Biochemical and Biophysical Research Communications 489 (2017) 399–403

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Bicarbonate alters cellular responses in respiration assays

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ARTICLE INFO

Article history:
Received 23 May 2017
Accepted 26 May 2017
Available online 27 May 2017

Keywords:
Bicarbonate
HEPES
Buffer
Mitochondria
Respiration

ABSTRACT

Metabolic assay buffers often omit bicarbonate, which is susceptible to alkalinisation in an open environment. Here, we assessed the effect of including bicarbonate in respirometry experiments. By supplementing HEPES-buffered media with low concentrations of bicarbonate, we found increased respiration in adipocytes and hepatocytes, but not myotubes. This was observed across multiple respirometry platforms and was independent of effects on enhanced insulin sensitivity, pH drift, or mitochondrial function. Permeabilised cell experiments suggest that bicarbonate increases substrate availability, likely by acting as a cofactor for carboxylase enzymes. This emphasises the importance of buffer choice in experimental biology.

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1. Introduction

The study of cell biology *in vitro* requires an intricate buffer system that is convenient, minimises fluctuations caused by experimental manipulations, and reflects physiological conditions. The criteria for an ideal pH buffer were originally outlined by Good *et al.*, who characterised a range of buffers compatible with biological experiments [1]. This was followed by Eagle, who examined the immense impact of pH buffer choice on cell growth [2]. This led to the development of standardised formulae for cell culture media and as media became commercially available, research into buffer optimisation diminished. However, recent studies have demonstrated there is still a need to better balance the physiological and logistical aspects of buffers used in cell biology experiments (e.g. [3]).

In metabolic experiments, the typical bicarbonate pH buffer used in cell culture media is susceptible to alkalinisation when not maintained at 5–10% CO_2 and thus does not permit frequent manipulations in an open environment outside the CO_2 incubator. Consequently, bicarbonate is often substituted by HEPES, which meets Good's buffer criteria [1] and is in standard mitochondrial assay buffers for cultured cells [4]. Furthermore, a popular tool in bioenergetics experiments is the XF Analyzer system [5], for which pH-buffer-free media is recommended by the manufacturer to facilitate extracellular pH flux measurements (Seahorse BioScience).

However, as the endogenous buffering system in mammals, bicarbonate is essential for metabolism for several reasons. First, it serves as a nutrient for carboxylation reactions, such as by acetyl-CoA carboxylase in lipogenesis. Second, bicarbonate alters cellular signalling responses, for example improving insulin sensitivity [6]. Third, it permits pH fluctuations in the cytosol, which can regulate metabolism (e.g. glycolysis [7]). Consequently, we assessed the potential for including bicarbonate in bioenergetic experiments. We found that bicarbonate increased mitochondrial respiration across multiple instruments in a cell-specific manner, with evidence suggesting this is due to enhanced substrate availability. This emphasises the importance of buffer choice in experimental biology.

2. Materials and methods

2.1. Cell culture

3T3-L1 fibroblasts were passaged and differentiated into

Abbreviations: 2DOG, 2-deoxyglucose; DMEM, Dulbecco's modified Eagle's medium; FCCP, trifluorocarbonylcyanide phenylhydrazone; PBS, phosphate-buffered saline.

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http://dx.doi.org/10.1016/j.bbrc.2017.05.151
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adipocytes as described previously [8], using Dulbecco's modified Eagle's medium (DMEM, bicarbonate-buffered) supplemented with 10% (v/v) foetal bovine serum and 2 mM GlutaMAX (Media A). For intact-cell experiments, adipocytes were used between days 9 and 12 after the initiation of differentiation. L6 myoblasts were cultured and differentiated into myotubes as described previously with slightly modifications [9]. Myoblasts were passaged using α-minimal essential medium (α-MEM) containing GlutaMAX supplemented with 10% (v/v) foetal bovine serum and differentiated into myotubes by switching to α-MEM containing GlutaMAX supplemented with 2% (v/v) horse serum. L6 myotubes were used between days 5 and 7 after the initiation of differentiation. AML12 cells were cultured in high glucose (4.5 g/L) DMEM, supplemented with 10% fetal bovine serum (ThermoFisher, USA). Unless otherwise stated, the DMEM used in cell culture and experiments was buffered solely by bicarbonate and cell culture reagents were obtained from Life Technologies (Scoresby, VIC, Australia).

2.2. Respirometry using the XFp and XF24 analyzers (Seahorse Biosciences, MA, USA)

On Day 7–8 of differentiation, 3T3-L1 adipocytes were trypsinised with 5× Trypsin/EDTA (Life Technologies) in phosphate-buffered saline (PBS) and seeded onto XFp cell culture plates coated with Matrigel (Corning, distributed by Sigma-Aldrich (Castle Hill, NSW, Australia)) and cultured in Media A prior to experimentation. L6 myoblasts were seeded directly onto Matrigel-coated XFp culture plates, then cultured and differentiated into myotubes. Prior to the assay, cells were washed 3 times with PBS, once with bicarbonate-free DMEM (Sigma-Aldrich) buffered with 30 mM Na-HEPES, pH 7.4 (DMEM/HEPES), and then incubated in DMEM/HEPES supplemented with 0.2% (w/v) BSA, 25 mM glucose, 1 mM GlutaMAX and 1 mM glutamine (Media B), for 1.5 h in a non-CO_2 incubator at 37 °C. Cells were then washed once with PBS, once with DMEM/HEPES, and assayed in the XFp Analyzer in Media B without BSA and supplemented with or without 10 mM $NaHCO_3$. During the assay, respiration was assayed with mix/wait/read cycles of 3/0/2 min for 3T3-L1 adipocytes and 2/0/2 min for L6 myotubes. Following assessment of basal respiration, the following compounds (final concentrations in parentheses) were injected sequentially: insulin (100 nM), oligomycin (10 μg/ml), BAM15 (10 μM), rotenone/antimycin A (5 μM/10 μM). All of these reagents were obtained from Sigma-Aldrich, except BAM15, which was provided as a gift from Kyle Hoehn (UNSW, Australia). After the assay, the media was aspirated and DNA content was measured by Hoechst staining as described previously [10], except following freeze-thaw lysis in 50 μl water per well, 50 μl TNE buffer (10 mM Tris-HCl pH 7.4, 2 M NaCl, 1 mM EDTA) was added to the wells. Lysates were then transferred to a 96-well plate before staining with 100 μl Hoechst solution, containing 10 μg/ml Hoechst-33342 (Life Technologies, instead of Hoechst-33258) in a 1:1 mixture of water and TNE buffer, and subsequent fluorescence measurement.

For AML12 experiments, cells were seeded into XF24 tissue culture plates at a cell density of 60,000 cells/well. Cells were cultured in the XF24 plates for 48 h and then assayed. Prior to respirometry analysis, cells were washed once with PBS and then incubated for 1 h in bicarbonate-free DMEM/HEPES media in a non-CO_2 incubator at 37 °C. Fresh DMEM/HEPES media supplemented with or without 10 mM $NaHCO_3$ was then added to the cells and the plate was placed inside the XF24 analyzer. During the assay, respiration was assayed with mix/wait/read cycles of 2/1/2 min. Following assessment of basal respiration, the following compounds (final concentrations in parentheses) were injected sequentially; insulin (100 nM), oligomycin (5 μM), trifluorocarbonylcyanide phenylhydrazone (FCCP, 5 μM), rotenone/antimycin A (5 μM/5 μM). At the conclusion of the assay, cells were washed once with PBS, lysed in RIPA buffer and protein content was determined via the BCA protein assay (ThermoFisher, USA).

For each experiment, $NaHCO_3$ was added fresh and the final pH was adjusted to 7.4 at 37 °C, such that the media with and without $NaHCO_3$ were within 0.05 pH units of each other.

2.3. Respirometry using the O2K electrode (Oroboros Instruments, Innsbruck, Austria)

High-resolution O_2 consumption measurements were conducted at 37 °C using the OROBOROS O2K Oxygraph (Oroboros Instruments, Innsbruck, Austria). For all experiments involving non-permeabilised cells, cells were washed with PBS, trypsinised with 0.25% trypsin/EDTA, pelleted at 2000 RPM and then resuspended in a bicarbonate-free DMEM media containing 25 mM glucose, 2 mM glutamine, and with or without 10 mM $NaHCO_3$ at a cell concentration of 1.0×10^6 cells/ml. To begin the respirometry assays, cells were first loaded into the O2K chambers and allowed to equilibrate to 37 °C. The pH of each condition ($NaHCO_3$ vs control) was then assessed via a pH electrode and subsequently clamped at 7.3 using dilute NaOH and/or HCl. The chambers were then sealed and basal respiration was assessed for approximately ~15 min. Maximal respiratory flux was determined with FCCP (5 μM). The pH of each condition was assessed at the completion of each experiment to ensure there was no deviation from 7.3.

For experiments designed to directly assess the mitochondrial oxidative phosphorylation system, cell pellets were suspended in respiration buffer [20 mM HEPES (pH 7.1), 110 mM sucrose, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 1 g/L BSA], supplemented with 0.03 mg/ml digitonin and 10 mM sodium bicarbonate or 10 mM NaCl. To begin, cells were first loaded into the O2K chambers and allowed to equilibrate to 37 °C. The pH of each chamber was then assessed via a pH electrode and subsequently clamped at 7.1 using dilute KOH and/or HCl. The chambers were sealed and State 1 respiration was assessed for approximately ~10 min in the absence of added carbon substrates. Subsequent additions of glutamate/malate (10 mM/2 mM), ADP (2 mM) and succinate (10 mM) were performed to determine the maximal capacity of oxidative phosphorylation. All permeabilised cell experiments included hexokinase (1 U/ml) and glucose (5 mM) to clamp the ADP concentration at 2 mM. Cytochrome C (0.01 mM) was added at the completion of all protocols to assess the integrity of the inner mitochondrial membrane. The pH of each condition was measured again at the completion of each experiment to ensure there was no deviation from 7.1.

2.4. Glucose uptake and consumption assays

To measure glucose uptake, the glucose analog 2-deoxyglucose (2DOG) was used. Cells were washed with PBS and incubated in DMEM supplemented with 0.2% (w/v) BSA and 2 mM GlutaMAX. After 1.5 h, cells were washed with PBS and assayed for 2DOG uptake as described previously [11], using Krebs-Ringer phosphate buffer supplemented with 30 mM Na-HEPES with or without 10 mM $NaHCO_3$. 2DOG uptake was normalised to protein content, measured using the BCA protein assay (ThermoFisher Scientific, North Ryde, NSW, Australia).

To measure glucose consumption following treatments, the plate of cells was chilled on ice and media was removed. Media was centrifuged at 2000 × *g* to remove debris and glucose content was measured using the glucose oxidase kit (ThermoFisher Scientific). Naïve treatment media was included as a control. Glucose consumption was determined by subtracting the glucose content of the conditioned media from the naïve media, and then normalised to

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