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Bicarbonate Modulates Oxidative and Functional Damage in Ischemia-Reperfusion

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Abstract

The carbon dioxide/bicarbonate ($\text{CO}_2/\text{HCO}_3^-$) pair is the main biological pH buffer. However, its influence on biological processes, and in particular redox processes, is still poorly explored. Here we study the effect of $\text{CO}_2/\text{HCO}_3^-$ on ischemic injury in three distinct models (cardiac HL-1 cells, perfused rat heart and *C. elegans*). We found that, while different concentrations of $\text{CO}_2/\text{HCO}_3^-$ do not affect function under basal conditions, ischemia-reperfusion or similar insults in the presence of higher $\text{CO}_2/\text{HCO}_3^-$ resulted in greater functional loss associated with higher oxidative damage in all models. Since the effect of $\text{CO}_2/\text{HCO}_3^-$ was observed in all models tested, we believe this buffer is an important determinant of oxidative damage following ischemia-reperfusion.

Introduction

CO_2 , formed in a multitude of intracellular reactions, is hydrated in a reaction catalyzed by carbonic anhydrase to carbonic acid (H_2CO_3), which de-protonates generating bicarbonate (HCO_3^-). The $\text{CO}_2/\text{HCO}_3^-$ pair, with a pKa of 6.4, is the main physiological buffer, due mostly to the high concentration in biological compartments (extracellular fluid pH ranges are ~7.2; Ezraty et al., 2011; Guais et al., 2011).

Interestingly, despite its ubiquity and abundance, biological activities of the $\text{CO}_2/\text{HCO}_3^-$ pair have received very little attention, probably because there is little ability to control concentrations *in vivo*. Bicarbonate buffer, which is composed of ~1.3 mM CO_2 in equilibrium with 25 mM HCO_3^- in serum and 14 mM HCO_3^- intracellularly, has well-demonstrated redox effects (see Medinas et al., 2007 for a review). The first suggestion in this sense came from Hodgson and Fridovich in 1976, who reported that xanthine oxidase-catalyzed luminescence was dependent on the presence of carbonate. After that, a series of studies demonstrated that the presence of $\text{CO}_2/\text{HCO}_3^-$ stimulates the oxidation, peroxidation and nitration of different biomolecules (Bonini et al. 2004; Zhang et al. 2003; Liochev and Fridovich, 2004; Radi, 2004; Arai et al. 2005; Medinas et al. 2009; Surmeli et al. 2010;

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Zhou et al. 2011). The mechanism through which $\text{CO}_2/\text{HCO}_3^-$ stimulates these oxidations has been elucidated for peroxynitrite-mediated processes but remains uncovered in most cases due to methodological difficulties involving the detection of highly reactive intermediates, such as the carbonate radical (see Medinas et al., 2007 for a review).

Most studies addressing the role of $\text{CO}_2/\text{HCO}_3^-$ in biological oxidations have been exclusively conducted in *in vitro* or, less commonly, *in vivo* systems to which oxidants were added exogenously promoting overt oxidative stress followed by an evaluation of the effects of HCO_3^- (Ezraty et al., 2011). This still leaves open the question if $\text{CO}_2/\text{HCO}_3^-$ levels are relevant for oxidative injury resulting from reactive oxygen species (ROS) generated endogenously *in vivo* under physiological or pathological conditions. The question is highly relevant since, due to their reactive and diverse nature, ROS effects are mostly resultant from localized intracellular reactions (Winterbourn, 2008, Cardoso et al., 2012). In addition, quantities of added oxidants may differ very significantly from those produced intracellularly, even under pathological conditions. The demonstration that $\text{CO}_2/\text{HCO}_3^-$ levels affect tissues under physiologically relevant conditions would provide evidence, albeit indirect, of the participation of carbonate radicals in biologically relevant processes (Medinas et al., 2007).

In order to address this point, we chose to study the effects of $\text{CO}_2/\text{HCO}_3^-$ in ischemia-reperfusion (IR). IR occurs in important pathological conditions such as heart attack and stroke and involves a burst in ROS production and oxidative damage, mainly during reperfusion, that are determinant toward the final outcome of tissue damage (Martin et al., 1996; Vanden Hoek et al., 1996; Facundo et al., 2006). Furthermore, because of the nature of these pathologies, which involve changes in local tensions of diluted gasses and modifications from oxidative to fermentative metabolism, $\text{CO}_2/\text{HCO}_3^-$ levels are expected to change during IR, and may, thus, have an important role in determining the extent of post-ischemic lesions.

The effects of $\text{CO}_2/\text{HCO}_3^-$ levels on functional and oxidative damage following IR were tested in three distinct models, under conditions in which external pH was clamped despite the changes in $\text{CO}_2/\text{HCO}_3^-$ concentrations. Our results show that $\text{CO}_2/\text{HCO}_3^-$ levels contribute strongly toward post-ischemic functional loss and oxidative damage.

Materials and Methods

Materials

All chemicals were of the highest purity available from Sigma (St. Louis, MO), unless otherwise specified. BCECF was purchased from Molecular Probes (Eugene, OR). Antibody sources are described in Western blot section.

Isolated Heart Perfusion

Heart perfusion was conducted as described previously (Facundo et al., 2006). Briefly, hearts were rapidly removed from male Sprague-Dawley rats (~300 g, 2–3 months), and Langendorff-perfused with oxygenated Krebs-Henseleit buffer (described below). Hearts were eliminated from the study if the time between rat death and the beginning of perfusion

was longer than 3 min. All studies were conducted in accordance with guidelines for animal care and use established by the *Colégio Brasileiro de Experimentação Animal* and approved by the local animal ethics committee.

After isolation, the hearts were stabilized for 50 min then submitted to a 30 min ischemia and 60 min reperfusion. The reperfusion was conducted with buffers containing 0, 5 and 10% CO₂. The buffer for 0% CO₂ contained (in mmol/L) 118 NaCl, 1.2 KH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 1.25 CaCl₂, 10 glucose, and 20 Na⁺-HEPES, pH 7.4 gassed with pure O₂, at 37°C, for 5% (in mmol/L) 118 NaCl, 17 NaHCO₃, 1.2 KH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 1.25 CaCl₂, 10 glucose, and 20 Na⁺-HEPES, pH 7.4, at 37°C gassed with 95% O₂ + 5% CO₂, and for 10% (in mmol/L) 118 NaCl, 25 NaHCO₃, 1.2 KH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 1.25 CaCl₂, 10 glucose, and 20 Na⁺-HEPES, pH 7.4, at 37°C gassed with 90% O₂ + 10% CO₂. L-NAME (200 µM), when present, was added 10 min before ischemia and remained in the perfusate until the end of the reperfusion time.

Hemodynamic data were obtained using an electrode connected to a Powerlab Langendorff apparatus from ADInstruments. The pressure transducer was connected to a latex balloon and placed inside the left ventricle, as described previously (Facundo et al., 2006).

Infarcted Area

Quantification of the infarcted area was conducted as previously described (Fishbein et al., 1981; Budas et al., 2010). Briefly, after reperfusion the heart was sliced and incubated in 1% triphenyltetrazolium chloride for 15 min. The infarcted area was quantified using Image J and is presented as a percentage of the total area of the slice. Each heart was sliced in 3 places and the areas from both sides were quantified by an unblinded scorer and averaged.

Cardiac HL-1 cell cultures and simulated cellular IR

Cardiac HL-1 cells were kindly donated by Professor William C. Claycomb. These cells maintain their cardiac phenotype during extended passages and present ordered myofibrils, cardiac-specific junctions and voltage-dependent currents that are characteristic of a cardiac myocyte phenotype (Claycomb et al., 1998). For routine growth, HL-1 cells were maintained in T-75 flasks at 37°C in an atmosphere of 5% CO₂ in Claycomb medium (Sigma) supplemented with 0.1 mM norepinephrine, 100 U/mL and 100 U/mL penicillin/streptomycin, 2 mM glutamine, and 10% fetal bovine serum. Experiments were conducted at 100% confluence, after trypsinization and resuspension in a standard buffer (pH 7.4) containing (in mmol/L): 137 NaCl, 20 Na-Hepes, 22 glucose, 5 Na-pyruvate, 20 taurine, 5 creatine, 5.4 KCl, 1 MgCl₂, and 1 CaCl₂.

Cell IR was simulated as previously described (Facundo et al., 2005; Facundo et al., 2006). Briefly, 10⁶ cells/mL were submitted to simulated ischemia by metabolic inhibition using 50 mM KCN and 2 mM 2-deoxyglucose added to standard cell buffer devoid of glucose and pyruvate for 90 min, followed by 5 min centrifugation and resuspension of the cell pellet in experiment buffer for simulated reperfusion. Control HL-1 cardiomyocytes were incubated with standard buffer solution during the entire experimental period, and submitted only to centrifugations and washes. The standard buffer was gassed with 100% O₂ for the 0% CO₂

condition, and 25 mM NaHCO₃ was added to a buffer gassed with a mixture of 90% O₂ + 10% CO₂ for 10% CO₂ conditions.

Cell viability

Cell viability was assessed by relative fluorescence of 50 μM ethidium bromide (Sigma-Aldrich) using a Hitachi F4500 spectrofluorometer at excitation and emission wavelengths of 365 and 580 nm, respectively (Karsten, 1980; Facundo et al., 2005; Facundo et al., 2006). Cells were permeabilized with 0.1% Triton at the end of the each experiment to promote 100% cell death. The autofluorescence of ethidium bromide was subtracted from total fluorescence in the presence of cells, ethidium bromide and Triton. Data are expressed as the percentage of total cells.

Intracellular pH measurements

pH measurements were conducted using the highly sensitive intracellular probe BCECF, with modifications of a described method (Rink et al., 1982; Johnson and Nehrke, 2010). Cells were trypsinized, washed and resuspended in experimental buffer (described in the cell IR protocol) twice. Cells (10⁶/mL) were incubated with 5 μM of BCECF for 90 min, pelleted and resuspend in experimental buffer. The readings were conducted using a Hitachi F4500 spectrofluorometer with fixed emission at 535 nm. The excitation was scanned from 400 to 550 nm. After the measurement of the baseline fluorescence, calibration was conducted adding 10 mg/mL nigericin to allow from proton exchange across the plasma membrane and adding NaOH and HCl to promote maximal alkalinization and acidification. The intracellular pH was calculated as described by the maker. Briefly, the formula used was $[H^+] = K_a \cdot ((R-R_a)/(R_B-R)) \cdot (F_{A(\lambda_2)}/F_{B(\lambda_2)})$, where R is the $F_{(\lambda_1)}/F_{(\lambda_2)}$ ratio of fluorescence intensities (F) measured at two wavelengths $\lambda_1 = 490$ nm and $\lambda_2 = 440$ nm and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration, respectively.

C. elegans culture and strains

C. elegans were cultured using standard techniques at 20°C on normal growth media (NGM) agar plates (Brenner, 1974). Synchronized young adults were used in the experiments. Strains were: Bristol N2 (wild-type); KWN85 (*him-5(e1490)*V, *uIs22 (Pmec-18::GFP)*V).

C. elegans anoxia-starvation (AS)

IR in *C. elegans* was simulated by promoting anoxia-starvation (AS) followed by reoxygenation and feeding, as previously described (Scott et al., 2002; Wojtovich et al., 2011; Wojtovich et al., 2012). *C. elegans* young adults were collected from NGM plates, washed 3 times and resuspended in M9 media (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, 1 mM MgSO₄, pH = 7.0) supplemented with 20 mM HEPES. The animals were incubated in 100 μL of M9 in an open eppendorf tube at 26°C for 20 h under either 100% N₂ or 90% N₂ and 10% CO₂. After AS, *C. elegans* were moved to a seeded plate with a minimal amount of M9 and left to recover for another 24 h, then scored by an unblinded observer for viability and sensitivity to touch.

C. elegans neuron imaging

Animals were transferred to a 2% M9 agarose pad containing 0.1% tetramisole and 0.1% tricaine (EMS, Hatfield, PA) and were imaged within 20 minutes of being placed under a coverslip. A Nikon Eclipse TE2000-U microscope (Nikon USA, Melville, NY), Polychrome V monochromator (TILL Photonics, Germany) and Cooke Sensicam CCD (PCO-TECH Inc., Romulus, MI) were coordinated using TILLvisION software to obtain fluorescent images (470-nm excitation/535-nm emission) under a 100× oil objective.

Western blots

Western blots used 12% denaturing gels. Gels were transferred (4 hours, 400 mV) onto PVDF membranes. Protein was quantified by the Bradford technique. For carbonylation detection, 5 µg of protein were used per lane. Detection of 3-nitrotyrosine and methionine sulfoxide residues used 10 µg of protein.

The samples from hearts and cells were prepared by homogenizing the tissue or the cells in the presence of a RIPA buffer (135 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1:10 Sigma proteinase inhibitor cocktail, pH 8) and frozen at −80°C until use. For *C. elegans* samples, the live worms were selected after reperfusion and resuspended in buffer previously described by Budas et al., 2010 (0.2 M Tris-HCl, 100 mM DTT, 20% glycerol, 10% SDS and 1:10 Sigma proteinase inhibitor cocktail, pH 8), and submitted to 3 freeze/thaw cycles (liquid nitrogen/boiling water) and frozen at −80°C until use.

For the carbonylation Western blots, samples were treated as described before (Nakamura and Goto, 1996; da Cunha et al., 2011) or the Oxyblot kit from Milipore was used and the reactions were done as described by the manufacturer. Briefly, we added SDS to the samples to reach a final concentration of 12% then submitted the proteins to a reaction with 2,4-dinitrophenylhydrazine (DNPH) for 30 min followed by the addition of a neutralization buffer. For detection, we used 1:5000 anti-DNP antibody from Sigma, and 1:7000 anti-rabbit from Calbiochem. For other Western blots, we added the protein with sample buffer (20 µg). Antibody concentrations were: anti-nitrotyrosine from Upstate, 1:5000; and anti-mouse from Calbiochem, 1:5000; anti-methionine sulfoxide from Upstate, 1:5000 and anti-rabbit from Calbiochem, anti-phospho-Akt(Ser473) from Cell Signaling, 1:5000.

The blots were scanned and analyzed using Image J. Images were converted to 8 bits color and intensities of the whole lane were included. Blots were compared to the 0% CO₂ control or to the 0% CO₂ ischemic group. In the 3-nitrotyrosine blot, we ran a standard amount of nitrated protein to quantify modified tyrosine.

Statistics

All experiments presented were replicated at least three times, and statistical analysis was conducted using GraphPad Prism 5. Panels A, B, D and E from Figure 2 were analyzed using 2-way ANOVA followed by Bonferroni correction, while all other data were analyzed using Student t-tests. Correlations were analyzed using linear fits. Differences were considered significant if $p < 0.05$.

Results

Our aim in this work was to evaluate the impact of $\text{CO}_2/\text{HCO}_3^-$ on oxidative and functional tissue damage under the pathologically relevant condition of IR. Since $\text{CO}_2/\text{HCO}_3^-$ is a vital buffer, and we wished to focus on the effects of $\text{CO}_2/\text{HCO}_3^-$ itself, and not changes in pH, all extracellular solutions used in this study were buffered using HEPES, and the pH was carefully adjusted after gassing. Additionally, we questioned if, despite the clamped pH, changes in extracellular $\text{CO}_2/\text{HCO}_3^-$ concentrations could result in alterations in intracellular pH. In order to address this question, we used cardiac HL-1 cells, a cell line which maintains cardiac phenotypes and has been extensively used to study cardiac IR (Fig. 1, Claycomb et al., 1998; White et al., 2004, Facundo et al., 2006). Cells were loaded with the intracellular pH probe BCECF, and intracellular pH was measured in the absence or presence of $\text{CO}_2/\text{HCO}_3^-$ (indicated as the percentage of gassed CO_2 , 0% or 10%). We found that intracellular pH was indistinguishable under both incubation conditions (Fig. 1A). Thus, the conditions established allow for the evaluation of the biological role of $\text{CO}_2/\text{HCO}_3^-$ independently of changes in physiological intracellular pH.

We then submitted the cells to simulated IR (see Materials and Methods) in the presence of 0 and 10% CO_2 (Fig. 1B). We found that after IR, cells incubated in buffer containing $\text{CO}_2/\text{HCO}_3^-$ (full bars, 95 min IR) had significantly lower viability compared to cells incubated in the absence of $\text{CO}_2/\text{HCO}_3^-$ (open bars). Indeed, cell viability in the absence of CO_2 was similar to that of cells submitted to 95 min incubation and centrifugations, but not IR. Cell viability before the ischemic intervention (0 min) and under non-ischemic conditions (95 min) was similar in both $\text{CO}_2/\text{HCO}_3^-$ -containing and 0% CO_2 groups, indicating that changes in $\text{CO}_2/\text{HCO}_3^-$ levels do not affect cell viability under physiological conditions, but exacerbate cell death after IR.

In order to verify if the loss of cell survival was associated with oxidative damage, we measured protein carbonyls in cell lysates. We found that incubation and centrifugation of samples during 95 min in the absence of IR increased carbonyl levels slightly relative to baseline in both 0 and 10% CO_2 (Fig. 1C, 95 min). However, after 95 min IR, very significant increments in protein carbonyl levels were observed, and this increase was substantially larger in 10% CO_2 samples. Together, these results demonstrate that the presence of $\text{CO}_2/\text{HCO}_3^-$ substantially affects cell survival and oxidative damage after IR in cardiac cells.

Given the striking results of changes in $\text{CO}_2/\text{HCO}_3^-$ concentrations in cells submitted to IR, we sought next to evaluate the effects of these on ischemic hearts. Langendorff-perfused rat hearts were either maintained for 150 min without any intervention (non-ischemic) or submitted to IR as described in Materials and Methods (Fig. 2). We found that different gassed CO_2 concentrations (0, 5 or 10%) did not affect non-ischemic heart beat rates (BPM, Fig. 2A) or left ventricular developed pressure (Fig. 2B), a measure of cardiac function. Furthermore, different CO_2 concentrations did not affect activating Akt phosphorylation, a known determinant of infarct injury (results not shown). On the other hand, ischemic hearts perfused with 10% CO_2 presented severely decreased BPM (Fig. 2D) and change in developed pressure (Fig. 2E) during reperfusion; the difference was significant both when

comparing the curves point by point (as shown in the figures) and integrating the area under the curve at reperfusion ($p < 0.05$ comparing 0 and 10% CO_2 using a t-test, for both BPM and developed pressure). Indeed, 10% CO_2 hearts displayed an infarcted area which was double of that observed in 0% CO_2 IR hearts (Fig. 2F). Overall, these results confirm, in a whole heart model, that $\text{CO}_2/\text{HCO}_3^-$ levels are determinant toward functional cardiac recovery after IR.

In order to evaluate if the changes in cardiac function observed were associated with oxidative damage, we measured protein carbonyl levels. While carbonyls were unaltered under different incubation conditions in non-ischemic hearts (results not shown), in IR hearts, protein carbonyl levels increased in proportion to the percent of gassed CO_2 (Fig. 3A), and were more than 50% higher in 10% CO_2 relative to the absence of this gas. Similar increases in methionine sulfoxide (Fig. 3B) and nitro-tyrosine (Fig. 3C) residue levels were also observed in 10% CO_2 tissues. These protein modifications were undetectable in non-ischemic heart samples perfused with any concentration of CO_2 . Again, our results suggest that, while different $\text{CO}_2/\text{HCO}_3^-$ do not overtly affect hearts under physiological conditions, they are determinant in functional and oxidative damage following IR.

The detection of increased nitro-tyrosine radicals in hearts perfused with CO_2 indicates the participation of nitric oxide-derived species in cardiac damage enhanced by CO_2 . Indeed, peroxynitrite in the presence of CO_2 is very efficient in promoting tyrosine nitration due to the production of nitrogen dioxide and the carbonate radical anion (reviewed in Medinas et al, 2007). In order to investigate a potential role for nitric oxide-derived oxidants in this process, we measured the effect of L-NAME, an inhibitor of nitric oxide synthases, on CO_2 -enhanced cardiac damage following IR (Fig. 4). We found that cardiac damage increases promoted by CO_2 persisted in the presence of L-NAME. While this result suggests nitric oxide synthases are not involved in the effects of CO_2 , a role for nitric oxide cannot be excluded since it can be produced through nitrite reduction during ischemia (Zweier et al., 1995; Webb et al., 2004).

We next evaluated the effect of $\text{CO}_2/\text{HCO}_3^-$ on protein carbonyl formation in *C. elegans* during anoxia/starvation (AS), as a model for worm IR. Behavior and cell morphology were also assessed in the surviving worms. We found that CO_2 had little apparent effect in the absence of AS (results not shown), while survival following AS was not altered by 0 or 10% CO_2 either (AS, Fig. 5A). Protein carbonyls under AS conditions tended, non-significantly, to increase in 10% CO_2 (Fig. 5B). Interestingly however, surviving animals exhibited subtle but significant differences in behavior, manifested as an increased defective response to light body wall touch as a function of CO_2 during hypoxia (Fig. 5C). The behavioral response to body wall touch is mediated by six mechanosensory neurons whose processes run just under the hypodermis of the animal. In order to investigate if the decrease in function in these animals was accompanied by damage to these neurons, an integrated transgene was used to label the touch cells with GFP, and two of these neurons (PLML and PLMR) were examined in detail, as described (Materials and Methods). Neuronal abnormalities that were scored included the appearance of GFP inclusions in the processes, tortuous processes and breaks, all of which have been shown to accumulate as a result of hypoxia (Dasgupta et al., 2007). The incidence of such abnormalities was significantly increased by AS in 10% CO_2

compared to 0% CO₂ (Fig 6) demonstrating that, in a whole organism model, higher CO₂/HCO₃⁻ promoted more significant tissue and functional damage following AS.

Discussion

Considering its role as the main biological buffer, it is surprising so little recent attention has been given to the biological activity of CO₂/HCO₃⁻ (Medinas et al., 2007; Guais et al., 2011). In particular, metabolic and redox effects of this buffer are expected. In this work, we evaluated the results of different tensions of CO₂, incurring in different CO₂/HCO₃⁻ levels.

Using cardiac cells, perfused rat hearts and *C. elegans*, we found that increased CO₂/HCO₃⁻ heightened the injury associated with IR (Figs. 1–3, Khandoudi et al., 1995). Previous studies have determined that increased levels of CO₂ result in increased heart beat rates (Stinson and Mattsson, 1970), but no change in pumping function (Wexels and Mjøs, 1987). However, these changes were completely reversed by normalizing pH, indicating that they are related to pH, and not to other possible biological activities of CO₂/HCO₃⁻. These data, in fact, correlate well with our finding that changes in CO₂/HCO₃⁻ in the presence of clamped perfusion pH do not alter the basal function of perfused rat hearts (Fig. 2). On the other hand, Lavani et al. (2007) found that reperfusion in the presence of high CO₂ tensions resulted in protection against cardiac damage. This result differs from ours, in which we found that higher cardiac damage in the presence of high CO₂ tensions. Since Lavani et al (2007) did not correct for pH changes, and acidic pH is strongly protective in cardiac ischemia (Kitakaze et al., 1988; Schäfer et al., 2000), it seems reasonable to propose that their effects also are attributable to pH changes promoted by altered CO₂ tensions. Our work separated the pH effect of CO₂ from other biological effects by clamping pH with high concentrations of other buffers. Although we could not ascertain that this extracellular pH clamping maintained intracellular pH in perfused hearts and *C. elegans*, measured intracellular pH was identical in cells incubated in the presence and absence of CO₂/HCO₃⁻ (Fig. 1A), indicating that changes in pH are not necessary for the detrimental effects of CO₂.

Under these conditions, it was possible to focus on the redox effects of CO₂/HCO₃⁻ under basal conditions and IR. The presence of CO₂ in solution allows for the generation of the highly reactive carbonate radical from the reaction of CO₂ with peroxynitrite. CO₂ also reacts with H₂O₂, producing peroxymonocarbonate, which is a better two-electron oxidant than H₂O₂ and decomposes to the carbonate radical in the presence of biologically ubiquitous metal ions (Ramirez et al, 2005; Medinas et al., 2010). The carbonate radical does not produce any known stable target-adducts and is therefore difficult to detect *in vivo* and even *in vitro* (Medinas et al., 2007). Peroxymonocarbonate and other oxidants may also be derived from bicarbonate. Thus, we investigated if changing CO₂/HCO₃⁻ altered markers of tissue redox state.

Levels of protein carbonyls, the only modification detected in the absence of IR, were not altered by CO₂/HCO₃⁻ under non-ischemic conditions in any of the models studied. This result is not unexpected, since bicarbonate-derived oxidants are produced secondarily to reactions promoted by other reactive oxygen and nitrogen species, which are much more abundant following IR. Indeed, we found that in both cardiac cells and perfused hearts (Figs.

1 and 3), levels of oxidized proteins following IR increase markedly with the presence and increasing levels of $\text{CO}_2/\text{HCO}_3^-$. In fact, a linear correlation was detected between carbonylated protein ($r^2 = 0.995$, $p = 0.01$) and methionine sulfoxide ($r^2 = 0.9881$, $p = 0.06$) and CO_2 levels. Changes in protein modifications were not significantly increased in *C. elegans*, although they tended to be higher; it should be pointed out that AS in *C. elegans* requires 20 h after reoxygenation to produce notable functional effects, and the long reperfusion time may result in the removal of many modified proteins. Despite the lack of strong evidence for changes in redox state in the *C. elegans* system, $\text{CO}_2/\text{HCO}_3^-$ affected the functional recovery of the worms following AS (Figs. 5 and 6), once again demonstrating the importance of bicarbonate in ischemic damage.

Overall, our results show that over a wide range of experimental models (cells, organs and whole organisms), the presence of $\text{CO}_2/\text{HCO}_3^-$ promotes a strong decrease in function following IR, in a manner correlated with tissue oxidative damage. This demonstrates that $\text{CO}_2/\text{HCO}_3^-$ levels are determinant toward the outcome of pathologically relevant conditions of oxidative imbalance, and may explain the protective effect of modulating carbonic anhydrases (Ahmad S. 2000; Räisänen et al., 1999). While $\text{CO}_2/\text{HCO}_3^-$ are unavoidable in biological systems, our data provides a gain in understanding of the mechanisms involved in tissue damage following ischemic insults we hope will be important for future development of therapeutic interventions. Furthermore, our results provide evidence, albeit indirect, for the participation of bicarbonate radicals in pathologically relevant biological processes, and indicate that more attention should be focused on the redox biology of the $\text{CO}_2/\text{HCO}_3^-$ buffer.

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Abbreviations

DNPH	2,4-dinitrophenylhydrazine
BCECF	2'-7'-bis(carboxyethyl)-5(6)-carboxyfluorescein
BPM	beats per minute
DTT	dithiothreitol
IR	ischemia-reperfusion
AS	anoxia-starvation
NGM	normal growth media
PLML	posterior lateral microtubule cell left
PLMR	posterior lateral microtubule cell right

ROS	reactive oxygen species
SDS	sodium dodecyl sulfate

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Fig. 1. Cardiac HL-1 cells present increased oxidative damage and loss of viability when submitted to IR in the presence of CO₂
Panel A: Intracellular pH was measured as described in Materials and Methods, in the presence or absence of CO₂, after 95 min stabilization. **Panel B:** Cell viability was measured in the absence (open bars) or presence (full bars) of 10% H₂CO₃/HCO₃⁻. Cell viability was measured as described in Materials and Methods, at 0 and 95 min, in the absence or presence of IR, as indicated. **Panel C:** Protein carbonyl levels were detected as described in Materials and Methods and are shown as percent of 0% CO₂ levels at 0 min. *, p < 0.05 relative to non-ischemic, 95 min; #, p < 0.05 relative to 95 min IR in 0% CO₂.

Fig. 2. Perfused rat hearts present increased functional loss when submitted to IR in the presence of 10% CO₂
 Beats per minute (BPM, Panels A and D), left ventricular developed pressure (Panels B and E) and infarct areas (Panels C and F) were measured as described in Materials and Methods for non-ischemic (Panels A–C) or IR (Panels D–F) hearts perfused with 0% (□), 5% (◆) or 10% CO₂ (●). * $p < 0.05$ relative to IR with 0% CO₂

Fig. 3. Increases in CO₂ are accompanied by enhanced oxidative damage in IR hearts

The amount of carbonylated proteins (A), methionine sulfoxide (B) and nitro-tyrosine (C) was quantified as described in Materials and Methods following IR conducted under the conditions of Fig. 2. *, $p < 0.05$ relative to 0% CO₂; #, $p < 0.05$ relative to 5% CO₂.

Fig. 4. L-NAME does not inhibit functional loss promoted by CO₂ in IR hearts

Beats per minute (BPM, Panels A), left ventricular developed pressure (Panels B) and infarct areas (Panels C) were measured as in Fig. 2, with the addition of 200 μ M L-NAME to the perfusion media. *, $p < 0.05$ relative to 0% CO₂.

Fig. 5. 10% CO₂ decreases touch responses in *C. elegans* following IR, without affecting survival

Panel A: The percentage of worms that exhibit 24-hr post-AS survival in the presence or absence of CO₂ was measured as described in Material and Methods. The N2 strain is the canonical wild type genetic background, and KWN85 contains an integrated transgene that labels mechanosensory neurons with GFP. **Panel B:** Proteins were extracted from living *C. elegans* after AS in the presence or absence of CO₂, and protein carbonyls were detected using an Oxyblot. **Panel C:** The response to touch stimuli of living *C. elegans* after AS in the presence or absence of CO₂ was measured as described in Material and Methods. *, $p < 0.01$ relative to 0% CO₂.

Fig. 6. 10% CO₂ increases touch neuron modifications

Touch neuron (PLML and PLMR) abnormalities such as tortuous processes and breaks (Panel A) or the accumulation of GFP aggregates in the processes (Panel B) were monitored in surviving anesthetized *C. elegans* after IR in the presence or absence of CO₂, as described in Material Methods. *, $p < 0.05$ relative to 0% CO₂.