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The CO-releasing molecule CORM-3 protects adult cardiomyocytes against hypoxia-reoxygenation by modulating pH restoration.

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ABSTRACT

Several studies have reported that CORM-3, a water-soluble carbon monoxide releasing molecule, elicits cardioprotection against myocardial infarction but the mechanism remains to be investigated. Numerous reports indicate that inhibition of pH regulators, the Na⁺/H⁺ exchanger (NHE) and Na⁺/HCO₃⁻ symporter (NBC), protect cardiomyocytes from hypoxia/reoxygenation injury by delaying the intracellular pH (pHi) recovery at reperfusion. Our goal was to explore whether CORM-3-mediated cytoprotection involves the modulation of pH regulation. When added at reoxygenation, CORM-3 (50 μM) reduced the mortality of cardiomyocytes exposed to 3h of hypoxia and 2h of reoxygenation in HCO₃⁻-buffered solution. This effect was lost when using inactive iCORM-3, which is depleted of CO and used as control, thus implicating CO as the mediator of this cardioprotection. Interestingly, the cardioprotective effect of CORM-3 was abolished by switching to a bicarbonate-free medium. This effect of CORM-3 was also inhibited by 5-hydroxydecanoate, a mitochondrial ATP-dependent K⁺ (mK_{ATP}) channel inhibitor (500 μM) or PD098059, a MEK1/2 inhibitor (10 μM). In additional experiments and in the absence of hypoxia-reoxygenation, intracellular pH was monitored in cardiomyocytes exposed to cariporide to block NHE activity. CORM-3 inhibited alkalinisation and this effect was blocked by PD098059 and 5-HD. In conclusion, CORM-3 protects the cardiomyocyte against hypoxia-reoxygenation injury by inhibiting a bicarbonate transporter at reoxygenation, probably the Na⁺/HCO₃⁻ symporter. This cardioprotective effect of CORM-3 requires the activation of mK_{ATP} channels and the activation of MEK1/2.

Key words: hypoxia-reoxygenation, cardiomyocytes, carbon monoxide, pH

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ABSTRACT

Several studies have reported that CORM-3, a water-soluble carbon monoxide releasing molecule, elicits cardioprotection against myocardial infarction but the mechanism remains to be investigated. Numerous reports indicate that inhibition of pH regulators, the Na^+/H^+ exchanger (NHE) and $\text{Na}^+/\text{HCO}_3^-$ symporter (NBC), protect cardiomyocytes from hypoxia/reoxygenation injury by delaying the intracellular pH (pHi) recovery at reperfusion. Our goal was to explore whether CORM-3-mediated cytoprotection involves the modulation of pH regulation. When added at reoxygenation, CORM-3 (50 μM) reduced the mortality of cardiomyocytes exposed to 3h of hypoxia and 2h of reoxygenation in HCO_3^- -buffered solution. This effect was lost when using inactive iCORM-3, which is depleted of CO and used as control, thus implicating CO as the mediator of this cardioprotection. Interestingly, the cardioprotective effect of CORM-3 was abolished by switching to a bicarbonate-free medium. This effect of CORM-3 was also inhibited by 5-hydroxydecanoate, a mitochondrial ATP-dependent K^+ (mK_{ATP}) channel inhibitor (500 μM) or PD098059, a MEK1/2 inhibitor (10 μM). In additional experiments and in the absence of hypoxia-reoxygenation, intracellular pH was monitored in cardiomyocytes exposed to cariporide to block NHE activity. CORM-3 inhibited alkalinisation and this effect was blocked by PD098059 and 5-HD. In conclusion, CORM-3 protects the cardiomyocyte against hypoxia-reoxygenation injury by inhibiting a bicarbonate transporter at reoxygenation, probably the $\text{Na}^+/\text{HCO}_3^-$ symporter. This cardioprotective effect of CORM-3 requires the activation of mK_{ATP} channels and the activation of MEK1/2.

Key words: hypoxia-reoxygenation, cardiomyocytes, carbon monoxide, pH

INTRODUCTION

Small amount of carbon monoxide (CO) gas is continuously produced in mammals via the endogenous degradation of heme by a family of constitutive (HO-2) and inducible (HO-1) heme oxygenase enzymes [5]. Several studies demonstrated the anti-apoptotic and cardioprotective properties of the endogenous HO-1/CO system [3, 33]. In this context, Motterlini et al. [21] have developed a group of compounds known as CO-releasing molecules (CO-RMs) that mimic the HO-1/CO pathway by liberating CO under appropriate conditions in biological systems. Among them, CORM-3, a water-soluble CO-releasing molecule, elicits cardioprotection through a mechanism involving mitochondrial ATP-dependent potassium channels (mK_{ATP}) [4]. This was confirmed *in vivo* as an intravenous infusion of CORM-3 significantly reduced infarct size in mice subjected to coronary artery occlusion [11]. However, the mechanism of its beneficial action remains to be investigated.

It is well known that reperfusion injury is partly due to a rapid normalization of intracellular pH (pHi) during revascularisation [13]. This pHi recovery involves the activation of two major transporters, the Na^+/H^+ exchanger (NHE) and the Na^+/HCO_3^- cotransporter (NBC) [31]. Inhibition of NHE by cariporide has been shown to protect cardiomyocytes by delaying pHi recovery at the time of reperfusion [35]. Inhibition of NBC also affords cardioprotection [15]. In this setting, previous studies demonstrated that prolonged acidosis at reperfusion is protective by inhibiting mitochondrial permeability transition pore (mPTP), reducing calcium overload and excessive contractile activation [22, 27, 32]. Concomitantly, during reperfusion, survival signaling pathways may be triggered and among them, the activation of RISK (Reperfusion Injury Salvage Kinase) pathway including cascades of protein kinases (*e.g.*, ERK1/2, p38 kinase, Akt) or mK_{ATP} channels are involved. Several studies suggested a link between regulation of pHi transporters, prolonged acidosis and activation of these survival kinase pathways [6, 10], while other reports indicate that ERK1/2

is activated following a transient production of ROS and participates to the regulation of NBC [2, 8, 9]. Accordingly, the aim of this study was to explore whether the cytoprotective effect of CORM-3 involves modulation of pH at reoxygenation in a model of hypoxia-reoxygenation and whether it requires MEK1/2 that is upstream of ERK1/2, p38 pathways and mK_{ATP} channels.

METHODS

Animals

Male 8 to 12 week-old wild-type C57BL/6J mice (n=37) (R. Janvier, Le Genest St Isle, France) were housed in an air-conditioned room with a 12 h light-dark cycle and received standard rodent chow and drinking water *ad libitum*. The authors have been granted a license from the institutional office “Préfecture du Val de Marne” (France) to conduct animal research (agreement A94-028-028). The investigations were performed in accordance with the European Directives (2010/63/EU).

Simulated ischemia-reoxygenation in adult mice cardiomyocytes

Adult mouse cardiomyocytes were isolated according to the technique previously described [25]. We obtained a cellular pellet which was washed 6 times with buffer containing 5% bovine calf serum and increasing concentrations of Ca^{2+} . With this method, we routinely obtained a high percentage (80%) of rod-shaped myocytes with clear striations and without visible vesicles. Only one mouse (*i.e.*, one individual isolation) was used for each set of experience. Cardiomyocytes obtained from different hearts were never pooled.

The freshly isolated cardiomyocytes were immediately re-suspended in serum-free/glucose-free medium, allowed to settle for 10 min and the supernatant was withdrawn and replaced with the serum-free/glucose-free medium bubbled with N_2 (pH 7.4). Petri dishes (35 mm) were filled with 1 mL of this final cellular suspension and incubated for 3 hours in an O_2/CO_2 incubator containing a humidified atmosphere of 1% O_2 , 5% CO_2 and 94% N_2 . These conditions aimed at simulating ischemia [25]. This simulated ischemia will be referred as “hypoxia” throughout the text of this report. After hypoxia (3 h), cells were reoxygenated

by adding 1 mL of a medium supplemented with bovine calf serum (10%) and glucose (11 mM) bubbled with O₂ (*i.e.*, final concentrations of 5% bovine calf serum and 5.5 mM glucose in 2 mL) and were placed under normoxia during 2 hours (CO₂ incubator).

To provide control conditions, freshly isolated cardiomyocytes were washed twice with a medium supplemented with bovine calf serum (5%) and glucose (5.5 mM). Petri dishes were filled with 1 mL of this final cellular suspension and cells were placed under normoxia. Reoxygenation was mimicked by adding 1 mL of the same medium. Experiments were performed at 37°C.

Hypoxia-reoxygenation sequences were performed either in 1) HCO₃⁻-containing medium (mM : NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄ 1.2, NaHCO₃ 12, KHCO₃ 10, HEPES 10, taurine 30, CaCl₂ 1; 37°C); under these conditions, both pH_i regulatory systems NHE and NBC are operative, or 2) in HCO₃⁻-free medium (mM : NaCl 113, KCl 4.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄ 1.2, HEPES 10, taurine 30, CaCl₂ 1 ; 37°C) which implies NBC inhibition.

CORM-3 or inactive CORM-3 (iCORM-3) (50 μM) was added at the time of reoxygenation. CORM-3 was synthesized as previously described [4] and stock solutions (5 mM) were prepared freshly the day of the experiments by solubilizing the compound in distilled water. iCORM-3 was prepared by dissolving CORM-3 in phosphate buffered saline and allowing liberation of CO over 2 days at room temperature. Residual CO was eliminated by bubbling the previous solution with N₂. The concentration of CORM-3 was defined as the concentration producing maximal protection during preliminary experiments investigating 25, 50 and 100 μM. This is in agreement with a previous report showing cardioprotection with the concentration of 50 μM [4].

To explore signalling pathways involved in cardioprotection of CORM-3, additional experiments were performed by exposing cells to reoxygenation in the presence of the mK_{ATP} channel inhibitor 5-HD (5-hydroxydecanoate, 500 μ M), the MEK1/2 inhibitor PD098059 (10 μ M) or the p38 kinase inhibitor SB202190 (10 μ M).

Measurement of cell viability

At the end of the sequence of hypoxia-reoxygenation or normoxia, cardiomyocyte survival was measured using 0.4% trypan blue solution (Sigma, Saint Quentin Fallavier, France). Cardiomyocytes were visualized under microscope. Ten fields were analyzed at $\times 20$ magnification and at least 300 cells were counted in each dish. The number of viable (unstained) and non viable (blue stained) cells was recorded and the percentage of viability was defined as the ratio between number of unstained myocytes and total cells. Survival was expressed as the percentage of the normoxic control values which were set at 100%.

pHi measurements

Freshly isolated cardiomyocytes were plated in Petri dishes pre-coated with laminin (12.5 μ g/mL) and containing 2 mL of HCO₃⁻ medium supplemented with glucose and ITS (mg/mL: recombinant human insulin 10^{-3} , human transferrin $0.55 \cdot 10^{-3}$, sodium selenite $0.5 \cdot 10^{-6}$). Cells were allowed to attach for 2 h in humidified 5% CO₂- 95% air at 37°C and then washed once to remove unattached cells before being used. All experiments were carried out at 37°C.

Adherent cardiomyocytes were incubated for 30 min with 5 μ M BCECF-AM followed by washout. Dye-loaded cells were placed into a heated perfusion chamber (Warner Instruments Inc, Hamden, CT, USA) mounted on an Olympus IX-81 motorized fluorescent inverted microscope (Olympus, Rungis, France). BCECF fluorescence was detected using excitation wavelengths of 440 and 495 nm and a wavelength emission of 535 nm. Fluorescent images

were acquired using ORCA-ER camera (Hamamatsu, Hamamatsu city, Japan). Fluorescence was integrated over a region of interest ($\approx 200 \mu\text{m}^2$) for each cardiomyocyte and the 495-to-440 nm fluorescence ratio was obtained using Cell-M software (Olympus, Rungis, France) [34].

The fluorescence ratio was converted to pH by in situ calibration using the high K^+ -nigericin method [34]. The K^+ ionophore nigericin was used to collapse the H^+ gradient, so that $(\text{K}^+_{\text{i}})/(\text{K}^+_{\text{o}}) = (\text{H}^+_{\text{i}})/(\text{H}^+_{\text{o}})$. Fluorescence ratio readings were taken for the cells exposed to high- K^+ medium (mM: KCl 140, MgCl_2 1, 2,3-butanedione monoxime 10, nigericin 0.01) over a range of pH values obtained with one of the following organic buffers (mM): MES 20 (pH 4 to 6.5), HEPES 20 (pH 7 to 8.5) and CAPSO 20 (pH 9). A calibration curve was established by measuring the fluorescence ratio values between pHi 4 and 9. The response was linear for pHi 6.5 to 8 and followed the equation $\text{pHi} = \text{pKa} - \log((R_{\text{max}} - R)/(R - R_{\text{min}}))$ where R is the 495/440 nm fluorescence ratio; R_{max} and R_{min} are the maximum and minimum ratio values obtained at pH 9 and 4, respectively. The typical parameter values were 1.79 (R_{max}), 0.37 (R_{min}) and 7.26 (pKa).

Ammonium pulse

As described above, the experiments were performed in HCO_3^- buffered medium. Under this condition, both pHi regulatory systems NHE and NBC are operative. The activity of the two transporters was assessed by evaluating the pHi recovery from an ammonium prepulse-induced acute acid load [26]. Transient exposure (4 min) of cardiomyocytes to 30 mM NH_4Cl followed by 20 min of washout with HCO_3^- buffered medium (bubbled continuously with 5% CO_2 and 95% O_2 , pH 7.4) was supplemented by several combinations of compounds. Cariporide (30 μM) was added to block NHE activity. In these conditions, the effects of CORM-3 (50 μM) alone or combined with 5-HD (500 μM) and PD098059 (10 μM) were

investigated. The intensity of pHi recovery was evaluated by a delta of pHi between the lower pHi value observed and the higher pHi value reached after the ammonium prepulse. Images were acquired every min during the 20 min of the pHi recovery period.

Statistics

All values are expressed as mean \pm SEM. Comparisons were performed using a Kruskal-Wallis analysis followed by Mann-Whitney test. Non-parametric tests were chosen regarding the low number of samples. Bonferroni correction was applied to correct for multiple comparisons. Statistical significance was defined as a value of $p < 0.05$.

RESULTS

Protective effects of CORM-3

The presence of CORM-3 reduced the mortality of cardiomyocytes exposed to 3h of hypoxia and 2h of reoxygenation in HCO_3^- -buffered solution (Figure 1A). The inactive iCORM-3 (50 μM), which is depleted of CO and used as negative control, did not protect cardiomyocytes against hypoxia-reoxygenation (HR) injury (absolute cell viability: $46\pm 2\%$ and $47\pm 1\%$ for HR and iCORM-3, respectively).

Bicarbonate-dependent cytoprotective effect of CORM-3

In this set of experiments, cardiomyocytes were incubated in HCO_3^- -free medium during the sequence of hypoxia-reoxygenation. Interestingly, the cardioprotective effect of CORM-3 was abolished by switching to the bicarbonate-free medium (Figure 1B).

The effect of CORM-3 on pHi recovery was investigated in cells superfused with HCO_3^- -buffered solution containing cariporide (Figure 2A). Following ammonium removal, while pHi recovered in the absence of cariporide, the delta of pHi recovery was significantly reduced by the addition of cariporide. When cells were exposed to cariporide plus CORM-3, this pHi recovery was almost abolished.

Signalling pathways involved in the protective effect of CORM-3

As shown in Figure 3A, the cardioprotective effect of CORM-3 was abolished by the presence of 5-HD. Similarly, the beneficial effect of CORM-3 on cardiomyocyte viability was significantly reduced by PD098059 (Figure 3B). We verified in additional experiments that 5-HD and PD098059 had no proper effects on cardiomyocyte submitted to HR (absolute cell

viability: $49\pm 4\%$, $51\pm 1\%$ vs $48\pm 2\%$ for PD098059 + HR and 5-HD + HR vs HR alone, respectively). Both HR+CORM-3-5HD and HR+CORM3-PD0908059 conditions were significantly different from corresponding HR+CORM3. In contrast, SB202190 did not modify cell viability (Figure 3C). SB202190 *per se* had no effect on cardiomyocyte viability (absolute cell viability vs HR: $49\pm 2\%$ vs $48\pm 2\%$, respectively). We also verified that 5-HD, PH098059, SB202190 had no effect *per se* on cell viability during normoxia (absolute cell viability: $56\pm 5\%$, $60\pm 3\%$; $57\pm 5\%$, 61 ± 2 for normoxia *per se*, 5HD, SB202190 and PD098059, respectively

Finally, cardiomyocytes were exposed to cariporide, CORM-3 plus 5-HD or PD098059 and pHi recovery at reoxygenation was evaluated (Figure 4). When cells were treated with 5-HD or PD098059 plus CORM-3 in the presence of cariporide, the change in pH was similar to control conditions, (absence of these inhibitors and the presence of cariporide).

DISCUSSION

Using an *in vitro* model of hypoxia-reoxygenation in adult mouse cardiomyocytes, we found that CORM-3 protected cells against reoxygenation damage at a concentration of 50 μM . The concentration of CORM-3 has been chosen based on a previous study which demonstrated the cytoprotection of CORM-3 against hypoxia-reoxygenation injury on rat H9C2 cardiac cells for this range of concentrations [4]. The fact that the inactive compound (iCORM-3) did not show any protection against reoxygenation injury strongly emphasizes that CO liberated by CORM-3 is required to induce this cardioprotective effect.

In our experimental conditions, the cardioprotective effect of CORM-3 was abolished in the absence of bicarbonate in the extracellular medium indicating a bicarbonate-dependent protective mechanism, suggesting an effect on pH restoration at reoxygenation. As NHE usually operates in parallel with NBC, it was first necessary to inhibit the activity of NHE (*i.e.*, a bicarbonate independent contributor) to better understand the effects of CORM-3 on pH restoration at reoxygenation. In these conditions, pHi recovery from an ammonium prepulse-induced acute acid load was almost abolished when cardiomyocytes were exposed to cariporide plus CORM-3. As cariporide inhibits NHE, it is reasonable to speculate that bicarbonate transporters as NBC are involved in the cardioprotection mediated by CORM-3. Although not directly demonstrated, we believe that CORM-3 inhibited NBC, *i.e.*, slowing the pHi recovery at reperfusion and thus protecting cardiomyocytes through inhibition of mPTP opening. Indeed, the regulation of pHi in cardiomyocytes depends on the activity of at least five transporters. NHE and NBC mediate acid-extrusion while $\text{Cl}^-/\text{HCO}_3^-$ (CBE) and Cl^-/OH^- (CHE) exchanges mediate acid loading. A lactate/ H^+ cotransport (monocarboxylate transporter, MCT) contributes to lactate washout at reperfusion [32]. The role of NHE in ischemia-reperfusion injury has been widely investigated. The inhibition of this exchanger has

been shown to protect from contractile dysfunction in correlation with reduced cellular Na^+ and Ca^{2+} levels [18-20].

The contribution of NBC to pHi recovery at reperfusion has been probably underestimated in several studies due to the use of HCO_3^- -free medium. Indeed, investigations performed in isolated hearts and cardiomyocytes demonstrated an important contribution of NBC to proton efflux at reperfusion [31]. Vandenberg et al. [31] reported pharmacological evidence suggesting that NBC contributed even more than NHE to the pHi recovery of reperfusion in ferret hearts. The coupling of NBC to Na^+ -coupled acid extrusion is of interest. Schafer et al. [28] demonstrated in adult rat cardiomyocytes that simultaneous inhibition of NBC and NHE during reoxygenation prevented pHi recovery, attenuated Ca^{2+} oscillations and abolished hypercontracture. Thus, NHE but also NBC contribute to Na^+ and Ca^{2+} overload at reperfusion.

Ten Hove et al. [30] showed that inhibition of one of these transporters did not increase the activity of the other one. Therefore, inhibition of only one transporter should be sufficient to slow the recovery of pHi at reperfusion and to induce cardioprotection. One study showed that mRNA levels of the electrogenic NBC form was increased by aortic constriction and that acid extrusion through NBC was increased in hypertrophied myocytes [36]. Furthermore, overexpression of NBC (mRNA and proteins) has been demonstrated in human cardiomyopathy [15]. This highlights NBC as an interesting target during ischemic disease. Indeed, a previous study suggested that selective inhibition of NBC induces cardioprotection as Kandoudhi et al. [15] demonstrated that administration of anti-NBC antibody to ischemic-reperfused rat hearts markedly protects systolic and diastolic functions during reperfusion. It should be stressed that our study did not determine the exact nature of the transporter targeted by CORM-3. It should be noted that another HCO_3^- dependent acid extruder, the Cl^-

$\text{HCO}_3^-/\text{Na}^+$ dependent cotransport (NDCBE or NBCn2) has been discovered in neonatal mouse heart and might participate to H^+ equivalent efflux [24].

This study also demonstrates that the effects of CORM-3 on bicarbonate-mediated pH recovery and hypoxia-reoxygenation viability involve mitochondrial K_{ATP} channels opening MEK1/2 and as a result probably ERK1/2 activity. Indeed, CORM-3 significantly reduces the variation of pH observed under cariporide (and *vs* control conditions), this means that 5-HD and PD098059 abolished the effects of CORM-3 on pH recovery. Indeed, we can speculate that CORM-3 triggered the opening of mK_{ATP} channels, which led to transitory mitochondrial ROS release in cytosol. This acted as the signalling molecules that induced the activation of MEK1/2 and subsequent inhibition of NBC. It should be acknowledged that we did not use a selective inhibitor of mitochondrial K_{ATP} but the involvement of the K_{ATP} channels in the cardioprotective effect of CORM-3 was already demonstrated by Clark et al. [4]. Furthermore, it has been demonstrated that CO activates K_{ATP} channels and that heme binding to a subunit of this protein is required for the CO-dependent increase in channel activity [15]. Opening of mK_{ATP} channels is known to induce transient production of ROS by mitochondria [7, 12, 23]. Furthermore, it has been demonstrated that CO-RMs and CO induce a transient and moderate production of mitochondrial ROS and particularly in cardiomyocytes [16, 17, 29, 38]. In line with our proposed mechanism, several studies showed that ERK 1/2 is activated following a transient production of ROS and participates in the regulation of NBC [2, 8, 9]. Finally, p38 MAPK was not involved in the cytoprotective effects of CORM-3 as SB202190 did not modify cell viability. This kinase is known to regulate NBC and to be involved in the cytoprotective effect of CO but it was in a context of hepatic and pulmonary ischemia-reperfusion injury and not cardiac injury [1, 37]. Finally, some limitations should be also acknowledged. The proper effects of CORM3, iCORM, 5HD and PD098059 on pH_i were not investigated as our objective was to isolate the NBC activity independently from

NHE. Some of our conclusions are also based only on the use of inhibitors and western blot experiments (e.g., MEK1/2 phosphorylation) would have strengthen this study.

In conclusion, CORM-3 protects cardiomyocytes against hypoxia-reoxygenation injury by inhibiting a bicarbonate transporter at reoxygenation, most likely the $\text{Na}^+/\text{HCO}_3^-$ symporter. This cardioprotective effect of CORM-3 requires the activation of mitochondrial K_{ATP} channels and the activation of MEK1/2 pathway.

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CONFLICT OF INTEREST

None.

FIGURES LEGENDS

Figure 1: Effect of CORM-3 (50 μM) on cardiomyocyte survival in presence (panel A, n = 8) or absence (panel B, n= 6) of HCO_3^- . Cardiomyocytes were exposed to 3 h hypoxia followed by 2 h reoxygenation at pH 7.4 (HR). Control cells were incubated under normoxic conditions for 5 h (N). * $p < 0.05$ vs HR.

Figure 2: Effect of CORM-3 (50 μM) on cardiomyocyte intracellular pH (pHi) recovery from an ammonium prepulse induced acute acid load (n = 7). Panel A: schematic evolution of pHi in control conditions. Panel B: the experiments were performed in control conditions, in the presence of cariporide alone and with the combination of cariporide and CORM-3. * $p < 0.05$ vs Control. † $p < 0.05$ vs Cariporide alone.

Figure 3: Effects of 5-hydroxydecanoate (5HD, panel A), PD098059 (panel B) and SB202190 (panel C) on the cytoprotective action of CORM-3 (n = 5-6). Cardiomyocytes were exposed to 3 h hypoxia followed by 2 h reoxygenation at pH 7.4 (HR). Control cells were incubated under normoxic conditions for 5 h (N). * $p < 0.05$ vs HR. † $p < 0.05$ for HR + CORM-3 vs HR + CORM-3 + 5-HD or PD098059.

Figure 4: Effects of 5-hydroxydecanoate 5HD (panel A) and PD098059 (panel B) on the those of CORM-3 on cardiomyocyte pHi recovery from an ammonium prepulse induced acute acid load (n = 4-5).

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Figure 1

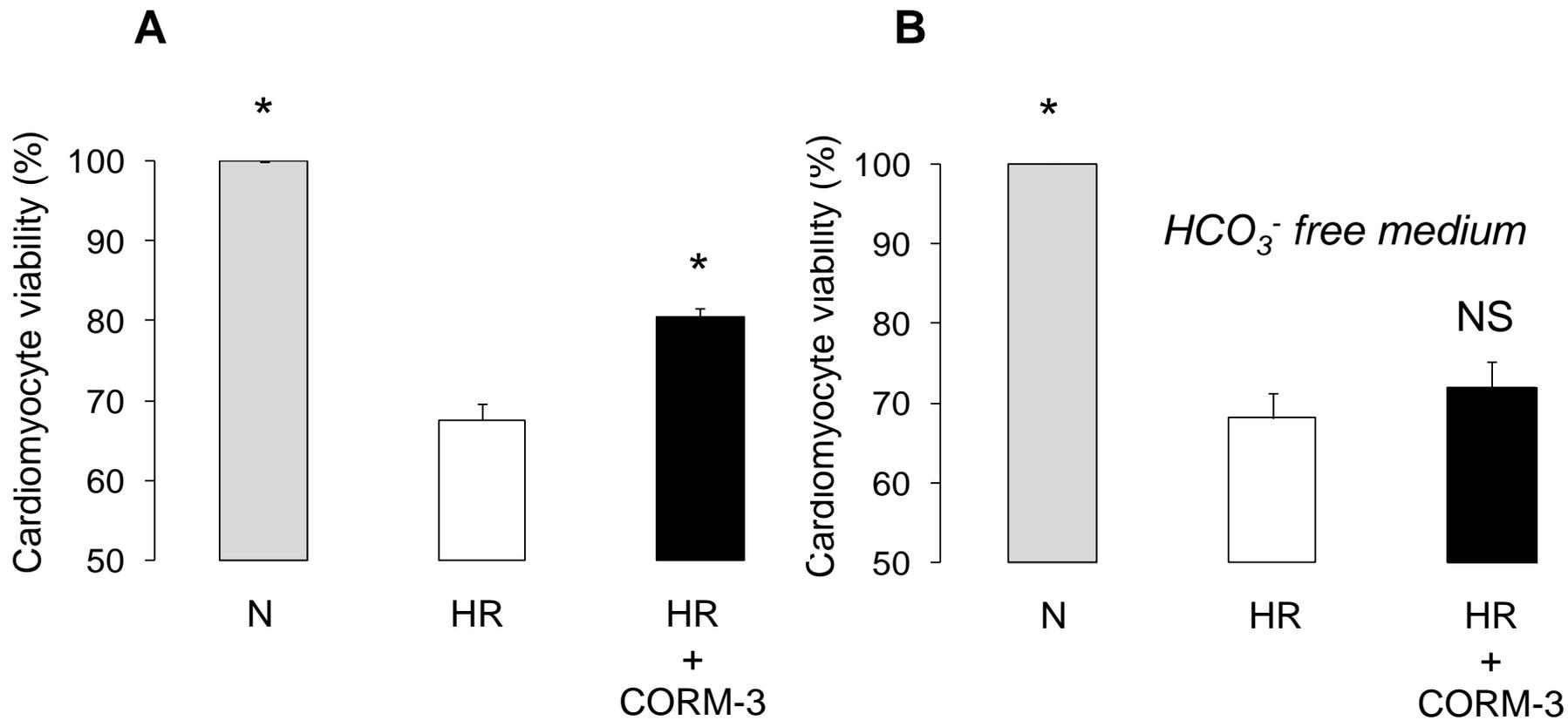
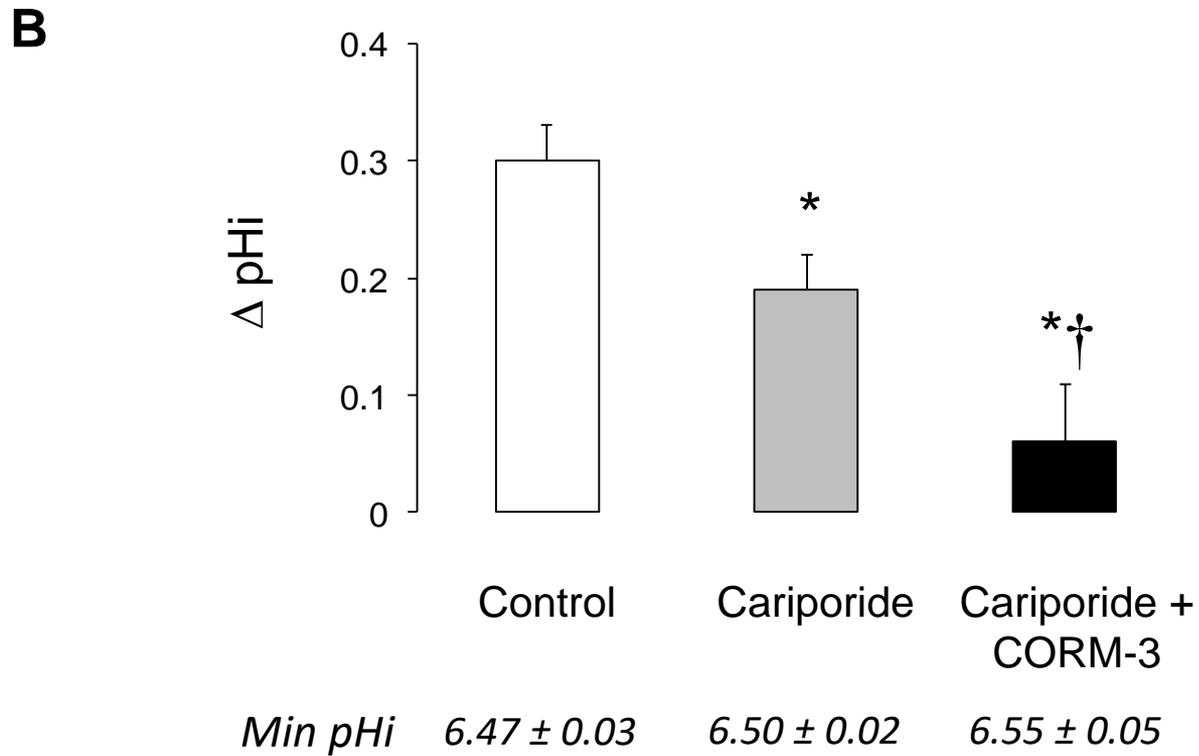
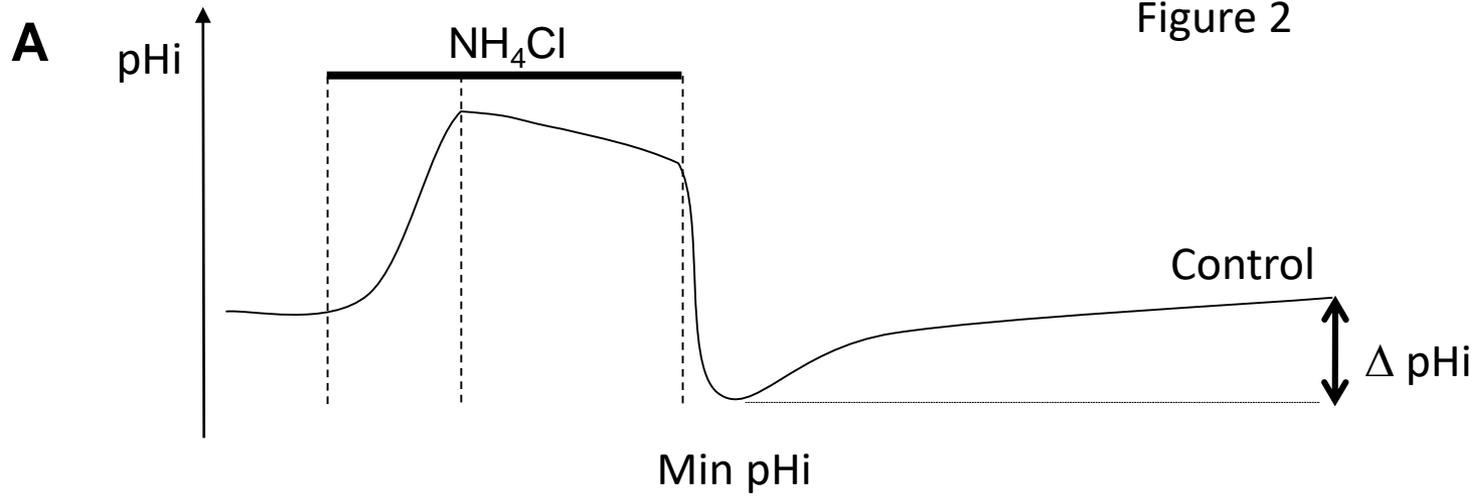


Figure 2



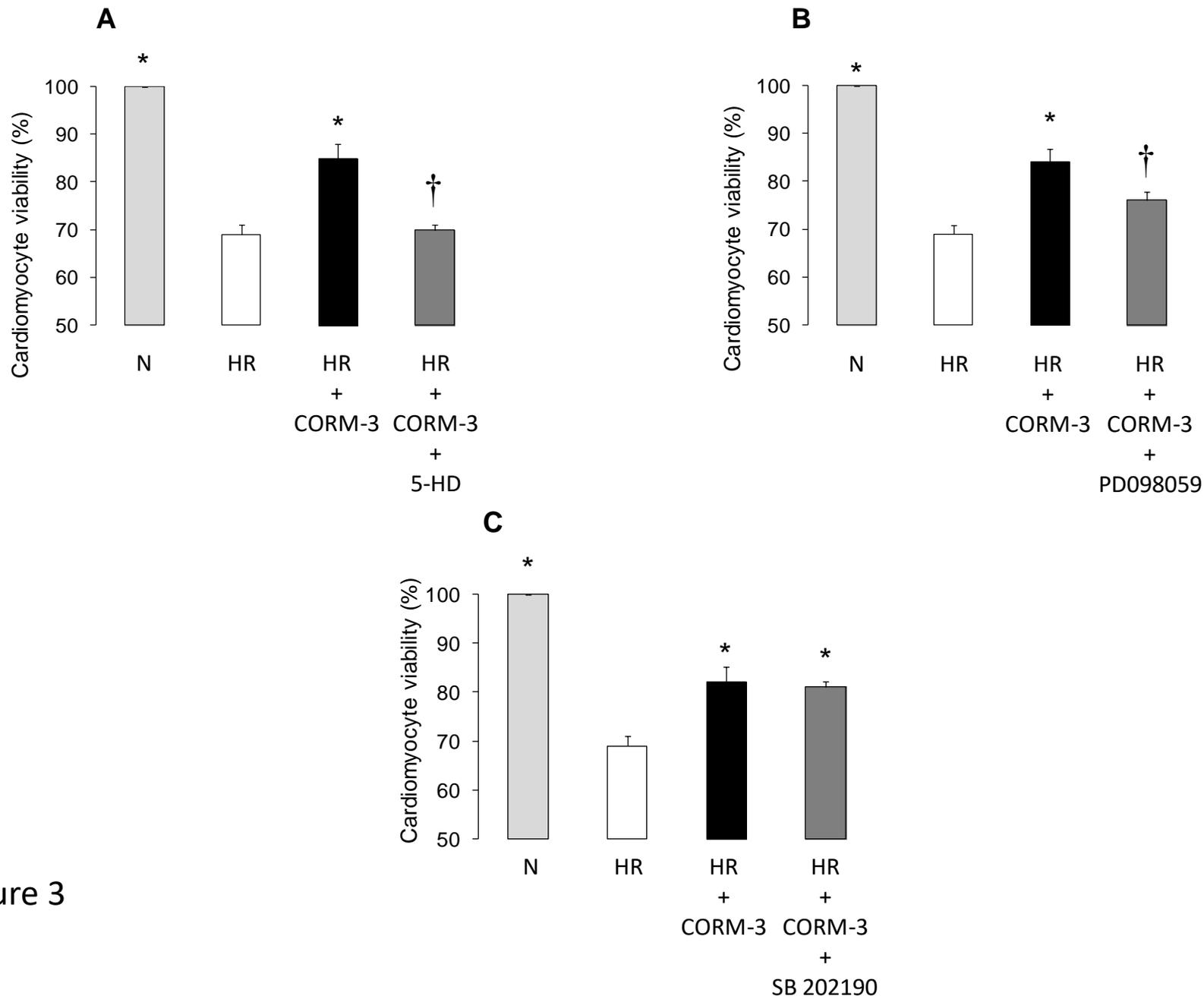


Figure 3

Figure 4

