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Original Contribution

Bicarbonate modulates oxidative and functional damage in ischemia–reperfusion

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ABSTRACT

The carbon dioxide/bicarbonate (CO_2/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 CO_2/HCO_3^- on ischemic injury in three distinct models (cardiac HL-1 cells, perfused rat heart, and *Caenorhabditis elegans*). We found that, although various concentrations of CO_2/HCO_3^- do not affect function under basal conditions, ischemia–reperfusion or similar insults in the presence of higher CO_2/HCO_3^- resulted in greater functional loss associated with higher oxidative damage in all models. Because the effect of CO_2/HCO_3^- was observed in all models tested, we believe this buffer is an important determinant of oxidative damage after 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 deprotonates, generating bicarbonate (HCO_3^-). The CO_2/HCO_3^- pair, with a pK_a of 6.4, is the main physiological buffer, due mostly to its high concentration in biological compartments (extracellular fluid pH is ~7.2 [10,14]).

Interestingly, despite its ubiquity and abundance, biological activities of the CO_2/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 [23] for a review). The first suggestion in this sense came from Hodgson and Fridovich in 1976 [15], 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 CO_2/HCO_3^- stimulates the oxidation, peroxidation, and nitration of various biomolecules [2,3,21,24,27,34,42,43]. The mechanism through which CO_2/HCO_3^- stimulates these oxidations has been elucidated for peroxynitrite-mediated processes but remains uncovered in most cases because of methodological difficulties involving the detection of highly reactive intermediates, such as the carbonate radical (see [23] for a review).

Most studies addressing the role of CO_2/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^- [10]. This still leaves open the question if CO_2/HCO_3^- levels are relevant for oxidative injury resulting from reactive oxygen species (ROS)[1] generated endogenously in vivo under physiological or pathological conditions. The question is highly relevant because, owing to their reactive and diverse nature, ROS effects mostly result from localized intracellular reactions [6,39]. In addition, quantities of added oxidants may differ very significantly from those produced intracellularly, even under pathological conditions. The demonstration that CO_2/HCO_3^- levels affect tissues under physiologically relevant conditions would provide evidence, albeit indirect, of the participation of carbonate radicals in biologically relevant processes [23].

To address this point, we chose to study the effects of CO_2/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

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 medium; PLML, posterior lateral microtubule cell left; PLMR, posterior lateral microtubule cell right; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate

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reperfusion, that is a determinant of the final outcome of tissue damage [12,22,35]. Furthermore, because of the nature of these pathologies, which involve changes in local tensions of diluted gasses and modifications from oxidative to fermentative metabolism, CO_2/HCO_3^- levels are expected to change during IR and may, thus, have an important role in determining the extent of postischemic lesions.

The effects of CO_2/HCO_3^- levels on functional and oxidative damage after IR were tested in three distinct models, under conditions in which external pH was clamped despite the changes in CO_2/HCO_3^- concentrations. Our results show that CO_2/HCO_3^- levels contribute strongly toward postischemic functional loss and oxidative damage.

Materials and methods

Materials

All chemicals were of the highest purity available from Sigma (St. Louis, MO, USA), unless otherwise specified. BCECF was purchased from Molecular Probes (Eugene, OR, USA). Antibody sources are provided under Western blots.

Isolated heart perfusion

Heart perfusion was conducted as described previously [12]. Briefly, hearts were rapidly removed from male Sprague–Dawley rats (~300 g, 2–3 months of age) 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 and then subjected to 30 min ischemia and 60 min reperfusion. The reperfusion was conducted with buffers containing 0, 5, and 10% CO_2. The buffer for 0% CO_2 contained (in mmol/L) 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 10 glucose, and 20 Na^+-Hepes, pH 7.4, gassed with pure O_2, at 37°C; that for 5% (in mmol/L) 118 NaCl, 17 $NaHCO_3$, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 10 glucose, and 20 Na^+-Hepes, pH 7.4, at 37 °C gassed with 95% O_2 + 5% CO_2; and that for 10% (in mmol/L) 118 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 10 glucose, and 20 Na^+-Hepes, pH 7.4, at 37 °C gassed with 90% O_2 + 10% CO_2. 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 [12].

Infarcted area

Quantification of the infarcted area was conducted as previously described [5,13]. Briefly, after reperfusion the heart was sliced and incubated in 1% triphenyltetrazolium chloride for 15 min. The infarcted area was quantified using ImageJ and is presented as a percentage of the total area of the slice. Each heart was sliced in three 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 [7]. For routine growth, HL-1 cells were maintained in T-75 flasks at 37 °C in an atmosphere of 5% CO_2 in Claycomb medium (Sigma) supplemented with 0.1 mM norepinephrine, 100 U/ml penicillin, 100 U/ml 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 $MgCl_2$, and 1 $CaCl_2$.

Cell IR was simulated as previously described [11,12]. Briefly, 10^6 cells/ml were subjected 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 subjected only to centrifugations and washes. The standard buffer was gassed with 100% O_2 for the 0% CO_2 condition, and 25 mM $NaHCO_3$ was added to a buffer gassed with a mixture of 90% O_2 + 10% CO_2 for 10% CO_2 condition.

Cell viability

Cell viability was assessed by relative fluorescence of 50 μM ethidium bromide (Sigma–Aldrich) using a Hitachi F4500 spectrofluorimeter at excitation and emission wavelengths of 365 and 580 nm, respectively [11,12,17]. 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 a modification of a described method [16,30]. Cells were trypsinized, washed, and resuspended in experimental buffer (described in the cell IR protocol) twice. Cells (10^6/ml) were incubated with 5 μM BCECF for 90 min, pelleted, and resuspended in experimental buffer. The readings were conducted using a Hitachi F4500 spectrofluorimeter 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 alkalization and acidification. The intracellular pH was calculated as described by the maker. Briefly, the formula used was $[H^+] = K_a((R - R_A)/(R_B - R))(F_{A(\lambda 2)}/F_{B(\lambda 2)})$, where R is the $F_{(\lambda 1)}/F_{(\lambda 2)}$ ratio of fluorescence intensities (F) measured at two wavelengths, λ1, 490 nm, and λ2, 440 nm, and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration, respectively.

Caenorhabditis elegans culture and strains

C. elegans were cultured using standard techniques at 20 °C on normal growth medium (NGM) agar plates [4]. Synchronized young adults were used in the experiments. The strains used were Bristol N2 (wild type) and KWN85 (*him-5*(*e1490*)V, uIs22 (P*mec-18*::GFP)V).

C. elegans anoxia–starvation (AS)

IR in *C. elegans* was simulated by promoting AS followed by reoxygenation and feeding, as previously described [32,40,41].