act as a regulator. Indeed, in de-energized conditions when DIC is inactive and only PiC works, we still observed an effect of CORM-3 although higher concentrations of the carbonyl complex are needed. The total inhibition of CO-mediated effects in the presence of N-ethylmaleimide at concentration that specifically inhibits PiC [19] confirms these results. However, when DIC is inactivated with specific inhibitors or in the absence of dicarboxylate, the effects of CO are highly reduced.

As illustrated in Figure 8, our results demonstrate that CO activates PiC, which leads to an increase in intramitochondrial phosphate and protons. DIC acts as a regulator: when it is working, it exacerbates the phosphate influx inside mitochondria and thus intensifies the effects of CO. The increase in intramitochondrial phosphate induces a subsequent mitochondrial swelling. The increases in swelling and in proton can both participate to the uncoupling effect induced by CO but the swelling effect seems to be mainly involved since in conditions where DIC was not stimulated the uncoupling effect was highly decreased. We cannot exclude other mechanisms participating to the uncoupling effect of CO as we previously showed that uncoupling proteins and adenine nucleotide transporter may be partially involved [10].

Surprisingly, the data on the effect of CO on intramitochondrial pHi was not in line with our hypothesis as we observed an increase in pHi after adding CORM-3 *i.e.* an extrusion of protons. This could be explained by the competition between BCECF and phosphate for protons occurring in our experimental conditions. Indeed, the increase in fluorescence could be due to the entry of phosphate rather than the extrusion of protons, confirming the effect of CO on phosphate transport. This interaction prevents us from concluding about a net influx of protons inside mitochondrial matrix. However, with regards to our results on PiC activity, on phosphate entry inside mitochondria and consecutive uncoupling effect, an intramitochondrial increase in protons concentration is highly likely.

Our data clearly involve phosphate transport in the induction of swelling but they do not exclude a possible involvement of an electrogenic transport of potassium and/or sodium in

this effect. Indeed, Wilson *et al*. [27] have recently shown that CORM-3 facilitates the transport of these cations across bacterial membranes. The mechanism by which phosphate could induce swelling remains unclear. A possible role of lipid peroxidation induction at high Pi concentrations [25] has been proposed. In fact, Pi may stimulate lipid peroxidation leading to an increase in mitochondrial reactive oxygen species production and mitochondrial swelling. This phenomenon may participate to the effect of CORM-3 as we have previously shown that CORM-3 induces an increase in mitochondrial H_2O_2 production [10]. Experiments are in progress to investigate this hypothesis. An important question emerging from these results is how CO could activate PiC directly since PiC does not appear to contain any molecular entity able to bind CO (such as a transition metal or a heme moiety). The activation of PiC could be due to an indirect interaction through a mediator containing such a structure. Further investigations are necessary to identify and characterize such a mediator.

In conclusion, this study demonstrates that CO activates PiC leading to an increase in phosphate and proton transports inside mitochondria which can both contribute to the non-classical uncoupling effect caused by CORM-3.

5. Acknowledgements

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7. Legends for Figures

Figure 1: CO induces mitochondrial matrix alkalinization.

A: Mitochondrial pHi was studied by following BCECF fluorescence. After 50 s of stabilization, the addition of pyruvate/malate (5 mM each) generated membrane potential and thus matrix alkalinization. Then (arrow) increasing concentrations of CORM-3 (10 µM, line 1; 20 µM, line 2; 40 µM, line 3; and 100 µM, line 4), of its inactive form iCORM-3 (100 µM, line 5), of 20 µM CORM-3 with the CO scavenger deoxymyoglobin (20 µM; line 6) or of 1µM tyrphostine A9 were added. Curves are representative of at least 4 independent experiments. Bar graphs represent changes in fluorescence between 200 and 600 seconds. *p< 0.05 *vs* iCORM-3 (line 5).

B: BCECF fluorescence was followed in mitochondria introduced in a CO-saturated buffer. Pyruvate/malate (5 mM/5 mM) was added at the beginning of the experiment. Deoxymyoglobin was used as CO scavenger. The following conditions were analyzed: respiration buffer (line 1), CO-saturated buffer (line 2), respiration buffer with 20 µM deoxymyoglobin (line 3) and CO-saturated buffer with 20 µM deoxymyoglobin (line 4). Curves are representative of at least 4 independent experiments. Bar graphs represent changes in fluorescence between 0 and 800 seconds. *p< 0.05 *vs* respiration buffer (line 1).

Figure 2: CORM-3-mediated uncoupling effect and matrix alkalinization need phosphate.

A: BCECF fluorescence was used to evaluate the role of phosphate on mitochondrial alkalinization induced by CORM-3. After addition of pyruvate/malate (5 mM each) that generates membrane potential and thus matrix alkalinization, 20 µM CORM-3 was added in respiration buffer (line 1), in phosphate- depleted buffer (line 2) or in respiration buffer in the

presence of 1 mM ADP (line 3). Curves are representative of at least 4 independent experiments. Bar graphs represent changes in fluorescence between 200 and 800 seconds. *p< 0.05 *vs* respiration buffer + CORM-3(line 1).

B: Membrane potential (ψ) in isolated heart mitochondria treated with CORM-3 was followed by measuring the uptake/release of the fluorescent dye rhodamine 123. The following conditions were analyzed: phosphate-depleted buffer (line 1), respiration buffer with 20 µM CORM-3 in the absence (line 2) or in the presence of cyclosporine A (line 4) or EGTA (line 5), or phosphate-depleted buffer with 20µM CORM-3 (line 3). Curves are representative of at least 4 independent experiments.

C and D: Substrate-dependent oxygen consumption (state 2) was recorded (C) and quantified (D) in isolated heart mitochondria. Various conditions were analyzed: respiration buffer (line 1), phosphate-depleted buffer (line 2), respiration buffer with 20 µM CORM-3 in the absence (line 3) or in the presence of 1 µM cyclosporine A (CsA) (line 5) or 100 µM EGTA (line 6), or phosphate-depleted buffer with 20µM CORM-3 (line 4). Each value represents the mean ± SEM of six independent preparations. *p<0.05 *vs* respective control.

Figure 3: CORM-3 induces a phosphate-dependent mitochondrial swelling.

A: CORM-3 induces a concentration-dependent swelling independent of mPTP opening. Mitochondrial swelling was measured in the presence of increasing concentrations of CORM-3 (2 µM, line 2; 10 µM, line 3; 20 µM, line 4; 40 µM, line 5 and 100 µM, line 6) or its inactive form iCORM-3 (100 µM, line 1). Cyclosporine A was introduced at the beginning of the experiment and then 100 µM CORM-3 was added (line 7). Curves are representative of at least 4 independent experiments. Bar graphs represent total swelling amplitude. *p< 0.05 *vs* iCORM-3 (line 1).

B: Effect of phosphate on CORM-3-induced swelling. Swelling was induced by 20 µM CORM-3 in the absence (line 1) or in the presence of 1 mM phosphate (line 2), 2.5 mM (line 3), 5 mM (line 4) or 10 mM (line 5). Curves are representative of at least 4 independent experiments. Bar graphs represent total swelling amplitude. *p< 0.05 *vs* absence of phosphate (line 1).

C: Mitochondrial swelling induced by 20 µM CORM-3 was evaluated in different buffers: sucrose-HEPES buffer (line 1), sucrose-HEPES buffer with 5 mM KCl (line 2), sucrose-HEPES buffer with 5 mM NaCl (line 3), sucrose-HEPES buffer with 5 mM KH_2PO_4 (line 4) and sucrose-HEPES buffer with 5 mM NaH_2PO_4 (line 5). Curves are representative of at least

4 independent experiments. Bar graphs represent total swelling amplitude. *p< 0.05 *vs* sucrose-HEPES buffer (line 1).

D: Effect of ADP on CORM-3-induced swelling. Swelling was induced by 20 µM CORM-3 (line 1), 20 µM CORM-3 with 1 mM ADP (line 2), 20 µM iCORM-3 with 1 mM ADP (line 3) or 20 µM CORM-3 with 1mM ADP in the presence of 1 µM oligomycin (line 4). When used, ADP and oligomycin were introduced at the beginning of the experiment. Curves are representative of at least 4 independent experiments. Bar graphs represent total swelling amplitude. *p< 0.05 *vs* iCORM-3 with ADP (line 3).

Figure 4: CORM-3 induces mitochondrial phosphate transport.

A: effect of increasing concentrations of CORM-3 on the accumulation of phosphate into mitochondria. *p< 0.05 *vs* iCORM-3

B: N-ethylmaleimide (NEM, 20 µM) inhibits the effect of CORM-3 (40 µM) on the mitochondrial accumulation of phosphate. *p< 0.05 *vs* iCORM-3; # *p< 0.05 *vs* CORM-3.

Figure 5: Mitochondrial phosphate carriers are involved in CORM-3-mediated effects.

A: Mitochondrial swelling induced by 20 µM CORM-3 was followed in the presence of various substrates: pyruvate/malate 5 mM each (line 1), pyruvate/glutamate 5 mM each (line 2), malate 10 mM (line 3) or succinate 10 mM (line 4). Malonate (20 mM, line 5) and N-ethylmaleimide (20 µM, line 6) were added at the beginning of the experiments when pyruvate/malate were used as substrates. Curves are representative of at least 4 independent experiments. Bar graphs represent total swelling amplitude. *p< 0.05 *vs* pyruvate/malate (line 1).

B: The effect of various inhibitors on mitochondrial matrix alkalinization induced by 20 µM CORM-3 was evaluated using the BCECF method. Inhibitors were added at the beginning of the experiment. The following conditions were studied: 20 µM CORM-3 (line 1), 20 µM CORM-3 with 20 mM malonate (line 2), 20 µM CORM-3 with 1 mM N-butylmalonate (line 3) or 20 µM CORM-3 with 20 µM N-ethylmaleimide (line 4). Curves are representative of at least 4 independent experiments. Bar graphs represent the variation of fluorescence between 200 and 600 seconds. *p< 0.05 *vs* CORM-3 (line 1).

C: Matrix alkalinization induced by 20 µM CORM-3 was evaluated using BCECF fluorescence in the presence of various substrates: pyruvate/malate 5 mM/5 mM (line 1), pyruvate/succinate 5 mM each (line 2), malate 10 mM (line 3), succinate 10 mM (line 4) or pyruvate/glutamate 5 mM each (line 5). Curves are representative of at least 4 independent

experiments. Bar graphs represent changes in fluorescence between 200 and 800 seconds. *p< 0.05 *vs* pyruvate/malate (line 1).
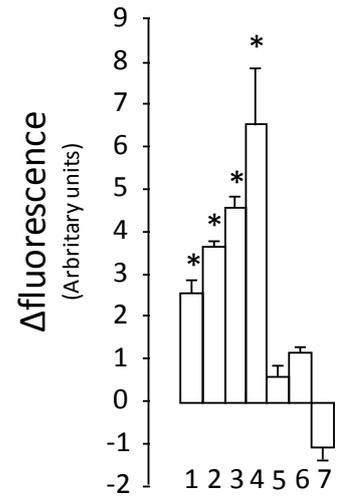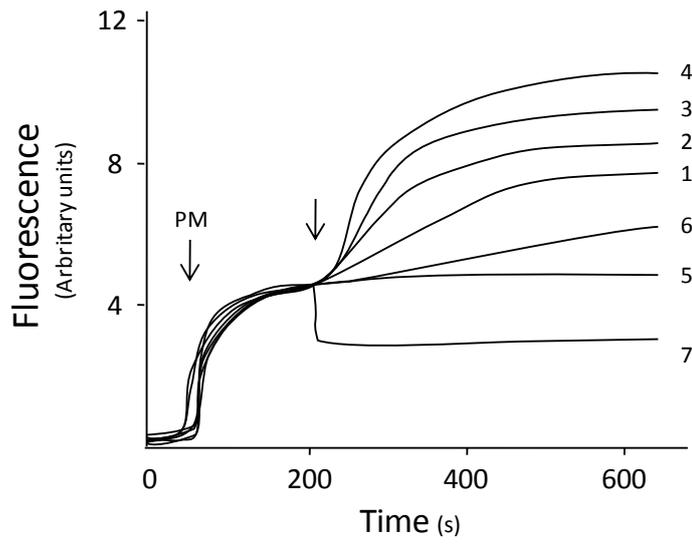
D: Substrate-dependent O_2 consumption (state 2) was quantified in isolated heart mitochondria in various conditions: pyruvate/malate (5 mM each, PM), pyruvate/glutamate (5 mM each, PG), malate (10 mM, M), succinate (10 mM, succ). When used, malonate (20 mM) or N-ethylmaleimide (NEM, 20 μM) were added at the beginning of the experiment. Each value represents the mean ± SEM of six independent preparations. *p<0.05 *vs* substrate alone. #p<0.05 *vs* PM + CORM-3.

Figure 6: Changes in BCECF fluorescence reflects the competition between BCECF and phosphate.

BCECF fluorescence of a broken-mitochondrial suspension was followed in the presence of increasing concentrations of phosphate (absence line 1; 0.5 mM, line 2; 1 mM, line 3; and 5 mM, line 4). Pyruvate/malate (5 mM/5 mM) were added at 300 s to confirm the loss of mitochondrial integrity (line 1). Curves are representative of at least 4 independent experiments. Bar graphs represent changes in fluorescence between 0 and 1000 seconds. *p< 0.05 *vs* absence of phosphate (line 1).

Figure 7: CORM-3 stimulates PiC activity.

Phosphate transport by PiC was assessed by swelling assay in de-energized conditions. When present, substrates (glutamate 5 mM or malate 5 mM) and PiC inhibitors (N-ethylmaleimide 40 μM, Mersalyl 40 μM or UQ0 40 μM) were added in the medium before the beginning of the experiment. CORM-3 (80 μM) or its inactive form iCORM-3 were used for these experiments. Swelling was induced by iCORM-3 (line 1), CORM-3 (line 2), CORM-3 with N-ethylmaleimide (line 3), CORM-3 with Mersalyl (line 4), CORM-3 with ubiquinone 0 (line 5), CORM-3 with glutamate (line 6) or CORM-3 with malate (line 7). Curves are representative of at least 4 independent experiments. Bar graphs represent total swelling amplitude. *p< 0.05 *vs* iCORM-3 (line 1).

Figure 8: Scheme summarizing the proposed molecular processes by which CO induces mitochondrial uncoupling.

CO stimulates PiC activity and thus increases the matrix concentration of phosphate and protons. When pyruvate/malate are used as substrates to energize mitochondria, DIC is activated further increasing matrix phosphate concentration. This leads to mitochondrial

swelling. Both mitochondrial swelling and the increase in intramitochondrial protons may participate to mitochondrial uncoupling *i.e.* a decrease in mitochondrial potential (Ψm) and an increase in mitochondrial respiratory chain (MRC) activity leading to generation of reactive oxygen species (ROS). Uncoupling proteins (UCP) and adenine nucleotide transporter (ANT) may also be involved in the uncoupling effect [10].
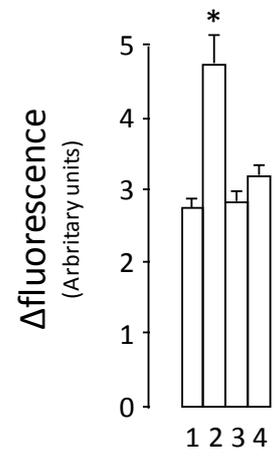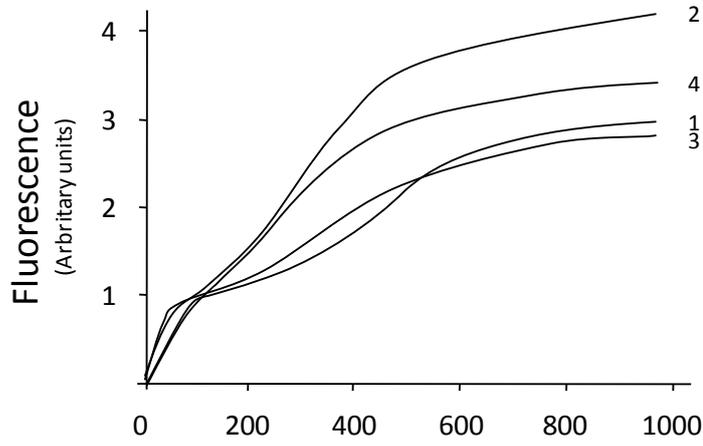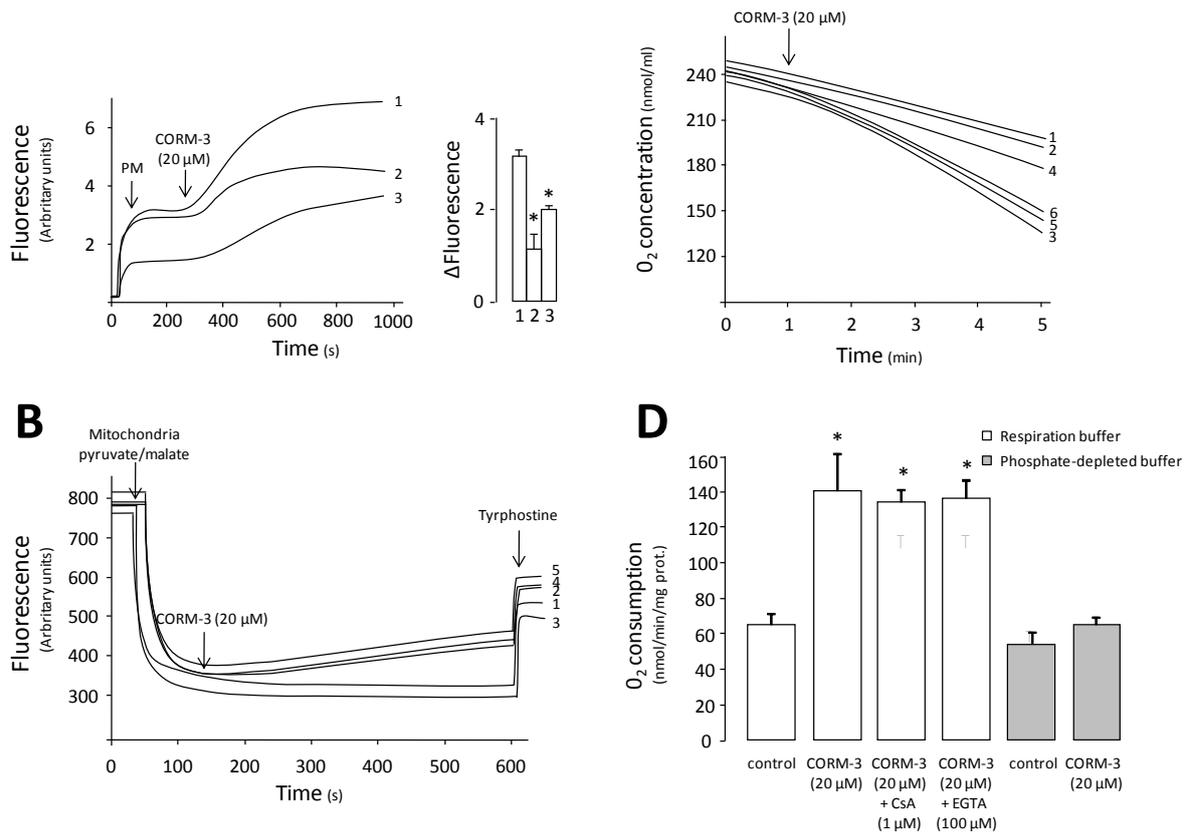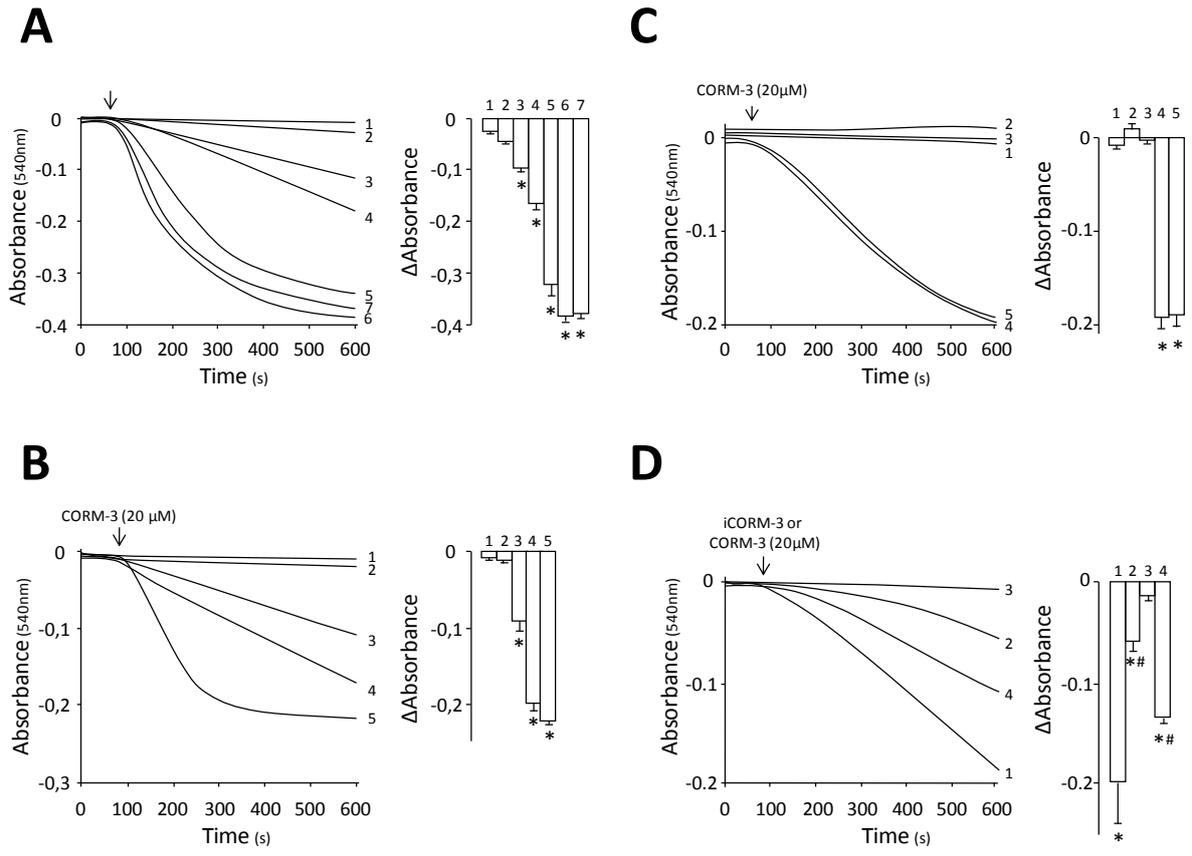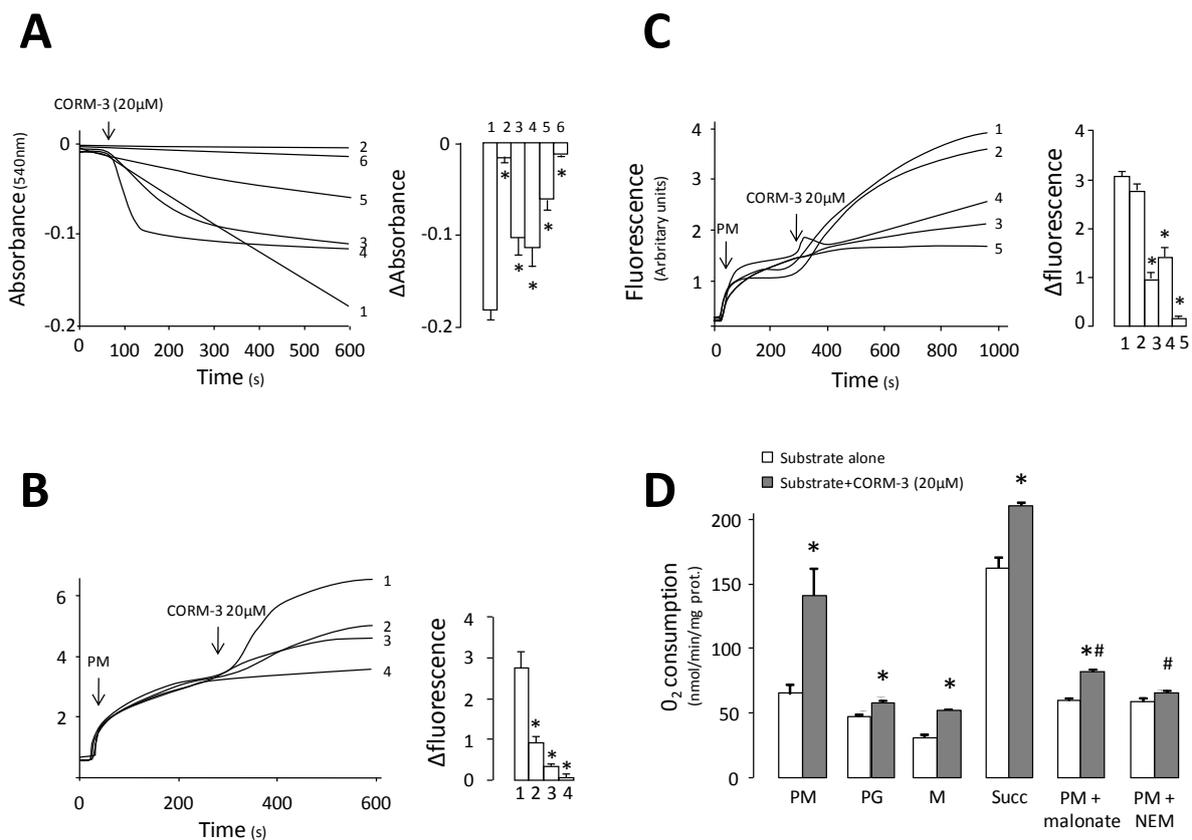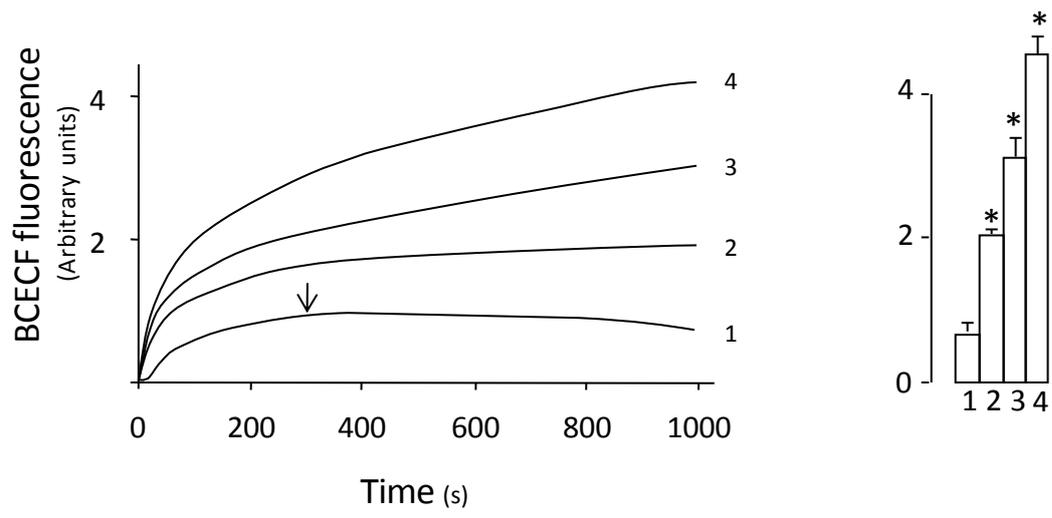
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8