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Lactate's effect on human neuroblastoma cell bioenergetic fluxes

Lezi E.[a,b,1], Russell H. Swerdlow[b,c,d,e,*]

[a] Department of Physical Therapy and Rehabilitation Sciences, University of Kansas Medical Center, Kansas City, KS, USA
[b] Department of Neurology, University of Kansas Medical Center, Kansas City, KS, USA
[c] Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS, USA
[d] Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA
[e] University of Kansas Alzheimer's Disease Center, University of Kansas Medical Center, Kansas City, KS, USA

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ABSTRACT

Lactate, once considered a metabolic dead-end, has been recently proposed to support neuron bioenergetics. To better understand how lactate specifically influences cell energy metabolism, we studied the effects of lactate supplementation on SH-SY5Y human neuroblastoma cell bioenergetic fluxes. Lactate supplementation increased cell respiration, there was no change in respiratory coupling efficiency, and lactate itself appeared to directly support the respiratory flux increase. Conversely, lactate supplementation reduced the glycolysis flux. This apparent pro-aerobic shift in the respiration:glycolysis ratio was accompanied by post-translational modifications and compartmental redistributions of proteins that respond to and modify bioenergetic fluxes, including cAMP-response element binding protein (CREB), p38 mitogen-activated protein kinases (p38 MAPK), AMP-activated protein kinase (AMPK), peroxisome-proliferator activated receptor gamma coactivator 1 β (PGC-1β), Akt, mammalian target of rapamycin (mTOR), and forkhead box protein O1 (FOXO1). mRNA levels for PGC-1β, nuclear respiratory factor 1 (NRF1), and cytochrome *c* oxidase subunit 1 (COX1) increased. Some effects depended on the direct presence of lactate, while others were durable and evident several hours after lactate was removed. We conclude lactate can be used to manipulate cell bioenergetics.

1. Introduction

Lactate sits at a bioenergetic flux pivot point. As the end metabolite of fermentation, it is released from cells and this has contributed to the perception that lactate is a waste product of glycolysis. Lactate, however, is also taken up by cells that subsequently use it to synthesize other metabolic intermediates. For example, the Cori cycle describes the relationship in which lactate exported by glycolytic white muscle cells and imported by hepatocytes through monocarboxylate transporters (MCTs) is used to support gluconeogenesis [1]. Lactate exported by glycolytic white muscle is also imported by red muscle MCTs [2]. Following its conversion to pyruvate and passage into mitochondria, lactate carbon can enter the Krebs cycle and support respiration [3–5].

A relationship similar to that which exists between the relatively anaerobic white muscle and aerobic red muscle is observed in the brain [6]. Astrocytes catabolize glucose to lactate or generate lactate through oxidation of glutamate during aerobic glycolysis [7] and export that lactate through MCT1 [8]. Aerobic neurons may import that lactate through MCT2 and oxidize it to generate ATP. When there is an increase in neuronal activity, this strategy may help to anatomically couple synaptic activity with energy production [9]. Although this notion is still controversial and opposing views and evidence have also been reported [10], in the brain lactate transport through the cell membrane is very likely to play a crucial role in memory function, since interfering with neuronal or glial lactate transporters impairs long-term memory formation and storage [11,12].

When systemically administered, lactate enters the brain and this can have functional consequences. For example, lactate intravenous infusion reverses encephalopathy that arises in the setting of hypoglycemia-associated bioenergetic distress [13,14]. Recently, we have shown that lactate generated during exercise accesses the brain and activates expression of peroxisome-proliferator activated receptor gamma coactivator 1 (PGC-1)-related coactivator (PRC) and vascular endothelial growth factor-A (VEGF-A) [15]. This suggests lactate may influence important brain

* Corresponding author at: Department of Neurology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA.
E-mail address: rswerdlow@kumc.edu (R.H. Swerdlow).
[1] Present address: Department of Molecular Genetics and Microbiology, Duke University, Durham, NC 27710, USA.

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physiological processes including mitochondrial biogenesis, angiogenesis, and neurogenesis.

To better understand how lactate impacts bioenergetic fluxes and the status of proteins and pathways that monitor those fluxes, we exposed SH-SY5Y human neuroblastoma cells to lactate, and found that lactate rapidly altered bioenergetic fluxes and some of these changes became more profound with prolonged exposure. Lactate changed the expression of genes and also the post-translational modification of proteins that affect (and are affected by) cell energy metabolism. Durable bioenergetic flux modifications that persisted well after lactate was removed from the cell cultures were also observed.

2. Materials and methods

2.1. Cell culture

Undifferentiated SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA) were cultured at 37 °C, 5% CO_2 in regular growth medium (pyruvate-free DMEM containing 25 mM glucose, purchased from Life Technologies, Grand Island, NY) and supplemented with 10% fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO) and 1% of a penicillin–streptomycin stock (Fisher Scientific, Pittsburgh, PA). Medium was changed twice a day in order to minimize the effects of endogenously produced lactate. Cells were cultured to 60–80% confluency in T75 culture flasks before use.

2.2. Lactate treatment

24 h after seeding the cells for experiments, growth medium was removed and cells were rinsed with and then incubated in FBS-free DMEM supplemented with 5 mM glucose and 1% penicillin–streptomycin (DMEM-5). After cells adapted overnight (18–20 h) to this medium, for the lactate (LAC) treatment groups the medium was changed to DMEM-5 supplemented with either 10 mM or 25 mM sodium lactate (#L7022, Sigma–Aldrich). These concentrations of lactate were selected as they are seen in human blood and muscles after vigorous exercise [16–18]. For the control group (CT), the medium was changed to DMEM-5 without sodium lactate. The pH of the medium for all groups was adjusted to pH 7.4 at the beginning of treatment.

2.3. Quantitative real-time, reverse-transcription PCR

Total RNA was prepared from SH-SY5Y cells using the TRI Reagent (Life Technologies). Reverse transcription was performed on total RNA (1 μg) using an iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time, reverse-transcription PCR (qPCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and ready-to-use TaqMan Gene Expression Assays (Applied Biosystems) to quantify the mRNA levels of PGC-1α, PGC-1β, PRC, nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), cytochrome *c* oxidase subunit 1 (COX1), and cytochrome *c* oxidase subunit 4 (COX4). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. qPCR amplifications were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were quantified using StepOnePlus Software v2.1 based on the comparative $\Delta\Delta CT$ method.

2.4. Immunoblotting

Nuclear and cytoplasmic protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. Protein concentrations were measured using a BCA protein assay reagent kit (Thermo Scientific). Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; Cell Signaling Technology, Beverly, MA) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Densitometry was performed using a Chem-iDoc XRS with Quantity One software (Bio-Rad).

Several bioenergetic pathway proteins were analyzed. Primary antibodies purchased from Cell Signaling Technology included antibodies to phospho-Thr172 AMP-activated protein kinase (AMPK) (1:1000 dilution; #2531), AMPK (1:1000 dilution; #2603), phospho-Ser133 cAMP-response element binding protein (CREB) (1:500 dilution; #9198), p38 mitogen activated protein kinase (p38 MAPK) (1:1000 dilution; #9212), phospho-Thr180/Tyr182-p38 (1:1000 dilution; #4511), Akt (1:1000 dilution; #5373), phosphor-Ser473 Akt (1:1000 dilution; #4060), mammalian target of rapamycin (mTOR) (1:1000 dilution; #2983), phospho-Ser2448 mTOR (1:1000 dilution; #2976), TFAM (1:500 dilution; #7495), forkhead box protein O1 (FOXO1) (1:500 dilution; #2880), and GAPDH (1:2000 dilution; #2118). Primary antibodies purchased from Abcam (Cambridge, MA) included antibodies to PGC-1β (1:500 dilution; #ab61249), and citrate synthase (CS) (1:500 dilution; #ab96600). Primary antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) included antibodies to CREB (1:200 dilution; #sc-25785) and NRF1 (1:200 dilution; #sc-33771). Antibodies to PGC-1α (1:1000 dilution; #PA5-22958) and histone deacetylase 1 (HDAC1) (1:1000 dilution; #PA1-860) were purchased from Thermo Scientific. An antibody to COX4 was purchased from Life Technologies (A21348, 1: 2000). GAPDH and HDAC1 were used as internal loading controls for cytoplasmic and nuclear fractions, respectively.

2.5. Bioenergetic flux assays

The oxygen consumption rate (OCR), glycolysis rate (measured as an extracellular acidification rate, ECAR), and CO_2 evolution rate (CDER) of cells were measured using Seahorse XF24 and XF24-3 Extracellular Flux Analyzers (Seahorse Bioscience, Billerica, MA), which give real-time measurements in 24-well plates. OCR is reported in units of pmol/min, ECAR in mpH/min, and CDER in relative units. Fluxes were measured using a cycling protocol consisting of 3 min mixing, 2 min waiting, and 3 min reading in one cycle. SH-SY5Y cells were seeded in 24-well Seahorse V7 plates (Seahorse Bioscience) at a density of 5×10^4 cells/well in SH-SY5Y regular growth medium. The assays were performed as previously described with minor modifications [19].

2.5.1. Lactate acute treatment

Unbuffered, lactate-free DMEM supplemented with 5 mM glucose and 1x GlutaMAX (Life Technologies) was used as the assay running medium (pH 7.4) for all groups. After simultaneously measuring the basal respiration and glycolysis rates over 3 reading cycles in a Seahorse XF24 Analyzer, sodium lactate was injected at a final concentration of 10 mM or 25 mM to the wells of the respective LAC groups. For the control group, assay running medium of the same volume was injected. The fluxes were then measured over 5 reading cycles. Next, we injected oligomycin at a final concentration of 0.5 μM to measure and determine the proton leak rate over 3 reading cycles. The proton ionophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which is an uncoupler of oxidative phosphorylation, was subsequently injected at a final concentration of 0.3 μM and the maximal respiration was determined over 3 reading cycles. Finally, the complex I inhibitor rotenone and complex III inhibitor antimycin A were injected at final concentrations of 1 μM and 0.2 μM,