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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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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 exchanges 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 hemoglobin 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 signaling function since CO is continuously produced in small quantities in tissues, regulates several physiological processes and exerts beneficial effects against pathophysiological conditions [1–3]. 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 is 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 [3,5–7].

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

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

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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 of 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, 10 mM 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, catalog number 57697).

2.4. Measurement of mitochondrial respiration

O_2 consumption was measured by a Clark-type oxygen micro-electrode 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 (Ψm) 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 was 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 13000 *g* for 10 min at 4 °C. The pellet containing mitochondria was resuspended in 1 mL of water and 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 Fig. 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 increase 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 (Fig. 1A). Although to a lesser extent, an increased pHi was also observed after addition of CO gas confirming the critical role of CO (Fig. 1B). We observed, however, that the variation of pHi induced by 20 μM CORM-3 was inhibited in the absence of phosphate (Fig. 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 (Fig. 2A). The data of Fig. 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 (Fig. 2B, C and D). 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

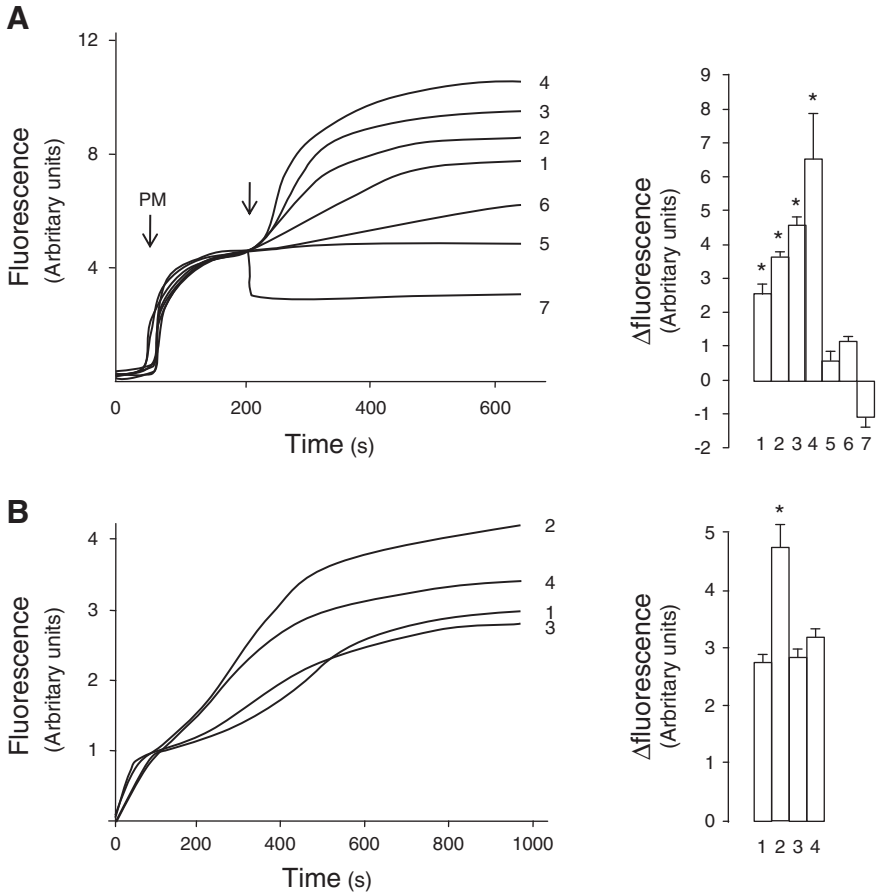

Fig. 1. CO induces mitochondrial matrix alkalinization. A: Mitochondrial pHi was studied by following BCECF fluorescence. After 50 s of stabilization, the addition of pyruvate/malate (5 mM each) generated membrane potential and thus matrix alkalinization. Then (arrow) increasing concentrations of CORM-3 (10, 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 1 μM tyrphostine A9 were added. Curves are representative of at least 4 independent experiments. Bar graphs represent changes in fluorescence between 200 and 600 s. *$p < 0.05$ *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 s. *$p < 0.05$ *vs* respiration buffer (line 1).

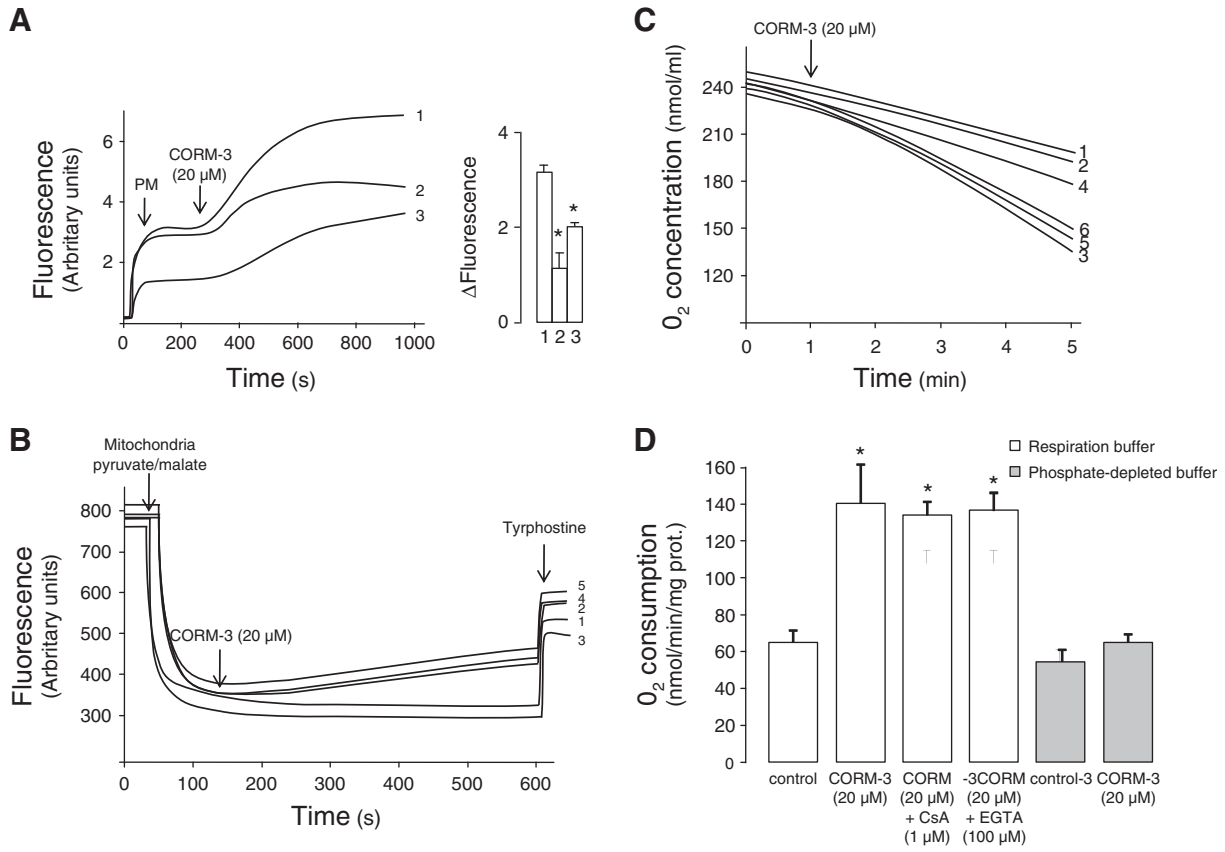

Fig. 2. CORM-3-mediated uncoupling effect and matrix alkalinization need phosphate. A: BCECF fluorescence was used to evaluate the role of phosphate on mitochondrial alkalinization induced by CORM-3. After addition of pyruvate/malate (5 mM each) that generates membrane potential and thus matrix alkalinization, 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 s. *$p < 0.05$ *vs* respiration buffer + CORM-3 (line 1). B: Membrane potential (Ψm) 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 20 μM 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 20 μM CORM-3 (line 4). Each value represents the mean ± SEM of six independent preparations. *$p < 0.05$ *vs* respective control.

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 critical factor for mPTP opening, reinforced this hypothesis. Indeed, neither the calcium chelator EGTA (Fig. 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 Fig. 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 (Fig. 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 (Fig. 3C). Addition of 1 mM ADP, which as previously mentioned consumes phosphate, reduced swelling and this was reversed in the presence of oligomycin (Fig. 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 (Fig. 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 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).

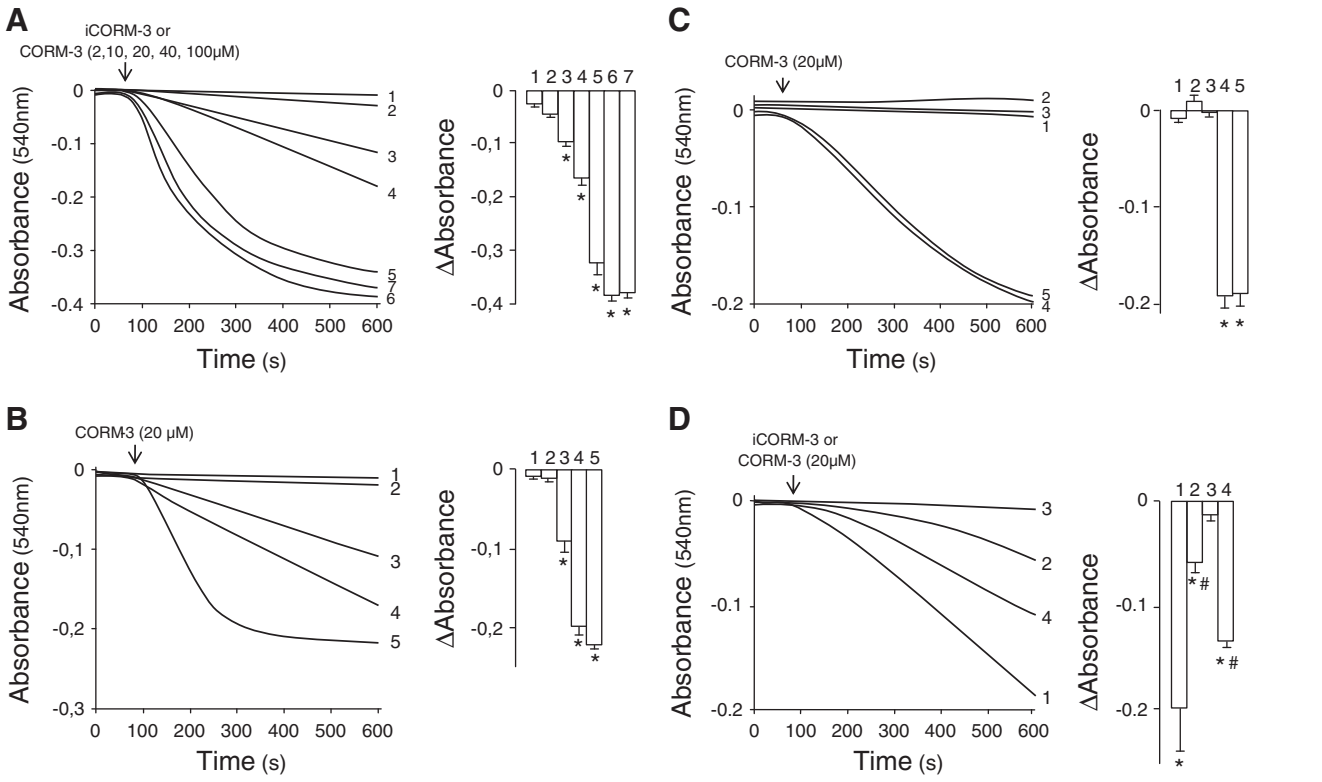

Fig. 3. CORM-3 induces a phosphate-dependent mitochondrial swelling. A: CORM-3 induces a concentration-dependent swelling independent of mPTP opening. Mitochondrial swelling was measured in the presence of increasing concentrations of CORM-3 (2 μM, line 2; 10 μM, line 3; 20 μM, line 4; 40 μM, line 5 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 (line 2), 2.5 mM (line 3), 5 mM (line 4) or 10 mM (line 5) phosphate. 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 1 mM 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).

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). Fig. 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 catalyzes 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 increase in intra-mitochondrial pH induced by CORM-3 (Fig. 5A and B). 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 (Fig. 5A and C). 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 (Fig. 5D). From these results we can conclude that DIC activity is involved in both CORM-3-induced phosphate entry into mitochondria and CORM-3-induced 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] (Fig. 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 (Fig. 4B) and totally suppressed the increase in mitochondrial O_2 consumption (Fig. 5D). However, the fact that N-ethylmaleimide also fully prevented mitochondrial matrix alkalinization (Fig. 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

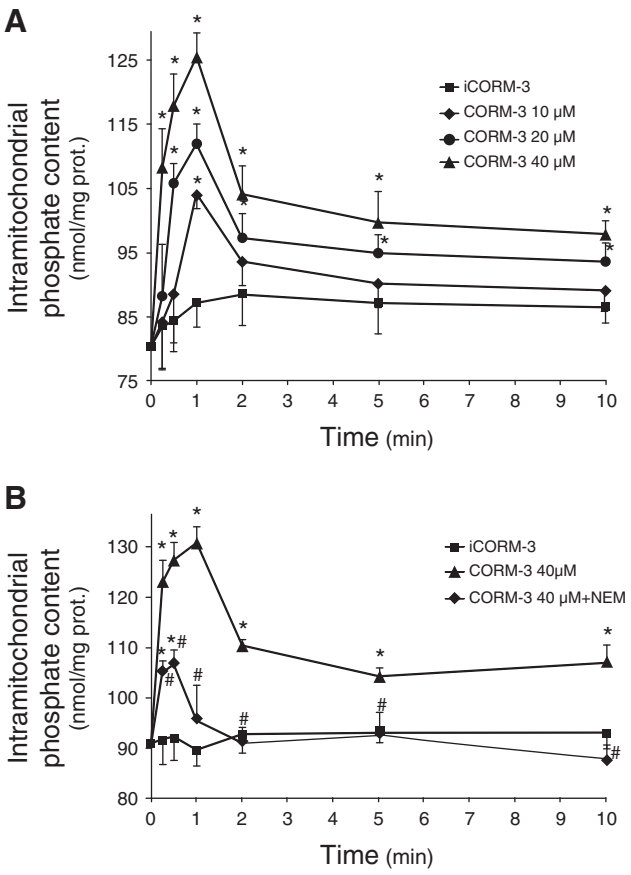

Fig. 4. CORM-3 induces mitochondrial phosphate transport. A: Effect of increasing concentrations of CORM-3 on the accumulation of phosphate into mitochondria. *$p < 0.05$ *vs* iCORM-3. B: N-ethylmaleimide (NEM, 20 μM) inhibits the effect of CORM-3 (40 μM) on the mitochondrial accumulation of phosphate. *$p < 0.05$ *vs* iCORM-3; #$p < 0.05$ *vs* CORM-3.

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 values, 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 and were broken by three cycles of freezing/thawing and BCECF fluorescence was followed in the presence of increasing concentrations of phosphate. As shown in Fig. 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 alkalinization described in Fig. 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. Fig. 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-transport 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 hemoglobin 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 the 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 the 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 exchanges 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

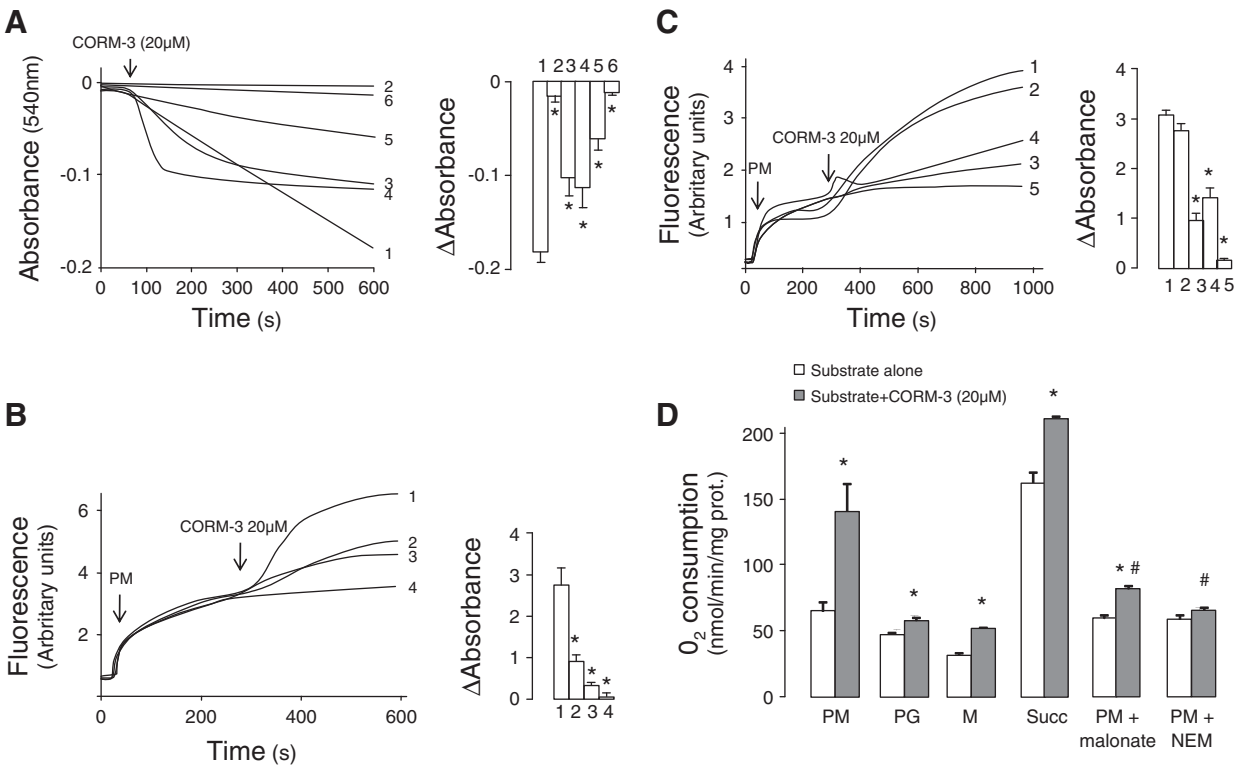

Fig. 5. Mitochondrial phosphate carriers are involved in CORM-3-mediated effects. A: Mitochondrial swelling induced by 20 μM CORM-3 was followed in the presence of various substrates: pyruvate/malate 5 mM each (line 1), pyruvate/glutamate 5 mM each (line 2), malate 10 mM (line 3) or succinate 10 mM (line 4). Malonate (20 mM, line 5) and N-ethylmaleimide (20 μM, line 6) were added at the beginning of the experiments when pyruvate and 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 s. *$p < 0.05$ *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 s. *$p < 0.05$ *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), and succinate (10 mM, Succ). When used, malonate (20 mM) or N-ethylmaleimide (NEM, 20 μM) was 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.

needed. The total inhibition of CO-mediated effects in the presence of N-ethylmaleimide at a 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.

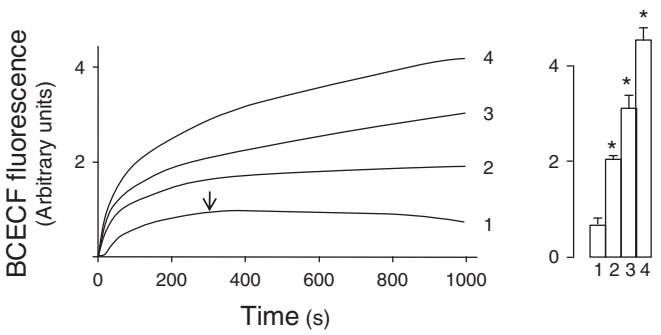

Fig. 6. Changes in BCECF fluorescence reflect the competition between BCECF and phosphate. BCECF fluorescence of a broken-mitochondrial suspension was followed in the presence of increasing concentrations of phosphate (absence, line 1; 0.5 mM, line 2; 1 mM, line 3; and 5 mM, line 4). Pyruvate and malate (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 s. *$p < 0.05$ *vs* absence of phosphate (line 1).

As illustrated in Fig. 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 a mitochondrial matrix. However, with regards to our results on PiC activity, on phosphate entry inside mitochondria and on consecutive uncoupling effect, an intramitochondrial increase in proton concentration is highly likely.

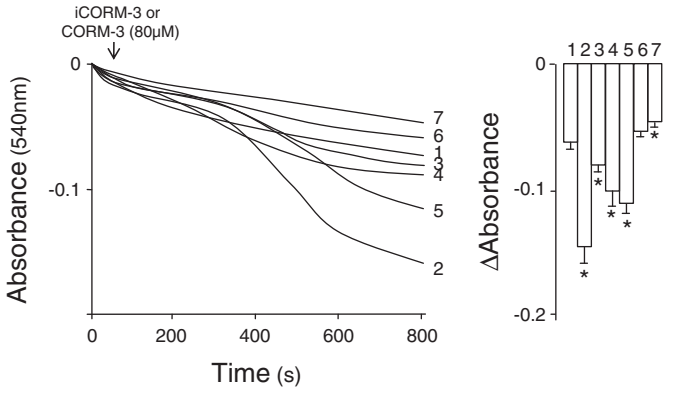

Fig. 7. CORM-3 stimulates PiC activity. Phosphate transport by PiC was assessed by swelling assay in de-energized conditions. When present, substrates (glutamate 5 mM or malate 5 mM) and PiC inhibitors (N-ethylmaleimide 40 μM, mersalyl 40 μM or UQ0 40 μM) were added in the medium before the beginning of the experiment. CORM-3 (80 μM) or its inactive form iCORM-3 was 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).

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 both of which can contribute to the non-classical uncoupling effect caused by CORM-3.

Acknowledgements

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References

[1] L. Wu, R. Wang, Carbon monoxide: endogenous production, physiological functions, and pharmacological applications, Pharmacol. Rev. 57 (2005) 585–630.
[2] M. Bilban, A. Haschemi, B. Wegiel, B.Y. Chin, O. Wagner, L.E. Otterbein, Heme oxygenase and carbon monoxide initiate homeostatic signaling, J. Mol. Med. 86 (2008) 267–279.
[3] R. Motterlini, L.E. Otterbein, The therapeutic potential of carbon monoxide, Nat. Rev. Drug Discov. 9 (2010) 728–743.
[4] S.W. Ryter, J. Alam, A.M. Choi, Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications, Physiol. Rev. 86 (2006) 583–650.
[5] R. Motterlini, J.E. Clark, R. Foresti, P. Sarathchandra, B.E. Mann, C.J. Green, Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities, Circ. Res. 90 (2002) 17–24.
[6] J.E. Clark, P. Naughton, S. Shurey, C.J. Green, T.R. Johnson, B.E. Mann, R. Foresti, R. Motterlini, Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule, Circ. Res. 93 (2003) 2–8.
[7] P. Sawle, R. Foresti, B.E. Mann, T.R. Johnson, C.J. Green, R. Motterlini, Carbon monoxide-releasing molecules (CO-RMs) attenuate the inflammatory response elicited by lipopolysaccharide in RAW264.7 murine macrophages, Br. J. Pharmacol. 145 (2005) 800–810.
[8] H.B. Suliman, M.S. Carraway, A.S. Ali, C.M. Reynolds, K.E. Welty-Wolf, C.A. Piantadosi, The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy, J. Clin. Invest. 117 (2007) 3730–3741.
[9] S. Lancel, S.M. Hassoun, R. Favory, B. Decoster, R. Motterlini, R. Neviere, Carbon monoxide rescues mice from lethal sepsis by supporting mitochondrial energetic metabolism and activating mitochondrial biogenesis, J. Pharmacol. Exp. Ther. 329 (2009) 641–648.

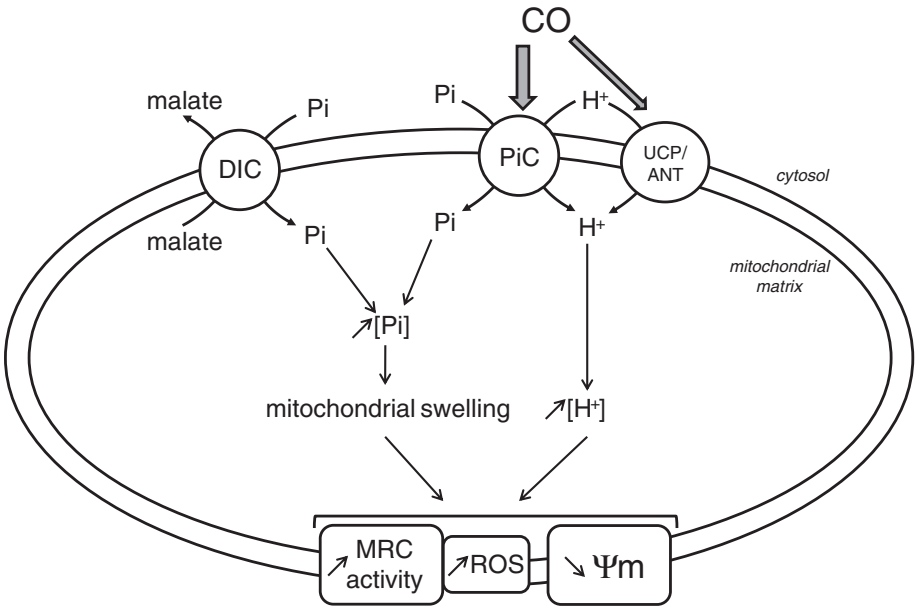

Fig. 8. Scheme summarizing the proposed molecular processes by which CO induces mitochondrial uncoupling. CO stimulates PiC activity and thus increases the matrix concentration of phosphate and protons. When pyruvate and malate are used as substrates to energize mitochondria, DIC is activated further 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].

[10] L. Lo Iacono, J. Boczkowski, R. Zini, I. Salouage, A. Berdeaux, R. Motterlini, D. Morin, A carbon monoxide-releasing molecule (CORM-3) uncouples mitochondrial respiration and modulates the production of reactive oxygen species, Free Radic. Biol. Med. 50 (2011) 1556–1564.
[11] F.N. Obame, R. Zini, R. Souktani, A. Berdeaux, D. Morin, Peripheral benzodiazepine receptor-induced myocardial protection is mediated by inhibition of mitochondrial membrane permeabilization, J. Pharmacol. Exp. Ther. 323 (2007) 336–345.
[12] F.N. Obame, C. Plin-Mercier, R. Assaly, R. Zini, J.L. Dubois-Randé, A. Berdeaux, D. Morin, Cardioprotective effect of morphine and a blocker of glycogen synthase kinase 3 beta, SB216763 [3-(2,4-dichlorophenyl)-4(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione], via inhibition of the mitochondrial permeability transition pore, J. Pharmacol. Exp. Ther. 326 (2008) 252–258.
[13] A.W. Leung, P. Varanyuwatana, A.P. Halestrap, The mitochondrial phosphate carrier interacts with cyclophilin D and may play a key role in the permeability transition, J. Biol. Chem. 283 (2008) 26312–26323.
[14] A.A. Baykov, O.A. Evtushenko, S.M. Avaeva, A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay, Anal. Biochem. 171 (1988) 266–270.
[15] P. Varanyuwatana, A.P. Halestrap, The roles of phosphate and the phosphate carrier in the mitochondrial permeability transition pore, Mitochondrion 12 (2012) 120–125.
[16] L.K. Seidlmayer, M.R. Gomez-Garcia, L.A. Blatter, E. Pavlov, E.N. Dedkova, Inorganic polyphosphate is a potent activator of the mitochondrial permeability transition pore in cardiac myocytes, J. Gen. Physiol. 139 (2012) 321–331.
[17] C. Indiveri, G. Prezioso, T. Dierks, R. Krämer, F. Palmieri, Kinetic characterization of the reconstituted dicarboxylate carrier from mitochondria: a four-binding-site sequential transport system, Biochim. Biophys. Acta 1143 (1993) 310–318.
[18] F. Palmieri, The mitochondrial transporter family (SLC25): physiological and pathological implications, Pflugers Arch. 447 (2004) 689–709.
[19] G. Fiermonte, L. Palmieri, V. Dolce, F.M. Lasorsa, F. Palmieri, M.J. Runswick, J.E. Walker, The sequence, bacterial expression, and functional reconstitution of the rat mitochondrial dicarboxylate transporter cloned via distant homologs in yeast and *Caenorhabditis elegans*, J. Biol. Chem. 273 (1998) 24754–24759.
[20] P. Costantini, V. Petronilli, R. Colonna, P. Bernardi, On the effects of paraquat on isolated mitochondria. Evidence that paraquat causes opening of the cyclosporine A-sensitive permeability transition pore synergistically with nitric oxide, Toxicology 99 (1995) 77–88.
[21] C.A. Piantadosi, Biological chemistry of carbon monoxide, Antioxid. Redox Signal. 4 (2002) 259–270.
[22] R. Foresti, M.G. Bani-Hani, R. Motterlini, Use of carbon monoxide as a therapeutic agent: promises and challenges, Intensive Care Med. 34 (2008) 649–658.
[23] J. Wang, J. Karpus, B.S. Zhao, Z. Luo, P.R. Chen, C. He, A selective fluorescent probe for carbon monoxide imaging in living cells, Angew. Chem. Int. Ed. Engl. 51 (2012) 9652–9656.
[24] B.W. Michel, A.R. Lippert, C.J. Chang, A reaction-based fluorescent probe for selective imaging of carbon monoxide in living cells using a palladium-mediated carbonylation, Am. Chem. Soc. 134 (2012) 15668–15671.
[25] A.J. Kowaltowski, R.F. Castilho, M.T. Grijalba, E.J. Bechara, A.E. Vercesi, Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions. A proposed model for phosphate-stimulated lipid peroxidation, J. Biol. Chem. 271 (1996) 2929–2934.
[26] P.L. Vághy, M.A. Matlib, A. Schwartz, Phosphate induced swelling, inhibition and partial uncoupling of oxidative phosphorylation in heart mitochondria in the absence of external calcium and the presence of EGTA, Biochem. Biophys. Res. Commun. 100 (1981) 37–44.
[27] J.L. Wilson, H.E. Jesse, B. Hughes, V. Lund, K. Naylor, K.S. Davidge, G.M. Cook, B.E. Mann, R.K. Poole, $Ru(CO)_3Cl$(glycinate) (CORM-3): a carbon monoxide-releasing molecule with broad-spectrum antimicrobial and photosensitive activities against respiration and cation transport in *Escherichia coli*, Antioxid. Redox Signal. 19 (2013) 497–509.