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Lactate's Effect on Human Neuroblastoma Cell Bioenergetic Fluxes

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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-1 β), Akt, mammalian target of rapamycin (mTOR), and forkhead box protein O1 (FOXO1). mRNA levels for PGC-1 β , 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.

Graphical abstract

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Keywords

Lactate; SH-SY5Y; mitochondrial respiration; glycolysis; 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, the 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 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 % CO₂ 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 hours after seeding the cells for experiments, growth medium was removed and cells were rinsed with and then incubated in FBS-free DMEM supplemented with 5mM glucose and 1 % penicillin-streptomycin (DMEM-5). After cells adapted overnight (18 – 20 hours) 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 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 ChemiDoc 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), phospho-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-1 β (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-1 α (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 CO₂ 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/minute, ECAR in mpH/minute, 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 μ M to measure and determine the proton leak rate over 3 reading cycles. The proton ionophore carbonylcyane p- trifluoromethoxyphenylhydrazone (FCCP), which is an uncoupler of oxidative phosphorylation, was subsequently injected at a final concentration of 0.3 μ M 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 μ M and 0.2 μ M, respectively, to terminate mitochondrial respiration and yield the non-mitochondrial oxygen consumption of the cells. The area under the curve (AUC) OCR at baseline, after injection of lactate or medium, post-oligomycin injection, and post-FCCP injection was calculated, and the non-mitochondrial AUC OCR value was subtracted from each.

2.5.2. Dynamic measurement of CO₂ evolution—To measure the CDER of SH-SY5Y cells using an XF24-3 Analyzer, DMEM containing 5 mM glucose and 25 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) was used as the assay running medium (pH 7.0). The experimental procedure was otherwise similar to the lactate acute treatment described in the previous section.

2.5.3. Mitochondrial stress test of lactate pre-treated cells—Cells were pre-treated with either 10 mM or 25 mM sodium lactate for 6 hours before being placed into an XF-24 Seahorse Analyzer. Unbuffered DMEM containing 5 mM glucose and 1x GlutaMAX was used as the base assay running medium (pH 7.4). For the LAC groups, the assay medium additionally contained either 10 mM or 25 mM sodium lactate. The basal OCR at 5 mM glucose (near-physiological concentration) was measured during the first 4 reading cycles. Oligomycin, FCCP, and rotenone/antimycin were subsequently and sequentially injected.

2.5.4. Glycolysis stress test of lactate pre-treated cells—Cells were pre-treated with either 10 mM or 25 mM sodium lactate for 6 hours before being placed into an XF-24 Seahorse Analyzer. Unbuffered DMEM containing 1 mM glutamine and no glucose served as the base assay running medium (pH 7.4). For the LAC groups, the assay medium also contained either 10 mM or 25 mM sodium lactate. Initially, the ECAR was measured under glucose starvation conditions for 3 reading cycles. Next, to determine the basal glycolysis rate we added glucose at a final concentration of 25 mM. For evaluating glycolysis capacity, oligomycin was subsequently injected at a final concentration of 1 μ M. Finally, we injected 2-deoxyglucose (2-DG) at a final concentration of 100 mM to again identify the non-glycolysis ECAR. The basal glycolysis rate was calculated by subtracting the non-glycolysis AUC ECAR from the post-glucose injection AUC ECAR. The glycolysis capacity was calculated by subtracting the non-glycolysis AUC ECAR from the post-oligomycin injection AUC ECAR.

2.6. Cytochrome c oxidase and citrate synthase activity assays

We evaluated COX and CS Vmax activities in SH-SY5Y cells that were treated for 6 hours with 25 mM lactate. Following this pre-incubation the cells were trypsinized and washed twice with ice-cold PBS, and then pelleted by centrifuging at $500 \times g$ for 3 minutes. Pellets were resuspended in ice-cold HBSS containing no magnesium and no calcium at a concentration of 30×10^6 cells/mL. COX and CS Vmax activities were determined as previously described [20]. COX Vmax activity was normalized both to protein concentration and to CS activity.

2.7. ATP and ADP/ATP assays

5×10^4 cells per well were plated in 96-well plates, and then treated with 25 mM lactate for 6 hours as described above. ATP levels and ADP/ATP ratios were determined using an EnzyLight™ ATP Assay Kit and ADP/ATP Ratio Assay Kit (Bioassay Systems, Hayward, CA), respectively, according to the manufacturer's instructions.

2.8. NAD⁺/NADH assay

60 mm dishes were each seeded with 1.5×10^6 cells and subsequently treated with 25 mM lactate for 6 hours as described above. NAD⁺/NADH levels were measured using a commercially available Fluorescent NAD⁺/NADH Detection Kit (Cell Technology Inc, Mountain View, CA) according to the manufacturer's instructions. NAD⁺ to NADH ratios were calculated to evaluate the redox state of the cells. Absolute NAD⁺ and NADH levels were also calculated individually after normalizing to protein content.

2.9. Statistical analyses

Data were summarized by means and standard errors. Mean values were compared by Student's independent *t*-test, paired *t*-test, or one-way analysis of variance (ANOVA) with Fisher's Least Significant Difference (LSD) post hoc testing. Statistical comparisons were performed using SPSS 18.0 (SPSS Inc., Chicago, IL). *p*-values less than 0.05 were considered statistically significant.

3. Results

We measured the acute effects of lactate on cell respiration. Shortly after adding 10 mM or 25 mM lactate to the culture medium, the OCR immediately increased by 35 % in the 10 mM group and by 30 % in the 25 mM group (Fig. 1A-B). This change was likely driven by an increase in the amount of ATP-coupled mitochondrial oxygen utilization, although differences in the ATP-coupled mitochondrial oxygen utilization between lactate-treated and untreated cells were not statistically significant (Fig. 1C). In the treatment groups, the lactate-associated OCR approximated the "maximum" OCR that was observed during FCCP-mediated uncoupling (Fig. 1D). The maximum OCRs across the groups remained comparable, though, as did the proton leak rates (Fig. 1E-F). On the other hand, the ECAR decreased immediately following lactate introduction (Fig. 1G-H).

We considered whether the observed mitochondrial OCR increases were directly or indirectly mediated by lactate. To address this question we measured the SH-SY5Y CDER

in the absence of glutamine. Glutamine is typically added to the growth media for SH-SY5Y cells as well as to the assay running media for bioenergetic flux assays, serving as a major source of energy besides glucose. An increase in glutamine utilization can support the increase in respiration that occurs when SH-SY5Y cells are deprived of glucose [21]. Even with no glutamine in the medium, the CDER significantly increased when lactate was added (Fig 2A-B). This suggests lactate directly supported the CDER increase, and by extension the OCR increase.

More pervasive OCR changes were observed when SH-SY5Y cells were analyzed 6 hours after lactate was added to the medium. Relative to control cells that were not treated with lactate, robust OCR increases were observed in both the 10 mM and 25 mM lactate-treated cells (Fig. 3A-B). Also, after 6 hours of lactate treatment the maximum OCR that was induced by FCCP was higher in the lactate treated cells than it was in the untreated cells (Fig. 3C). Although the absolute proton leak rate was higher in the 25 mM lactate treated cells, the amount of oxygen used to generate ATP and the amount of oxygen consumed due to proton leak remained proportional (Fig. 3D-F). In each group, approximately 75 % of the oxygen consumed by the mitochondria was used to generate ATP.

We also fully assessed glycolysis flux in SH-SY5Y cells exposed to lactate for 6 hours. Figure 4A shows that in the absence of glucose, the ECAR was equally low across groups. When glucose was introduced to establish the specific contribution of glycolysis to the ECAR, the resulting ECAR values were lower in the lactate treated cells than they were in the untreated cells (Fig. 4B). After adding oligomycin in order to elicit the maximum glycolysis rate, however, the ECAR values were equivalent across the groups (Fig. 4C). Under unstressed conditions, therefore, the “spare” glycolysis capacity was higher in the lactate treated cells (Fig. 4D).

The OCR changes we observed following the 6-hour lactate treatment could reflect differences in respiratory substrate delivery, changes to the respiratory infrastructure itself, or both. To help address this we prepared three groups of cells. One group of cells received no lactate pretreatment (CT), the second received a 25 mM lactate pretreatment for 6 hours before the lactate was removed (LAC-1), and the third received a 25 mM lactate pretreatment and remained in lactate during the rest of the experiment (LAC-2) (Fig. 5A). We then followed the OCRs for these three groups over a 6 hour period, and found that the resulting OCR rates were different across the groups. The control cells had the lowest OCR, the cells maintained in 25 mM lactate for the entire experiment (LAC-2) had the highest OCR, and the cells whose lactate was removed immediately prior to flux determination (LAC-1) had an intermediate OCR (Fig. 5B-C). Once the lactate was removed from the medium of the pre-treated cells (LAC-1), however, their ECAR quickly reverted to that of the control cells (Fig. 5D). Throughout the reading period, the ECAR for the cells continuously maintained in lactate (LAC-2) remained lower than the ECAR from the other two groups (Fig. 5D-E).

During this experiment, therefore, the OCR/ECAR ratios were also different across the groups. It was lowest in the control cells, intermediate in the lactate treated cells whose lactate was removed after the 6-hour pre-treatment (LAC-1), and highest in the cells

maintained in lactate throughout the experiment (LAC-2). Interestingly, for the cells maintained in lactate (LAC-2) the OCR/ECAR ratio appeared to increase over the course of the experiment (Fig. 5F-G). At the start of the reading period the OCR/ECAR ratio was 8, and by the end of the reading period it was 16.

To better understand why lactate treated cells maintained a higher OCR even after lactate was removed from their culture medium, we assessed lactate's effect on genes and proteins that increase mitochondrial mass (Fig. 6A-B). After 6 hours of 25 mM lactate exposure, PGC-1 β mRNA and protein levels were 30 % and 65 % higher, respectively, than the corresponding control cell values. PRC mRNA levels, conversely, fell by approximately 20–25 % in the 10 mM and 25 mM lactate-treated groups. The NRF1 mRNA level increased, although the amount of NRF1 protein did not change. Protein and mRNA levels for PGC-1 α and TFAM were comparable between groups.

After 6 hours of 25 mM lactate exposure, COX4 mRNA and protein levels were comparable across the groups (Fig. 7A-B). CS protein levels did not change, while COX1 mRNA increased in the 25 mM lactate-treated group (Fig. 7A-B). Compared to the control group, when normalized to total protein, COX and CS Vmax activities were both higher in the lactate-treated cells (25 % and 20 % increases, respectively) (Fig. 7C-D). The increase in the COX Vmax persisted even after normalizing its measured activity to the CS activity (Fig. 7E).

Mitochondrial mass and function are to some extent regulated by CREB, p38 MAPK, and AMPK. CREB promotes PGC-1 β transcription [22, 23], and CREB is activated by phosphorylation at its Ser133 residue. Following 6 hours of 25 mM lactate exposure, the phospho-CREB/CREB ratio increased by 135 % (Fig. 8A). p38 is known to directly phosphorylate and activate PGC-1 α , and it also can indirectly increase the expression level of PGC-1 β by activating CREB [24]. p38 activity correlates positively with the amount of phosphorylation present at its Thr180/Tyr182 residues. Following 6 hours of 25 mM lactate exposure, the phospho-p38/p38 ratio increased by 45 % (Fig. 8A). AMPK phosphorylation at its Thr172 residue was also increased by 35 % in the lactate group (Fig. 8A), suggesting that AMPK was activated in SH-SY5Y cells by lactate treatment. AMPK activation would predictably contribute to increased PGC-1 β expression in the lactate group by activating p38 MAPK [25, 26].

AMPK is itself activated in the presence of elevated AMP: ATP ratios. While we did not directly measure AMP: ATP ratios, we did measure ATP levels in the presence and absence of 25 mM lactate and found that ATP levels were comparable (Fig. 8B). We also measured ADP:ATP ratios, and found they decreased by 40 % in the lactate treated cells (Fig. 8C). Interestingly, we found that adding 2-DG to cells in the presence of lactate did not alter the ADP:ATP ratio, while adding 2-DG to cells in the absence of lactate increased the ADP:ATP ratio by 65 %.

We also measured lactate's effect on the cell redox state. While cell NAD⁺ levels did not change following a 6-hour, 25 mM lactate exposure, NADH levels actually declined by 30 % (Fig. 8D-E). Lactate, therefore, increased the NAD⁺/NADH ratio by 25 % (Fig. 8F).

A cell's bioenergetic status influences the state of its insulin signaling pathway (Fig. 9). We found that following 6 hours of 25 mM lactate treatment, Akt phosphorylation at its Ser473 residue was reduced by 40 %. mTOR phosphorylation at its Ser2448 residue was reduced by 10%. FOXO1 redistributed to the nucleus; the FOXO1 protein level declined by 20 % in the cytoplasm, and rose by 130 % in the nucleus. Taken together, these findings suggest lactate reduced insulin signaling pathway activity.

4. Discussion

We found that lactate increases undifferentiated SH-SY5Y cell respiration over its baseline state, and most likely directly supports this increase. Respiration that occurs following the addition of lactate to SH-SY5Y cultures remains coupled, and initially approximates the maximum respiratory capacity that arises through chemical-induced oxidative phosphorylation uncoupling. With prolonged lactate treatment, the mitochondrial infrastructure itself also begins to change such that the basal respiration rate and the maximal capacity at which respiration can occur increase; the lactate-induced basal respiration increase that arises following sustained exposure partly persists even after lactate levels in the medium return to normal. Conversely, lactate reduces the SH-SY5Y cell glycolysis flux. This is not a durable effect, though, since even after sustained lactate exposure, the glycolysis flux rapidly returns to its pre-treatment baseline when the lactate is removed.

Lactate-mediated changes in respiratory flux alter the status of proteins that monitor and regulate cell bioenergetics. Our data suggest lactate treatment activated CREB, p38 MAPK, and AMPK. These proteins tend to enhance respiration, and are known to collectively increase PGC-1 α gene expression and PGC-1 α protein activity [27]. Although we did not observe changes in PGC-1 α , we did observe increased mRNA and protein levels of PGC-1 β , a PGC-1 family member that also induces mitochondrial biogenesis [28]. Data from the literature suggest mitochondria produced through PGC-1 β -driven mitochondrial biogenesis are more tightly coupled, and hence more energy-efficient, than mitochondria produced through PGC-1 α -driven mitochondrial biogenesis [29–31]. Although it is currently unknown whether or not p38 MAPK and AMPK can directly activate PGC-1 β , CREB is reportedly able to promote PGC-1 β transcription [22, 23]. Given reports that AMPK phosphorylates and activates p38 MAPK, and that p38 MAPK activates CREB [24–26], our data suggest (but do not prove) that CREB, p38 MAPK, and AMPK may activate PGC-1 β to produce relatively coupled mitochondria. Figure 10 summarizes these proposed relationships.

An absolute increase in the maximum OCR following prolonged lactate exposure and persistence of the basal OCR elevation following lactate removal are consistent with either increased mitochondrial mass or increased respiratory chain efficiency. Relevant to this question, an observed increase in the mRNA levels of PGC-1 β , NRF-1 and COX 1, in conjunction with an increase in the CS Vmax activity, suggests lactate successfully activated at least a partial mitochondrial biogenesis. An increase in the COX Vmax activity that was evident after normalization to total cell protein and to CS activity could be consistent with both possibilities.

SH-SY5Y cells tend to show reciprocal OCR-ECAR relationships. We previously reported that preventing glycolysis flux via glucose starvation, or inhibiting glycolysis flux with 2-DG or iodoacetate, induces an OCR increase [32]. In those experiments, glutamine likely fueled respiration. Cell ATP levels fell, and AMPK phosphorylation increased. In the lactate experiments we now report, we determined that the lactate-associated OCR increase was not simply a consequence of reduced glycolysis, and was not strictly glutamine-dependent. Also, lactate-induced changes in the OCR/ECAR ratio were flexible enough to maintain a consistent ATP level.

ECAR measurements are determined by the rate at which cells release protons. Under most conditions, it largely reflects the rate at which lactate is produced during glycolysis and subsequently released. It is possible that high levels of lactate in the medium made it more difficult for cells to release lactate, that such an effect was responsible for the ECAR decrease, and that the glycolysis flux did not truly decline. This seems unlikely, however, since ECAR values sharply increased when oligomycin was used to inhibit mitochondrial ATP production.

Exogenous lactate can be co-transported into cells with H^+ via proton-linked MCTs. It is possible that the ECAR decrease observed with lactate exposure is due to the fall of intracellular pH, which decreases the substrate's affinity for phosphofructokinase (PFK), a rate-limiting enzyme for glycolysis [33, 34]. We, therefore, suspect that the basal glycolysis flux truly did decline, either due to an inhibition of PFK, or simply due to the law of mass action in which a build-up of intermediates at the end of a metabolic pathway retards the forward motion of that pathway. A decline in the glycolysis flux, in conjunction with a shift towards aerobic bioenergetics, may also have contributed to the observed reduction in insulin signaling. Conversely, a lactate-mediated reduction in insulin signaling may also slow glycolysis by decreasing the translocation of glucose transporters to the plasma membrane [35, 36], thereby reducing SH-SY5Y glucose uptake.

We previously found the OCR/ECAR ratio in differentiated SH-SY5Y cells to be approximately 8 [32]. Following prolonged lactate treatment, the OCR/ECAR ratio increased to that level and eventually surpassed it (after 12 hours of 25 mM lactate treatment it rose to 16). To some extent, therefore, lactate induced a reversal of Warburg metabolism. Also, lactate additionally reduced the activation state of the pro-growth insulin signaling pathway as manifested by a decrease in Akt phosphorylation, a decrease in mTOR phosphorylation, and a redistribution of FOXO1 to the nucleus. Lactate treatment in the present study, therefore, shifted the undifferentiated SH-SY5Y cells to a more differentiated state or at least a more differentiated-like metabolic state.

It is not immediately clear why AMPK phosphorylation increased with lactate treatment, especially since ATP levels did not fall. Intracellular reactive oxygen species (ROS) reportedly stimulate AMPK activity in the absence of a change in overall ATP levels [37]. Increased mitochondrial respiration may positively correlate to some extent with ROS production, and so ROS may have contributed to AMPK activation in lactate-treated cells. The ADP/ATP ratio also declined with lactate treatment. If lactate treated cells maintain a constant ATP level, but have less ADP, then an increase in AMP is suggested. If correct, an

increased AMP/ATP ratio could still potentially account for the AMPK phosphorylation increase. Lactate also prevented a glucose starvation-induced ADP/ATP ratio increase. This finding is potentially consistent with lactate's reported ability to mitigate hypoglycemia-associated brain dysfunction [38].

We were surprised to find lactate treatment increased, rather than decreased, cell NAD^+/NADH ratios since the conversion of lactate to pyruvate reduces NAD^+ to NADH. An increase in the NAD^+/NADH ratio case suggests lactate caused a decline in the rate of other reactions that produce NADH, an increase in NADH consumption, a shift from NADH to NADPH, or a combination of these possibilities. Data from our experiments can be used to argue in favor of each scenario. A reduction in the glycolysis flux would limit the reduction of NAD^+ to NADH. Increased respiration would increase the oxidation of NADH to NAD^+ . In view of the decrease of NADH concentration with lactate treatment, a shift from NADH to NADPH production would predictably occur, if glucose catabolism were shifted from glycolysis to the pentose phosphate shunt. Cells may use this strategy to regenerate glutathione and counter the presumably elevated ROS production rate that might result from a lactate-induced increase in mitochondrial respiration.

We previously demonstrated that at least for mice, exercising above the lactate threshold has pro-bioenergetic effects on the brain and that lactate itself may mediate at least some of these effects [15]. The cell culture data we now report are consistent with our animal data, and provide insight into how lactate may influence neuronal bioenergetics. While we acknowledge that our use of a tumor cell line in these experiments limits specific translational extrapolations and using differentiated human neuroblastoma cells might yield more informative results for follow-up research, the primary intent of the current study was not to use these cells to mimic human brains but rather to investigate the fundamental bioenergetic flux relationships at the cellular level. On the other hand, the data we now report have important implications for the emerging field of bioenergetic medicine [39]. Bioenergetic medicine is based on the assumption that bioenergetic intermediates can be used to alter bioenergetic fluxes, and that changing these fluxes in turn alters the function of proteins that monitor and regulate those fluxes. These changes then alter gene expression and through this produce relatively durable intracellular changes that persist even after levels of flux intermediates return to their baseline levels. The pharmacokinetic principles that underlie bioenergetic medicine, therefore, differ somewhat from those that apply to more traditional receptor-based pharmacologic approaches. Overall, data from this study argue approaches such as this can be used to manipulate cell bioenergetics, and that manipulating cell bioenergetics can itself induce extensive and durable functional changes.

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Figure 1. Acute lactate-mediated changes in mitochondrial respiration and glycolysis
 SH-SY5Y cell OCRs were measured using a Seahorse XF24 Analyzer. (A) At the first injection point assay medium or lactate at final concentrations of 10 mM or 25 mM were injected. Subsequently, final concentrations of 0.5 μ M oligomycin, 0.3 μ M FCCP, and 1 μ M rotenone plus 0.2 μ M antimycin were injected. (B) The AUC OCR between the 2nd and 3rd measured points, as well as between the 7th and 8th points, were calculated for each group. The AUC OCR between 7 and 8 was ratioed to the AUC OCR between 2 and 3 in order to evaluate the percentage change after the introduction of lactate. Both 10mM and 25mM

lactate quickly increased the SH-SY5Y cell OCR. **(C)** The ATP production or coupled AUC OCR was calculated by subtracting the post-oligomycin AUC OCR (between the 10th and 11th measured points) from the basal AUC OCR (between the 7th and 8th points). **(D)** In the control group, the FCCP-induced maximal respiration (the OCR at the 12th measured point) was significantly higher than the basal respiration (the OCR at the 8th measured point), while in both the 10mM and 25mM LAC groups the lactate-associated OCR approximated the FCCP-mediated maximal OCR. **(E)** The maximal respiration, calculated by subtracting the non-mitochondrial AUC OCR (between the 16th and 17th measured points) from the post-FCCP AUC OCR (between the 12th and 13th points) was comparable across the groups. **(F)** The proton leak-associated AUC OCR, calculated by subtracting the non-mitochondrial AUC OCR from the post-oligomycin AUC OCR (between the 10th and 11th measured points) was comparable across the groups. **(G-H)** The simultaneously measured ECAR showed an immediate decrease after lactate injection. In the lactate groups, the ECAR at the 8th measured point decreased by 25–35 % when compared to the pre-lactate injection ECAR (defined by the 3rd measured point). **AUC**, area under the curve; **BSL**, basal; **ECAR**, extracellular acidification rate; **LAC**, lactate; **OCR**, oxygen consumption rate; **MED**, assay running medium. **MR**, maximal respiration. $*p < 0.05$. $n = 5-7$. Note: The assay medium for all groups contained 5 mM glucose but no lactate.

Figure 2. Acute lactate-mediated CDER effects

(A) The carbon dioxide evolution rate (CDER) was measured with a Seahorse XF24-3 analyzer. Each point measured was normalized to the 3rd point. The relative CDER in the LAC group increased immediately after lactate injection, while in the CT group it remained unchanged for the first 3 points and then started to decline. (B) One hour after the first injection, the relative CDER (at the 9th measured point) of the LAC group was significantly higher than that of the CT group. $*p < 0.05$, $n = 5-7$. Note: The assay medium for all groups contained 5 mM glucose but no lactate.

Figure 3. Effect of a 6-hour lactate pre-treatment on SH-SY5Y mitochondrial respiratory function

Prior to the mitochondrial respiratory assay in a Seahorse XF24 Analyzer, SH-SY5Y cells in LAC groups were treated with either 10 mM or 25 mM lactate for 6 hours in the presence of 5 mM glucose. The assay was also performed in a 5 mM glucose environment, and for the LAC groups the assay medium also contained lactate. (A) OCRs from untreated cells, cells pre-treated with 10 mM lactate, or cells pretreated with 25 mM lactate for 6 hours were measured. At the times indicated, oligomycin (0.5 μ M, final concentration), FCCP (0.3 μ M,

final concentration), or rotenone (1 μ M, final concentration) plus antimycin (0.2 μ M, final concentration) were injected. **(B)** The 10 mM LAC and 25 mM LAC groups had significantly higher basal AUC OCRs than the CT group, while no difference was seen between the 10 mM and 25 mM LAC groups. **(C)** The FCCP-induced maximal AUC OCRs were comparable between the 10 mM and 25 mM LAC groups, and were both higher than that of the CT group. **(D)** When compared to the CT group, the oligomycin-mediated proton leak-associated AUC OCR was elevated in the 25 mM LAC group but not in the 10 mM LAC group. **(E)** The percentage of ATP production associated with the basal AUC OCR was comparable across the groups. **(F)** The percentage of the proton leak-associated AUC OCR was comparable across the groups. * $p < 0.05$. $n = 5-7$.

Figure 4. Effect of a 6-hour lactate pre-treatment on SH-SY5Y glycolysis

Prior to the glycolysis assay in a Seahorse XF24 Analyzer, SH-SY5Y cells in LAC groups were treated with either 10 mM or 25 mM lactate for 6 hours in the presence of 5 mM glucose. The assay medium for all groups did not contain any glucose but for the LAC groups lactate was present in the assay medium. **(A)** The ECARs of SH-SY5Y cells untreated, pre-treated with 10 mM lactate, or pre-treated with 25 mM lactate for 6 hours were measured. At the times indicated, glucose, oligomycin and 2-DG were injected at final concentrations of 25 mM, 1 μ M and 100 mM, respectively. **(B)** After glucose was introduced, the 10 mM and 25 mM LAC groups had a significantly lower basal glycolysis AUC ECAR when compared to the CT group, calculated by subtracting the non-glycolysis (post-2-DG) AUC ECAR from the post-glucose injection AUC ECAR. **(C)** Glycolysis capacity was calculated by subtracting the non-glycolysis AUC ECAR from the post-oligomycin injection AUC ECAR, and no inter-group differences were observed. **(D)** The glycolysis reserve, which represents a cell's spare glycolysis capacity, was calculated by subtracting the basal glycolysis AUC ECAR from the glycolysis capacity AUC ECAR. Both the 10 mM and 25 mM lactate treatments changed the glycolysis dynamics such that the glycolysis reserve was increased. **2-DG**, 2-deoxyglucose. * $p < 0.05$; ** $p < 0.001$. $n = 5-7$.

Figure 5. Effect of sustained lactate pre-treatment on mitochondrial respiration and the glycolysis rate

(A) Cells in the CT group received no lactate pretreatment, the LAC-1 group received a 25 mM lactate pre-treatment for 6 hours before the lactate was removed, and the LAC-2 group received a 25 mM lactate pre-treatment for 6 hours and remained in lactate during the rest of the experiment. We then measured the OCRs and ECARs for these three groups over a 6 hour period. (B-C) CT cells had the lowest OCR, LAC-2 cells had the highest OCR, and LAC-1 cells had an intermediate OCR. (D-E) Once the lactate was removed from the

medium of the pre-treated cells (the LAC-1 group), their ECAR quickly reverted to that of the CT cells. Throughout the reading period, the ECAR for the LAC-2 group (in which the cells were continuously maintained in lactate) remained lower than the ECAR from the other two groups. **(F-G)** The OCR/ECAR ratio was lowest in the CT group, intermediate in the LAC-1 group, and highest in the LAC-2 group. For cells in the LAC-2 group, the OCR/ECAR ratio increased over the course of the experiment. At the start of the reading period the OCR/ECAR ratio was 8, and by the end of the reading period it was 16. **M1**, first measured point; **M30**, 30th measured point. * $p < 0.05$; ** $p < 0.001$. $n = 5-7$.

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Figure 6. Effect of a 6-hour lactate treatment on levels of genes and proteins that regulate mitochondrial biogenesis

(A) PGC-1 β mRNA levels were significantly higher in the 25 mM LAC group but were comparable between the CT and 10 mM LAC groups. PRC mRNA levels were decreased in both LAC groups when compared to the CT group. NRF-1 mRNA levels were higher in the 25 mM LAC group but were comparable between the CT and 10 mM LAC groups. No inter-group differences were detected in mRNA levels of PGC-1 α or TFAM ($n = 6$). (B) Nuclear PGC-1 β protein levels were significantly higher in cells pre-treated with 25 mM lactate

when compared to untreated cells, but inter-group differences were not observed in the protein levels of PGC-1 α , NRF-1, or TFAM ($n = 8$). $*p < 0.05$.

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Figure 7. Effects of a 6-hour lactate pre-treatment on levels and activity of complex IV and citrate synthase

(A-B) COX1 mRNA levels were higher in the 25 mM LAC group but were comparable between the CT and 10 mM LAC groups. COX4 mRNA and protein levels were comparable across the groups ($n = 6$). CS protein levels were also comparable between lactate-treated cells and untreated cells ($n = 8$). (C-E) COX Vmax activities, when normalized to total protein content, were significantly higher in the LAC group when compared to the CT group. CS Vmax activities normalized to total protein content were also increased in the LAC group. After normalization to the CS activity, COX activities in the LAC group were

still significantly higher than those in the CT group ($n = 5$). **COX**, cytochrome *c* oxidase; **CS**, citrate synthase. $*p < 0.05$.

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Figure 8. Effect of a 6-hour lactate pre-treatment on energy-sensitive proteins, ADP/ATP levels, and intracellular redox state

(A) 6 hours of 25 mM lactate pre-treatment significantly increased cytosolic AMPK phosphorylation and p38 phosphorylation, and also increased nuclear CREB phosphorylation ($n = 8$). (B) Absolute ATP levels were comparable between cells treated with 25 mM lactate for 6 hours and untreated cells ($n = 7$). (C) ADP:ATP ratios decreased by 40 % in cells treated with 25 mM lactate for 6 hours. Adding 2-DG to cells in the presence of lactate did not alter the ADP:ATP ratio, while adding 2-DG to cells in the

absence of lactate increased the ADP:ATP ratio by 65 % ($n = 4$). **(D-F)** While cell NAD^+ levels did not change following a 6-hour, 25 mM lactate exposure, NADH levels significantly declined. Lactate treatment increased the NAD^+/NADH ratio by 25 % ($n = 6$). $*p < 0.05$; $**p < 0.001$.

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Figure 9. Lactate treatment decreased the activation state of the insulin signaling pathway
Following 6 hours of 25 mM lactate treatment, Akt phosphorylation at its Ser473 residue and mTOR phosphorylation at its Ser2448 residue were reduced. Lactate treatment also resulted in cytoplasmic to nuclear translocation of FOXO1; the FOXO1 protein level declined by 20 % in the cytoplasm, and rose by 130 % in the nucleus. * $p < 0.05$; ** $p < 0.001$. $n = 8$.

Figure 10. Summary of lactate-mediated respiration changes

Upon accessing the cytosol via MCTs, lactate enters mitochondria (either as pyruvate or as lactate) and lactate carbon subsequently enters the Krebs' cycle and enhances respiration. Either due to the presence of lactate itself, or else due to acute lactate-induced changes in respiration, proteins that stimulate PGC-1 family transcriptional coactivators (p38, AMPK, CREB, and FOXO1) are activated. This in turn leads to a durable, further enhancement of respiration.