

**EFFECTS OF EXERCISE TRAINING ON BRAIN BIOENERGETICS:
LACTATE AS A KEY MEDIATOR**

By

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Lezi E

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ABSTRACT

Exercise has been given credits for promoting health in general. Research show various exercise-induced benefits for brain in both humans and rodents, including improvement of cognitive function, increase of adult neurogenesis, and protection against the onset of neurodegenerative diseases. Despite these demonstrated beneficial effects, the underlying mechanisms are not clearly understood yet. Here, we hypothesize that muscle-generated lactate mediates some of the exercise-induced benefits on brain. Identification of the mechanisms will enable us to better apply exercise prescription clinically, and will also allow us to find a direction to develop exercise mimetics for older adults. The goal of this dissertation work is to evaluate effects of exercise training below and above the lactate threshold on brain health, explore the related mechanisms, and provide scientific evidence for developing exercise mimetics. In this work, we attempted to answer these questions from the perspective of brain bioenergetics.

Chapter II was aimed to assess the effects of 6-week moderate-intensity exercise below the lactate threshold on liver and brain bioenergetic infrastructures in young adult mice. In liver, exercise training induced an increase in monocarboxylate transporter 2 (MCT2) expression which is responsible for liver lactate uptake, and this change was accompanied by increased liver expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), sirtuin 1 (SIRT1), p38, and Complex IV subunit 4 (COX4), as well as increased AMP-activated protein kinase (AMPK) phosphorylation levels. Despite these changes, liver mitochondrial DNA (mtDNA) copy number and mitochondrial transcription factor A (TFAM) expression were reduced, suggesting the training shifted the liver's bioenergetic profile to promote gluconeogenesis, but not

oxidative phosphorylation. The only altered brain parameter observed was a reduction in tumor necrosis factor alpha (TNF- α) expression. Brain bioenergetics were unlikely modified by moderate-intensity exercise. Our study showed that liver lactate import appears to be favored over brain lactate import, possibly limiting the ability, if any, of exercise-generated lactate to modify brain bioenergetics. We speculate that exercise-mediated effects on brain may be robust only when lactate production exceeds the lactate threshold.

In order to test whether lactate mediates exercise-induced changes in brain, in *Chapter III*, we subjected young adult mice to 7-week supra-lactate threshold treadmill training (defined as training at exercise intensities above the lactate threshold). In liver, similar to what we found in studies described in *Chapter II*, training shifted the liver's bioenergetic profile to promote gluconeogenesis, and the expression levels of respiration-related genes were all down-regulated. In brain, PGC-1 related co-activator (PRC) expression and mtDNA copy number increased, suggesting mitochondrial biogenesis was induced by supra-lactate threshold treadmill training. Brain TNF- α expression fell, and vascular endothelial growth factor A (VEGF-A) expression increased. In another group of experiments, exogenously administered lactate over 14 days was found to reproduce some but not all of these observed liver and brain changes. Our data suggest that lactate is more than an exercise byproduct. It can mediate some of the exercise-induced changes in liver and brain, and lactate itself can act as a partial exercise mimetic.

In *Chapter IV*, the effect of supra-lactate threshold exercise on brain mitochondrial biogenesis was demonstrated in aged mice as well. 19-month old mice were subjected to supra-lactate threshold intensive treadmill training for 8 weeks. Brain

mitochondrial biogenesis was evidenced by increased brain PGC-1 α and citrate synthase (CS) expression, as well as increased mtDNA content. Similar to the observations in young adult mice, a slight increase in brain VEGF-A expression was found in exercise group but brain TNF- α expression was unchanged. Our exercise training protocol did not affect aging-associated increase of plasma chemokine levels. In this study, we also tested the correlations between brain mitochondrial biogenesis, neurogenesis-related factors, and systemic/brain inflammatory factors to better understand the networking between exercise-induced benefits in brain, while no significant associations were detected. Nonetheless, together with the results from *Chapter II* and *III*, we conclude that supra-lactate threshold exercise training induces brain mitochondrial biogenesis in both young and aged mice.

To investigate potential mechanisms of lactate's effects on brain bioenergetics, in *Chapter V*, we treated SH-SY5Y human neuronal cells with lactate. Our results showed that, lactate treatment significantly decreased glycolysis flux and had a prolonged enhancement of mitochondrial respiration. Exogenous lactate gradually shifted the bioenergetic metabolism towards a more aerobic state. These changes in bioenergetic fluxes were accompanied by increased expression of PGC-1 β , nuclear respiratory factor 1 (NRF-1), and COX1, while mtDNA content was unchanged, indicating some components of mitochondrial biogenesis were up-regulated. Expression of VEGF-A was also increased. These lactate-induced effects were likely mediated by activation of AMPK, p38 mitogen-activated protein kinase (MAPK), and Akt signaling pathways to modify bioenergetic infrastructures. We also demonstrated that lactate treatment decreased mammalian target of rapamycin (mTOR) activation while increased forkhead box protein

O1 (FOXO1) activation, implying a potential role for lactate in longevity. The results from this study provide novel insights into bioenergetics-based pharmacological therapies for neurodegenerative diseases with altered brain bioenergetic fluxes.

In summary, this dissertation work suggests that exercise training above the lactate threshold is effective in promoting mitochondrial biogenesis, inducing expression of angiogenic/neurogenic factors, and/or reducing inflammation in mouse brains at different ages. Lactate seems to be mediating some of these changes, and appears to have additional beneficial effects including the modification of brain bioenergetic fluxes towards a healthier state. The results from this work have extensive therapeutic implications for persons with perturbed brain energy metabolism, such as Alzheimer's and Parkinson's diseases and other neurodegenerative disorders.

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ABBREVIATIONS

2-DG	2-deoxyglucose
AD	Alzheimer's disease
AICAR	5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATP6	ATP synthase F0 subunit 6
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
BrdU	5'-bromo-2'-deoxyuridine
CCL11	C-C motif chemokine 11
COX1	Cytochrome <i>c</i> oxidase subunit 1
COX2	Cytochrome <i>c</i> oxidase subunit 2
COX4I1	Cytochrome <i>c</i> oxidase subunit 4 isoform 1
CREB	cAMP-response element binding protein
CS	Citrate synthase
CT	Control
CXCL	Chemokine (C-X-C motif) ligand
DCX	Doublecortin
DMEM	Dulbecco's Modified Eagle Medium
ECAR	Extracellular acidification rate
ELISA	Enzyme-linked immunosorbent assay
ERR- α	Estrogen-related receptor alpha
ETC	Electron transport chain
EX	Exercise

FCCP	Carbonylcyanide p-trifluoromethoxyphenylhydrazone
FOXO1	Forkhead box protein O1
FOXO3a	Forkhead box O3
G6Pase	Glucose 6-phosphatase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK3 β	Glycogen synthase kinase 3 beta
HBSS	Hank's Balanced Salt Solution
HDAC1	Histone deacetylase 1
HIF-1 α	Hypoxia-inducible factor alpha
HOMA-IR	Homeostasis model assessment of insulin resistance
IL-1 β	Interleukin-1 beta
LAC	Lactate
LSD	Fisher's Least Significant Difference post-hoc test
MCT	Monocarboxylate transporter
ME1	Malic enzyme 1
ME2	Malic enzyme 2
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
ND1	NADH dehydrogenase subunit 1
ND2	NADH dehydrogenase subunit 2
NeuN	Neuronal nucleus protein
NRF-1	Nuclear respiratory factor 1
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
p38 MAPK	p38 mitogen-activated protein kinase
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase

PCK1	Phosphoenolpyruvate carboxykinase 1
PD	Parkinson's disease
PDHA1	Pyruvate dehydrogenase alpha 1
PDHC	Pyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase kinase 4
PEPCK	Phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PGC-1	Peroxisomal proliferator-activated receptor-gamma co-activator 1
PRC	PGC-1 related co-activator
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SIRT1	NAD-dependent deacetylase sirtuin-1
TCA cycle	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TNF- α	Tumor necrosis factor alpha
VEGF-A	Vascular endothelial growth factor A
VEH	Vehicle
Vmax	Maximum initial velocity or rate of a reaction

Chapter I

Introduction

1. Exercise for brain health

Physical exercise has been shown to have systemic beneficial effects such as alleviating hyperglycemia (Richter *et al.*, 1992), hypercholesterolemia (Jia *et al.*, 2012), and hypertension (Stewart, 2004) in both human and animal studies. As discussed below, exercise training is also beneficial for some organs other than muscle, including liver and brain.

Exercise is a major challenge particularly for the liver due to its central role in maintaining glucose and lipid homeostasis as well as its function as energy supplier for the working muscle (Hoene & Weigert, 2010). Both human and animal studies have shown that aerobic exercise training could improve hepatic gluconeogenesis (Donovan & Sumida, 1997), reduce hepatic lipid level (Johnson *et al.*, 2009), and also increase the expression of antioxidant enzymes (Wilson & Johnson, 2000; Navarro *et al.*, 2004).

While the underlying neuroprotective mechanism of exercise is still not clearly understood or remain controversial, studies based on both human subjects and rodent models have shown that long-term exercise enhances learning/cognitive function, increases brain volume, and also protects against the onset of neurodegenerative diseases [for review, (Lautenschlager *et al.*, 2012; Marques-Aleixo *et al.*, 2012)].

There are several potential mechanisms mediating the beneficial effects of exercise for brain. It has been shown that exercise training increases the activity of several neurotrophic/vascular growth factors such as brain-derived neurotrophic factor (BDNF) (Vaynman *et al.*, 2004; Berchtold *et al.*, 2010; Wu *et al.*, 2011) and vascular endothelial growth factor (VEGF) (Fabel *et al.*, 2003b) to enhance neurogenesis and angiogenesis in the adult or aged brain. Long-term exercise training induces a decrease in central pro-

inflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin-1 beta (IL-1 β), and increases neuroprotective chemokines, including chemokine (C-X-C motif) ligand 1 (CXCL1) and CXCL12, that improve neuronal-glia communication, possibly contributing to enhancement of learning and memory [for review, (Packer *et al.*, 2010)]. Also, exercise may increase the activity of antioxidative enzymes to decrease free radical damage to neurons (Somani & Husain, 1996; Devi & Kiran, 2004). In terms of mitochondrial function, activities of complex I and IV on the brain mitochondrial electron transport chain (ETC) decrease upon aging, and regular treadmill exercise for 6 to 12 months is able to prevent the age-associated decline in the activities of these enzymes in mouse (Boveris & Navarro, 2008).

Here, in this dissertation, we focus on whether and how exercise training influences mitochondrial function and bioenergetic metabolism in the brain. We also discuss the possible networking between brain bioenergetics, neuronal plasticity and central/systemic inflammation under the condition of exercise.

2. Mitochondrial biogenesis and balanced bioenergetics for brain health

2.1. Regulation of mitochondrial biogenesis

Improved mitochondrial function has been recognized as an important factor in health benefits including prevention of chronic diseases such as cardiovascular disease and diabetes as well as neurodegenerative diseases (Fontan-Lozano *et al.*, 2008; Ren *et al.*, 2010; Duncan, 2011). Previous studies have shown that peroxisomal proliferator-activated receptor- γ co-activator 1 (PGC-1) family acts as a group of master regulators of mitochondrial biogenesis and cellular energy metabolism, by binding

and activating nuclear respiratory factor 1 (NRF-1) which in turn regulates the transcriptional activity of mitochondrial transcription factor A (TFAM) (Fig. I-1) (Scarpulla, 2008b; Vina *et al.*, 2009). TFAM is a nucleus-encoded protein that binds upstream of the promoters of mtDNA and promotes the transcription of mitochondrial DNA (mtDNA) (Uchiyama & Kang, 2012).

The expression of PGC-1 α , most studied member of PGC-1 family, is regulated by several mechanisms. AMP-activated protein kinase (AMPK) is a crucial sensor of the energy status of the cell, and it becomes activated when the AMP/ATP ratio is elevated. The activation of AMPK regulates PGC-1 α activity by inducing phosphorylation of PGC-1 α at Thr177 and Ser538 (Jager *et al.*, 2007). Silent mating type information regulation 2 homolog 1 (SIRT1) is an enzyme that mediates NAD⁺-dependent deacetylation of target substrates. It is generally understood that when NAD⁺ amounts or the NAD⁺/NADH ratio increases, SIRT1 is activated. Under conditions of rich nutrients or low NAD⁺ levels, PGC-1 α is heavily acetylated and biologically inactive (Rodgers *et al.*, 2005). Thus, SIRT1-mediated deacetylation and activation of PGC-1 α becomes an important response of the cell to enhance mitochondrial metabolism when energy demands increase (Fernandez-Marcos & Auwerx, 2011). p38 mitogen-activated protein kinase (MAPK) has also been shown to phosphorylate PGC-1 α and enhance its stability and thereby controls its activity (Puigserver *et al.*, 2001). In addition, cAMP response element-binding protein (CREB) is known to regulate hepatic gluconeogenesis by activating PGC-1 α (Herzig *et al.*, 2001).

PGC-1 β , another member from PGC-1 family, has high sequence similarity and similar tissue distribution as PGC1- α (Scarpulla, 2008b). PGC-1 β also binds NRF-1 and

is its potent co-activator (Lin *et al.*, 2002a). PGC-1 β plays important roles in controlling mitochondrial capacity in both cultured cells and transgenic mice (Kamei *et al.*, 2003; St-Pierre *et al.*, 2003; Sonoda *et al.*, 2007). Moreover, in neurons, PGC1- α and PGC-1 β regulate mitochondrial density in an additive manner, as when they are overexpressed together, the mitochondrial density increases more than it does when PGC1- α or PGC-1 β is overexpressed individually (Wareski *et al.*, 2009). Despite the functional similarities between these two PGC-1 family members, PGC-1 α robustly activates gluconeogenic gene expression (PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase) to a greater extent than PGC-1 β does. On the other hand, PGC-1 β is a stronger inducer of mitochondrial respiration-relevant gene expression (cytochrome *c*; ATP synthase) and promotes a much higher level of coupled respiration than PGC-1 α , which may make the fine-tuning of mitochondrial function possible in response to specific metabolic needs (Lin *et al.*, 2003; St-Pierre *et al.*, 2003; Lin *et al.*, 2005).

PGC-1 related co-activator (PRC), the most recently identified member of PGC-1 family, has been shown to associate with NRF-1 and NRF-2 as well (Scarpulla, 2008a). PRC also interacts with the estrogen-related receptor α (ERR- α) to induce mitochondrion-related gene transcription, and in general supports mitochondrial ATP production (Vercauteren *et al.*, 2009; Mirebeau-Prunier *et al.*, 2010). Silencing PRC results in mitochondrial dysfunction including abundant morphologically abnormal mitochondria, reduced ETC subunit expression and reduced ATP levels (Gleyzer & Scarpulla, 2011).

2.2. Biochemical methods to evaluate mitochondrial biogenesis

There are several ways to provide evidence for mitochondrial biogenesis or increased mitochondrial mass. Transcript or protein levels of cytochrome *c* oxidase (complex IV, or COX) subunits and ATP synthase subunits evaluated by quantitative reverse transcription-PCR or immunoblotting, respectively, should be elevated when mitochondrial biogenesis occurs. Another approach generally used is to assess the expression levels of key components of mitochondrial biogenesis pathway, such as PGC-1 α , PGC-1 β , PRC, NRF1, NRF2, and TFAM, as discussed above.

In addition to evaluation at mRNA or protein level, it is more common to use the ratio of mtDNA copy number relative to nuclear DNA copy number to test whether mitochondrial biogenesis is induced. Mitochondria have their own DNA that is about 16.5 kB. In humans, mtDNA codes for 37 genes including some subunits of complex I (NADH dehydrogenase), complex III (cytochrome *bc₁* complex), complex IV (cytochrome *c* oxidase), and ATP synthase (Fig. I-2). The quantity of DNA encoding for these proteins is supposed to be proportional to the number of mitochondria, but this should be corrected for the nuclear DNA level, such as the copy number of 18S ribosomal RNA (rRNA) (Medeiros, 2008).

2.3. Perturbed bioenergetic metabolism in neurodegenerative disorders

Mitochondrial dysfunction and perturbed bioenergetic balance increase with age in different organs (Navarro *et al.*, 2004; Bratic & Larsson, 2013), and these bioenergetic changes are also observed in the brains of some neurodegenerative diseases (Lin & Beal, 2006; Lezi & Swerdlow, 2012). For instance, decreased activity of complex I on mitochondrial ETC has been found in the brains of Parkinson's disease (PD). Similarly,

reduced complex IV activity is observed in several areas of brains as well as platelets of Alzheimer's disease (AD) patients. Complex I and II-III or IV dysfunctions are associated with familial or sporadic amyotrophic lateral sclerosis (ALS), respectively. In these neurodegenerative diseases, reduced mitochondrial respiratory function results in altered NAD⁺/NADH redox status, less ATP production and increased mitochondrial reactive oxygen species (ROS)/oxidative stress, possibly inducing or exacerbating mtDNA mutations. An accumulating body of evidence suggests that these serial adverse events may play a central roles in the pathogenesis or progression of the diseases (Lin & Beal, 2006; Swerdlow, 2011). Impaired mitochondrial biogenesis in the neurons undergoing degeneration further makes it difficult to compensate for the unbalanced mitochondrial bioenergetics observed in the diseases. Therefore, therapies targeting the perturbed brain bioenergetics have been proposed for neurodegenerative diseases, which will be discussed in 4.1. Exercise as a Bioenergetic Medicine Intervention.

3. Exercise and brain bioenergetics

It is well established that long-term exercise enhances mitochondrial biogenesis in muscles (Hood *et al.*, 2006). Although previous rodent studies have reported that exercise training is likely able to reduce the oxidative stress in the brain by increasing the activity of several antioxidants enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase [for review, (Marques-Aleixo *et al.*, 2012)], only a few studies have focused on the effects of exercise on mitochondrial biogenesis and other bioenergetic pathways in the brain.

Two rat studies by the same research group have shown that when young/adult rats were subjected to short-term exercise training a few days after inducing cerebral ischemia, the mRNA levels and/or protein levels of brain PGC-1 α , NRF1 and TFAM, as well as mtDNA content were increased when compared with the sedentary rats with ischemia (Zhang *et al.*, 2012b; Zhang *et al.*, 2012c). Another two groups recently published papers reporting that when they exercised aged rats or exercised young rats until early aging stage on treadmills, the PGC-1 α – NRF-1 – TFAM pathway was up-regulated and oxidative stress was reduced in the brain (Bayod *et al.*, 2011; Marosi *et al.*, 2012). Based on our literature search, there was only one mouse study showing increased PGC-1 α expression and mtDNA content after intensive treadmill training (25 m/min, 5 % incline, 1 hr/d, for 8 weeks) (Steiner *et al.*, 2011).

It appears that exercise indeed has an influence on mitochondrial function in the brain. However, what type/intensity of exercise training and how exercise-induced muscle activation affect non-muscle tissues, particularly brain, is not fully understood. This dissertation seeks to further explore the effects of moderate- and high-intensity exercise training on brain bioenergetics and the mechanisms mediating exercise-induced effects on non-muscle tissues, specifically, brain.

4. Bioenergetic medicine and exercise mimetics

4.1. Exercise as a bioenergetic medicine intervention

Bioenergetic medicine refers to the manipulation of a bioenergetic pathway to increase or decrease its associated fluxes to positively affect health. Bioenergetic medicine interventions intend to target mitochondrial respiratory flux as well as pathways

outside the mitochondria to indirectly modify mitochondrial function. (Swerdlow, 2013). Several bioenergetic flux-based therapy approaches have been proposed for neurodegenerative diseases, such as ketone body-based therapies, combination of malate, glucose and thiamine, and oxaloacetate treatment for AD and PD. These proposed therapies are based on their potential to modify NAD⁺/NADH redox status, glycolysis, or/and mitochondrial respiratory fluxes (Swerdlow, 2013), to provide a balanced bioenergetic state for neuron survival.

Exercise may also be considered as a type of bioenergetic medicine intervention, given its well-established effects of improving muscle mitochondrial respiratory function and energy metabolism. Although its bioenergetic modification ability for brain remains relatively unstudied, as mentioned before, it is significant that exercise training benefits the brain from other perspectives.

For these reasons and others, regular physical exercise is often recommended for middle-aged or older adults to improve insulin resistance or prevent aging-related cognitive function decline. However, the exercise tolerance (especially exercise of moderate- and high-intensity) and compliance/adherence rate are of concern. Thus, there appears to be a great need for developing exercise mimetics.

4.2. Exercise mimetics for muscle

Exercise mimetics for improving muscle function has been proposed recently. Here are several examples. 5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR), an analog of AMP, leads to AMPK activation, increased PGC-1 α expression and increased activity of citrate synthase in muscles. In addition, it reprograms muscles towards a more oxidative phenotype and improves running performance in mouse (Wang

et al., 2004; Narkar *et al.*, 2008). Similarly, muscle-specific overexpression of PGC-1 α triggers a fast- to slow-twitch transformation resulting in an increase in oxidative metabolism, mitochondrial biogenesis, and fatigue resistance (Lin *et al.*, 2002b). These studies have provided evidence for developing PGC-1 α stimulants as exercise mimetics for muscles. Angiotensin II receptor antagonists, such as telmisartan, have been demonstrated to improve decreased exercise capacity and impaired mitochondrial function of skeletal muscles in wild-type mice and mouse model of type 2 diabetes (Feng *et al.*, 2011; Takada *et al.*, 2013). Resveratrol is a type of natural phenol and has been given extensive attention during the last decade because of its various benefits for health, especially its potential to extend lifespan. Treatment of mice with high doses of resveratrol induces expression of oxidative genes and mitochondrial biogenesis in skeletal muscle and improves running endurance (Lagouge *et al.*, 2006). The possible mechanism may involve SIRT1-dependent deacetylation and activation of PGC-1 α .

4.3. Lactate as a potential exercise mimetic for brain

As discussed above, research has been conducted in exploring exercise mimetics for improving muscle function. On the other hand, it seems not easy to develop such mimetics for brain. Intensive investigation is needed first to explore and identify the mechanisms of exercise inducing benefits for the brain.

Lactate is known to be released by contracting muscles during exercise. It has long been believed to be a waste product from anaerobic glycolysis until two decades ago. In this section, we discuss the rationales for and potentials of lactate serving as an exercise mimetic for mammalian brain.

4.3.1. Lactate metabolism in brain

The transport of lactate occurs via monocarboxylate transporters (MCTs), proton-linked membrane carriers that transport monocarboxylates including lactate, pyruvate and ketone bodies across the cell membrane. In liver, two isoforms of MCTs, MCT1 and MCT2, are expressed (Bonen *et al.*, 2006), with MCT2 having higher affinity to lactate than MCT1 (Garcia *et al.*, 1995; Broer *et al.*, 1999). Lactate uptaken by the liver could be a substrate for gluconeogenesis and help maintain the blood glucose level. There are also MCTs in the brain, and several isoforms exist. MCT4 is expressed mainly by astrocytes, whereas MCT2 is primarily neuronal (Pierre & Pellerin, 2005).

In 1994, Pellerin and Magistretti proposed a hypothesis called astrocyte-neuron lactate shuttle (Pellerin & Magistretti, 1994). The concept posits that (1) neural activity increases the extracellular glutamate concentration, and (2) the glutamate is uptaken by astrocytes with concomitant stimulation of Na⁺-K⁺ ATPase and glutamine synthetase activity, which (3) stimulates anaerobic glycolysis (the conversion of glucose to lactate) in astrocytes that subsequently release lactate, which then (4) is used by neurons to fuel their activity (Fig. I-3). This hypothesis has been supported by recent studies (Suzuki *et al.*, 2011; Tarczyluk *et al.*, 2013).

Human subject studies using nuclear magnetic resonance spectroscopy techniques have reported that blood lactate also is an important energy source for the brain during exhaustive exercise. When the blood lactate level increases either by exercise or intravenous infusion of lactate, the brain can import, oxidize and utilize it via the tricarboxylic acid (TCA) cycle (Quistorff *et al.*, 2008; Gallagher *et al.*, 2009; van Hall *et al.*, 2009; Boumezbeur *et al.*, 2010; Wyss *et al.*, 2011).

4.3.2. Neuroprotective roles of lactate

Lactate has also been shown to have neuroprotective roles. Hypoglycemia has neurological consequences, such as atrophy and loss of neurons in hippocampus, and transient cognitive problems. Systemic lactate injection, intraperitoneally or intravenously, is able to prevent hypoglycemia or hyperinsulinemia induced neuronal death and cognitive dysfunction in both rodents and human subjects (Veneman *et al.*, 1994; Won *et al.*, 2012; Herzog *et al.*, 2013). Berthet *et al.* have demonstrated that after inducing oxygen and glucose deprivation, treatment of rat hippocampal slices with lactate containing medium significantly protected against neuronal death. In addition, after inducing *in vivo* cerebral ischemia in mice, an intracerebroventricular injection of lactate significantly decreased the lesion size, and improved neurologic outcome (Berthet *et al.*, 2009). Moreover, two recently published rat studies have demonstrated that the lactate (released by astrocytes) import into neurons is essential for long-term memory formation, and glucose and lactate are not functionally interchangeable (Newman *et al.*, 2011; Suzuki *et al.*, 2011).

4.3.3. Lactate induced modification of bioenergetic pathways

Little is known about lactate's benefits for brain other than serving as an alternative energy source. Lactate has been previously shown to induce the expression of mitochondrial respiration-related genes, including PGC-1 α and COX4 in L6 rat skeletal muscle cells (Hashimoto *et al.*, 2007). Although studies have shown the possibility of exogenous lactate being oxidized in the brain through TCA cycle (Gallagher *et al.*, 2009; van Hall *et al.*, 2009; Boumezbeur *et al.*, 2010), whether or not lactate induces changes in

mitochondrial respiratory function and bioenergetic infrastructure in brain has not been investigated straightforwardly.

5. Purpose and significance of the study

This dissertation aims to investigate whether and how, from the perspective of bioenergetics, exercise training influences non-muscle tissues, especially brain, in young and aged mice. We hypothesize that exercise-induced benefits for brain are mediated by lactate released from contracting muscles. We tested this hypothesis using the following approaches: exercising mice below and above the lactate threshold, peripherally administering mice with lactate, and treating human neuronal cells with lactate. The results from this work are significant for clarifying the neuroprotective effects of exercise, and providing scientific evidence for designing exercise protocols for older adults or patients with neurodegenerative diseases. In addition, a better understanding of mechanisms mediating the exercise-induced changes in the brain will enable us to develop exercise mimetics or bioenergetic medicine intervention specific for the brain in near future.

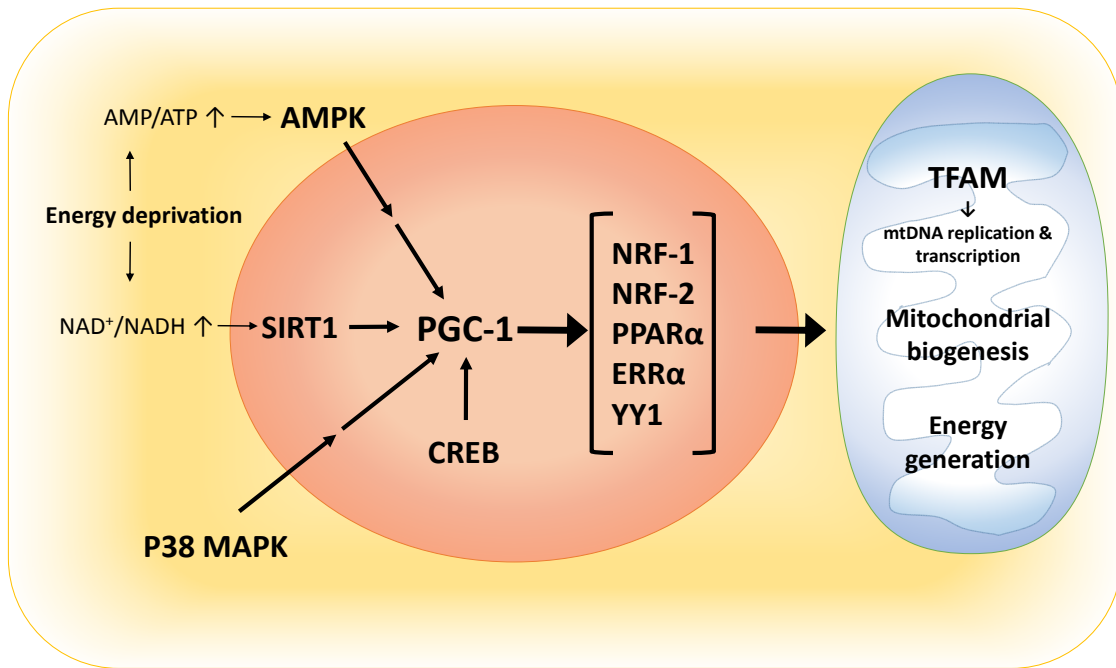


Figure I-1. Illustration summarizing PGC-1 mediated pathways governing mitochondrial biogenesis and function. Illustrated in the nucleus (orange sphere) are the key transcription factors (NRF-1, NRF-2, ERR α , PPAR α , and YY1) that are PGC-1 targets and act on nuclear genes controlling mitochondrial biogenesis and energy production. Some of the pathways involved in regulation of expression or function of PGC-1 are also shown. Abbreviations: AMPK, AMP-activated protein kinase; CREB, cAMP response-element binding protein; ERR α , estrogen related receptor α ; MAPK, mitogen-activated protein kinase; NRF, nuclear respiratory factor; PGC-1, peroxisomal proliferator activated receptor-gamma coactivator 1; PPAR α , peroxisomal proliferator activated receptor α ; SIRT1, sirtuin 1; TFAM, mitochondrial transcription factor A; YY1, Yin Yang 1.

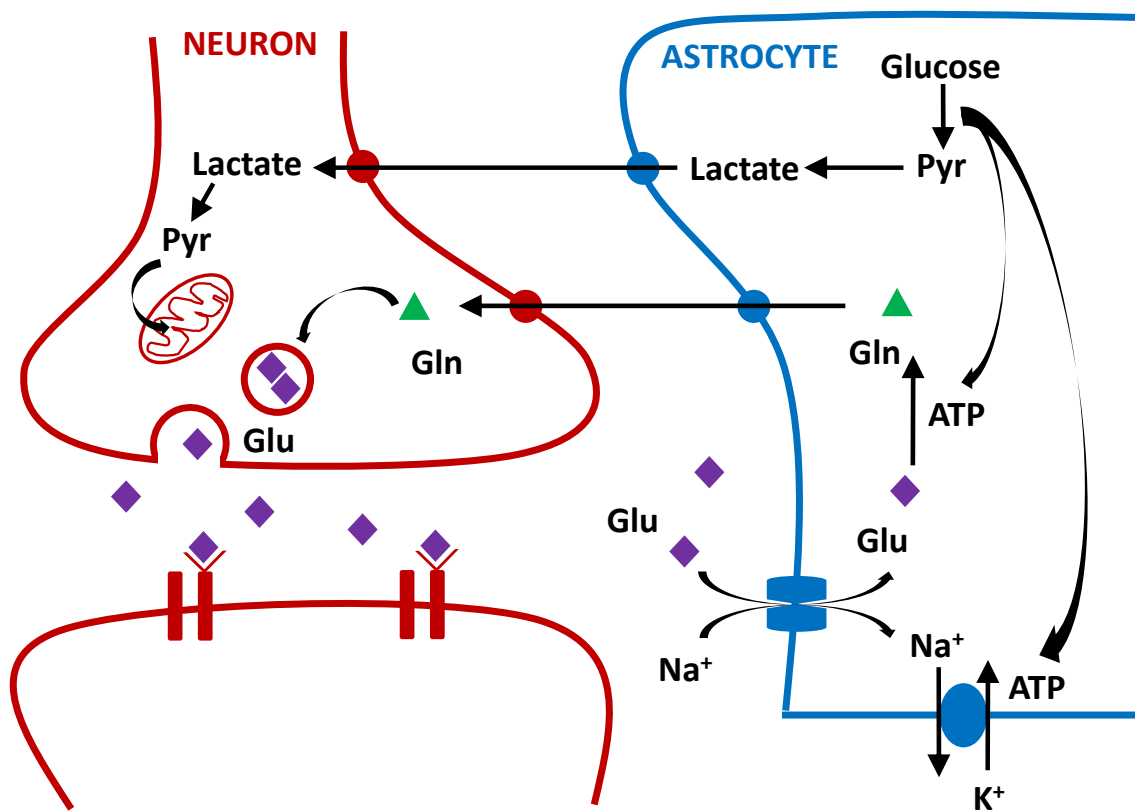


Figure I-3. Schematic illustration of astrocyte-neuron lactate shuttle. According to this hypothesis, neural activity increases the extracellular glutamate concentration. The sodium-coupled reuptake of glutamate by astrocytes stimulates $\text{Na}^+\text{-K}^+$ ATPase and it also activates ATP-dependent conversion of glutamate to glutamine by glutamine synthetase. These two events stimulate anaerobic glycolysis (the conversion of glucose to lactate) in astrocytes that subsequently release lactate, which then is uptaken by neurons. Uptaken lactate is converted to pyruvate by lactate dehydrogenase, and pyruvate enters mitochondria in neurons to participate in tricarboxylic acid cycle. Abbreviations: Gln, glutamine; Glu, glutamate; Pyr, pyruvate.

Chapter II

Effect of Exercise on Mouse Liver and Brain Bioenergetic Infrastructures

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Abstract

To assess the effects of exercise on liver and brain bioenergetic infrastructures, we exposed C57BL/6 mice to 6 weeks of moderate-intensity treadmill exercise. During the training period fasting blood glucose was lower in exercised mice than it was in sedentary mice, but serum insulin levels were not reduced. At week 6, trained mice showed a paradoxical decrease in plasma lactate levels during exercise, which was accompanied by an increase in the liver MCT2 protein level ($\sim 30\%$, $p < 0.05$). Exercise increased liver PGC-1 α expression (~ 2 -fold, $p < 0.001$), SIRT1 protein ($\sim 30\%$, $p < 0.05$), p38 protein ($\sim 15\%$, $p < 0.05$), COX4I1 protein ($\sim 50\%$, $p < 0.05$), and AMPK phosphorylation ($\sim 40\%$, $p < 0.05$). Despite this, liver mtDNA copy number ($\sim 30\%$, $p = 0.05$), TFAM expression ($\sim 15\%$, $p < 0.05$), COX2 expression ($\sim 10\%$, $p < 0.05$), CREB phosphorylation ($\sim 60\%$, $p < 0.05$), and BDNF expression ($\sim 40\%$, $p < 0.05$) were all reduced and cytochrome oxidase and citrate synthase activities were unchanged. The only altered brain parameter observed was a reduction in TNF- α expression ($\sim 35\%$, $p < 0.05$); TNF- α expression was unchanged in liver. Our data suggest lactate produced by exercising muscle modifies the liver bioenergetic infrastructure, and enhanced liver uptake may in turn limit the ability of exercise-generated lactate to modify brain bioenergetics. Also, it appears that at least in the liver a dissociated mitochondrial biogenesis, in which some components are strategically enhanced while others are minimized, can occur.

Introduction

Physical exercise has systemic benefits. It favorably affects blood glucose levels, cholesterol, and blood pressure (Richter *et al.*, 1992; Stewart, 2004; Jia *et al.*, 2012). Some exercise effects result from an increase in muscle mitochondrial mass and bioenergetic efficiency (Rockl *et al.*, 2008; Little *et al.*, 2010). Mitochondrial mass is increased through the process of mitochondrial biogenesis, which in turn is regulated by energy and redox-sensing pathways that converge on the peroxisomal proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (TFAM) co-transcription and transcription factors (Vina *et al.*, 2009). Among these proteins, PGC-1 α engagement is an upstream event. PGC-1 α activates NRF-1, and this initiates TFAM expression (Vina *et al.*, 2009). TFAM plays roles in both mitochondrial DNA (mtDNA) replication and gene expression.

Although muscle is the primary tissue utilized during exercise, both the liver and brain are also engaged and modified. Antioxidant enzyme expression and activities increase in both tissues (Somani & Husain, 1996; Wilson & Johnson, 2000; Devi & Kiran, 2004; Navarro *et al.*, 2004). In brain, brain derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VGEF) levels appear to rise, consequently driving angiogenesis and neurogenesis (Fabel *et al.*, 2003b; Vaynman *et al.*, 2004; Berchtold *et al.*, 2010). One recent study even reported high-intensity exercise increases brain PGC-1 α expression and several other markers of mitochondrial mass (Steiner *et al.*, 2011).

Why exercise-induced muscle activation modifies non-muscle tissues is not fully understood. This phenomenon presumably must be mediated by either a removal from or

release into the blood of specific molecules. Regarding the latter possibility, lactate constitutes a potential candidate since lactate produced by contracting muscles enters the bloodstream, from where it is taken up by liver, heart, and skeletal muscle monocarboxylate transporters (MCTs) (Bonen *et al.*, 2006).

Lactate figures prominently in both liver and brain bioenergetic metabolism. In the Cori Cycle, blood lactate is transferred to hepatocytes by MCT2 and enters gluconeogenesis. The glucose produced is released into the blood and helps prevent hypoglycemia during sustained exercise (Hoene & Weigert, 2010). When blood lactate levels are sufficiently elevated, lactate from blood can also enter the brain (Ide *et al.*, 2000; van Hall *et al.*, 2009). This could affect brain physiology, since lactate is used by neurons to support oxidative phosphorylation (Pellerin *et al.*, 2007), and lactate also appears to play a role in memory formation (Veneman *et al.*, 1994; Berthet *et al.*, 2009; Suzuki *et al.*, 2011).

The primary purpose of this study was to evaluate the effects of moderate exercise on mouse liver and brain bioenergetic infrastructures. To do this, we analyzed pathways and proteins that sense cell energy and redox states, mediate adaptive responses to cell energy and redox state changes, and execute these responses. As part of this analysis, we especially considered the potential role that muscle-generated lactate might play.

Methods

Animals

The animal work described in this study was approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center, and efforts were made to minimize animal discomfort. Twelve male, four-month old C57BL/6 mice were obtained from the Jackson Laboratory. All mice were maintained on an ad libitum diet. The mice were housed four per cage on a 12:12 hour light:dark schedule. After a 1 week accommodation period, the 4.25 month-old mice were randomly placed into two groups, a control/sedentary group (CT, $n = 6$) or an exercise group (EX, $n = 6$).

Exercise training

EX mice were exercised 2 sessions per day on a six-lane treadmill designed for mice (Columbus Instruments, Columbus, OH). Each session consisted of a 3 minute warm-up at 15 m/min plus 42 minutes at 18 m/min. This speed approximates the lactate threshold for untrained C57BL/6 mice (Billat *et al.*, 2005). The training was performed 5 days per week for 6 weeks. CT mice were not subjected to any exercise training. In order to avoid confounding factors such as sound and light, during the training sessions CT mice were placed in the same room as the EX mice. After the 6 week training, EX mice were sacrificed by decapitation 30 minutes after the last session. CT mice were also decapitated on the same day. Liver and brain tissue were immediately frozen in liquid nitrogen, and saved at -80°C for later analysis.

Glucose, insulin, and lactate level determination

Blood glucose levels were measured using a One-Touch Ultra Blood Glucose Monitoring System (LifeScan, Milpitas, CA, USA). Plasma samples were also prepared from tail vein blood that was collected in heparinized micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA). Plasma insulin levels were measured using an insulin ELISA kit according to the manufacturer's instructions (American Laboratory Products Company, Salem, NH). Blood glucose and plasma insulin levels were measured during a period of unrestricted food access (non-fasting), and after a 15 hour fast (fasting). Values for the homeostasis model assessment of insulin resistance (HOMA-IR) were calculated from the product of fasting serum glucose (mM) and insulin (microunits/ml) divided by 22.5 (Matthews *et al.*, 1985). Plasma lactate levels were assayed using a commercial L-lactate assay kit (Eton Bioscience Inc., San Diego, CA), and the values were normalized to the mean lactate level of CT mice at rest on the same day in order to minimize day-to-day variation.

Complex IV and citrate synthase activity assays

Crude mitochondrial fractions isolated from the left cerebral hemisphere and the right liver lobe were used to measure complex IV (cytochrome oxidase; COX) and citrate synthase (CS) V_{max} activities. To isolate mitochondria, brain and liver tissue were homogenized in MSHE buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.5% fatty acid free BSA, pH 7.2) using a Teflon homogenizer and centrifuged at 800 x g for 10 minutes. The supernatant was centrifuged again at 8000 x g for 10 minutes.

The resulting pellets containing mitochondria were re-suspended in MSHE buffer and frozen at -80°C for later use. COX and CS Vmax activities were determined as previously described (Ghosh *et al.*, 2007). COX Vmax activities were normalized to protein concentration, as well as to citrate synthase activity.

Immunoblotting

Protein lysates were prepared using the front half of the right cerebral hemisphere and the left lobe of the liver. For total protein lysates, Radioimmunoprecipitation Assay Buffer (Cell Signaling Technology, Danvers, MA) was used. The tissue was homogenized in the buffer and the homogenates were subsequently sonicated three times, for 5 seconds each time, at setting 4 using an F60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). To prepare crude membrane protein fractions, tissue was placed in ST buffer (250 mM sucrose, 10 mM Tris base, pH 7.5) and homogenized by 10 strokes of a Dounce homogenizer. The homogenates were centrifuged at 1000 x g for 5 minutes, and the supernatant was further centrifuged at 100,000 x g for 1.5 hours. The resulting pellets containing membrane proteins were re-suspended in ST buffer for further analysis. Protein concentration was measured using a BCA protein assay reagent kit (Thermo Scientific, Rockford, IL).

Several energy-sensitive proteins or proteins that influence energy metabolism were analyzed by immunoblotting. MCT2, a proton-linked plasma membrane transporter that transfers lactate into hepatocytes and neurons, was also analyzed. Primary antibodies to the following proteins were used: MCT2 (1:200 dilution; sc-166925, Santa Cruz

Biotechnology, Santa Cruz, CA); phospho-Thr172 AMP-activated protein kinase (AMPK) (1:1000 dilution; 2531, Cell Signaling Technology); AMPK (1:1000 dilution; 2603, Cell Signaling Technology); cAMP-response element binding protein (CREB) (1:1000 dilution; sc-25785, Santa Cruz Biotechnology), phospho-Ser133 CREB (1:500 dilution; 9198, Cell Signaling Technology), p38 (1:1000 dilution; 9212, Cell Signaling Technology), phospho-p38 (1:1000 dilution; 4511, Cell Signaling Technology), mammalian target of rapamycin (mTOR) (1:1000 dilution; 2983, Cell Signaling Technology), phospho-Ser2448 mTOR (1:1000 dilution; 2976, Cell Signaling Technology), NAD-dependent deacetylase sirtuin-1 (SIRT1) (1:500 dilution; 2028, Cell Signaling Technology); PGC-1 α (1:1000 dilution; 516557, Millipore, Billerica, MA); and cytochrome oxidase subunit 4 isoform 1 (COX4I1) (A21348, 1 : 2000; Invitrogen, Carlsbad, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000 dilution; 2118, Cell Signaling Technology) was used as a loading control for total protein lysates. Pan-cadherin (1:1000 dilution; ab16505, Abcam, Cambridge, MA) was used as a loading control for the membrane protein fraction. Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; Cell Signaling Technology) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Densitometry was performed using a ChemiDoc XRS with Quantity One software (Bio-Rad, Hercules, CA, USA).

Quantitative real-time RT-PCR

Total RNA was prepared from frozen brain and liver tissue using the TRI Reagent (Life Technologies, Grand Island, NY). Reverse transcription was performed with total RNA (1 μ g) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR (qPCR) was performed using the 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 MCT2, PGC-1 α , NRF-1, TFAM, COX2, COX4I1, tumor necrosis factor alpha (TNF- α), CREB, and BDNF. GAPDH was used as an internal loading control. Real-time RT-PCR amplification was determined utilizing an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were quantified using the StepOnePlus Software v2.1 based on the *comparative $\Delta\Delta CT$* method.

To quantify liver mtDNA, total DNA was extracted with a Dneasy Blood & Tissue kit (Qiagen, Valencia, CA). TaqMan Gene Expression Assays (Applied Biosystems) for two mtDNA-encoded genes, NADH dehydrogenase subunit 2 (ND2) and 16S ribosomal RNA (rRNA), and the nuclear 18S rRNA gene were used. The relative mtDNA to nuclear DNA copy number ratio was determined using the *comparative $\Delta\Delta CT$* method, in which ND2: 18S rRNA and 16S rRNA: 18S rRNA ratios were calculated.

Statistical analysis

Data were summarized by mean and standard error. Mean values were compared by two-sample *t*-test or paired *t*-test using SPSS 18.0. Pearson's correlation analysis was

performed to determine relationships between MCT2 and PGC-1 α . *p*-values less than 0.05 were considered significant.

Results

Effect on weight, glucose, and insulin

At the beginning of the study, body weights were comparable between the CT and EX groups. After 6 weeks, the CT mice gained significantly more weight (baseline: 29.28 g \pm 0.42; after 6 weeks: 30.82 g \pm 0.40, *p* < 0.05, Fig. 1A), while the EX mice maintained their weight over the 6 weeks (baseline: 29.70 g \pm 0.32; after 6 weeks of training: 29.95 g \pm 0.54, *p* > 0.05, Fig. 1A). After 3 weeks, the non-fasting plasma glucose trended down in EX mice (CT: 184.33 mg/dL \pm 5.95; EX: 167.67 mg/dL \pm 8.33, *p* = 0.06, Fig. 1B). At the 3.5-week point, fasting blood glucose levels were significantly lower in the EX mice (CT: 119.33 mg/dL \pm 5.75; EX: 95.08 mg/dL \pm 5.23, *p* < 0.05, Fig. 1B). At the beginning of the study, non-fasting plasma insulin levels and 15-hour fasting plasma insulin levels (both at rest) were comparable between the CT and EX groups (data not shown). After 6 weeks of training, we did not observe inter-group differences in either the non-fasting insulin level (CT: 1.16 ng/mL \pm 0.20; EX: 1.77 ng/mL \pm 0.27, *p* > 0.05, Fig. 1C) or the 15-hour fasting insulin level (CT: 0.42 ng/mL \pm 0.09; EX: 0.80 ng/mL \pm 0.35, *p* > 0.05, Fig. 1C). The HOMA-IR calculation suggested that 6 weeks of moderate intensity treadmill training did not increase insulin sensitivity (CT: 3.04 \pm 0.79; EX: 4.41 \pm 1.72, *p* > 0.05, Fig. 1D).

Plasma lactate and MCT2

After the 6 week training period, resting plasma lactate levels were comparable between the CT and EX groups ($p > 0.05$, figure not shown). At the end of the 6 weeks, we found that the plasma lactate level actually declined in the EX mice while they were running. The plasma lactate levels appeared to gradually return to its resting baseline level over the first 2 hours of the recovery period (Fig. 2). Protein levels of MCT2, a transporter responsible for cell lactate uptake, were significantly increased in EX mice livers (~30%, $p < 0.05$), although no inter-group difference was seen in mRNA expression (Fig. 3). Other investigators have also observed a similar dissociation between MCT2 protein levels and mRNA expression (Jackson *et al.*, 1997). Exercise training did not change brain MCT2 mRNA expression or protein levels (Fig. 3).

Mitochondrial enzyme activities

Liver and brain COX Vmax activities, when normalized to protein concentration, were comparable between the CT and EX groups (Table 1). Normalizing COX activity to CS activity, a parameter sometimes used to independently control for mitochondrial mass, did not change this relationship. CS activities in both liver and brain were also comparable between the groups.

Bioenergetics-related proteins

Liver but not brain PGC-1 α mRNA was significantly increased in EX mice (~2-fold, $p < 0.001$, Fig. 4A), while the liver and brain PGC-1 α protein levels were unchanged (Fig. 4B). Liver PGC-1 α mRNA and MCT2 protein levels were positively correlated ($r = 0.67$, $p < 0.05$, Fig. 5A). No correlation was seen between liver PGC-1 α and MCT2 protein levels, liver PGC-1 α and MCT2 mRNA levels, or liver PGC-1 α protein and MCT2 mRNA levels (Fig. 5B-D).

We assessed the status of several proteins that are known to regulate PGC-1 α . The protein level of SIRT1, which activates PGC-1 α by deacetylating it (Canto *et al.*, 2009), was increased in the livers but not brains of the EX mice (~30% increase, $p < 0.05$, Fig. 6A). AMPK, another important metabolic sensor that monitors intracellular AMP/ATP ratios (Hardie, 2007), showed increased Thr172 phosphorylation in the EX mice livers but not in brains. This increase was observed when normalized to either total AMPK or GAPDH (~40% increase, $p < 0.05$, Fig. 6B-C). Liver and brain total AMPK protein levels were comparable between groups (Fig. 6D).

We analyzed p38 MAPK activation by immunoblotting, since p38 MAPK has been shown to activate PGC-1 α (Cao *et al.*, 2005). When normalized to GAPDH, p38 phosphorylation in the EX mouse livers trended towards an increase (~15% increase, $p = 0.087$, Fig. 7A). When corrected for total p38, though, this trend was no longer apparent (Fig. 7B). The total p38 level, when normalized to GAPDH, was higher in EX mouse livers (~15% increase, $p < 0.05$, Fig. 7C), which suggests any apparent increase in liver p38 phosphorylation was a secondary consequence of increased total p38 protein. Brain total p38 levels were comparable between the EX and CT groups.

We assessed the activation status of mTOR, a kinase that regulates cell growth, size, and survival and which can form a complex with PGC-1 α (Cunningham *et al.*, 2007). There was no difference in mTOR Ser2448 phosphorylation, when normalized to either GAPDH or total mTOR, in either liver or brain (Fig. 7D-E). Liver and brain total mTOR protein levels were also comparable between the two groups (Fig. 7F).

CREB can reportedly activate PGC-1 α expression (Herzig *et al.*, 2001). Liver CREB mRNA expression was lower in the EX mice than it was in control mice (Fig. 8A), although total CREB protein levels were comparable (Fig. 8B). Ser133 CREB phosphorylation was reduced in the EX mouse livers when normalized to GAPDH (~60% decrease, $p < 0.05$), but when normalized to total CREB this exercise-induced phospho-CREB reduction did not remain statistically significant (Fig. 8C-D). No changes in brain CREB expression, total protein, or phosphorylation were observed. To further help address whether a functional reduction in liver CREB activity occurred, we measured the expression of BDNF, as CREB drives BDNF expression (Finkbeiner, 2000). BDNF mRNA levels were significantly lower in EX mouse livers (~40% decrease, $p < 0.05$, Fig. 8E). Contrary to what has been reported in other studies of exercised mice (Chen & Russo-Neustadt, 2009; Liu *et al.*, 2009; Rasmussen *et al.*, 2009), but consistent with our observed lack of brain CREB changes, we did not observe changes in brain BDNF expression.

NRF-1 and TFAM are regulated by PGC-1 (Vina *et al.*, 2009). Liver and brain NRF-1 mRNA levels did not differ between the groups (Fig. 9A). Liver TFAM expression, but not brain TFAM expression, was significantly reduced in EX mice (~15% decrease, $p < 0.05$, Fig. 9B). In accordance with this liver TFAM mRNA reduction, we

also observed that mtDNA content was lower in the EX mouse livers (16S rRNA/18S rRNA: ~25% decrease, $p = 0.1$; ND2/18S rRNA: ~30% decrease, $p = 0.05$, Fig. 10). The mRNA levels of cytochrome *c* oxidase subunit 2 (COX2), an mtDNA-encoded protein that constitutes part of the COX holoenzyme, was decreased in the EX group livers (~10% decrease, $p < 0.05$, Fig. 11A). Brain levels were statistically equivalent. Interestingly, levels of COX4I1 protein, a nuclear DNA-encoded constituent of the COX holoenzyme, were significantly increased in the EX mouse livers (~50% increase, $p < 0.05$, Fig. 11B). COX4I1 protein levels were similar in brain, and liver and brain COX4I1 mRNA expression between EX and CT mice was comparable (Fig. 11B-C).

Bioenergetic metabolism is known to influence inflammation and inflammatory markers (Salminen *et al.*, 2011). To assess the effects of exercise on a marker of inflammation, we measured liver and brain TNF- α expression. Although exercise did not alter liver TNF- α mRNA levels, TNF- α mRNA levels were reduced in EX mouse brains (~35% decrease, $p < 0.05$, Fig. 12).

Discussion

We found that when young male C57BL/6 mice were exercised regularly on a treadmill, at 18 m/min for 6 weeks, their ability to clear blood lactate increased. In conjunction with this adaptation, and likely contributing to it, liver MCT2 protein levels rose. Liver but not brain PGC-1 α expression increased. SIRT1, AMPK, p38, and CREB are all reported to activate PGC-1 α activity or expression, and our data suggest SIRT1, AMPK, and p38 but not CREB may have contributed to the observed PGC-1 α increase.

Although PGC-1 α is known to coordinate mitochondrial biogenesis, and protein levels of the nuclear DNA-encoded electron transport chain COX4I1 subunit rose in the liver, liver TFAM expression, COX2 expression, and mtDNA content were reduced. In the liver, therefore, exercise appeared to induce at most a relatively selective mitochondrial biogenesis in which the respiratory capacity was not enhanced. This is potentially consistent with the fact that from a bioenergetics perspective, a key liver function is glucose homeostasis (Herzig *et al.*, 2001). Under exercise conditions, it accomplishes this through gluconeogenesis. In our study, the liver bioenergetic infrastructure may have changed to facilitate gluconeogenesis at the expense of oxidative phosphorylation.

If correct, these data could help explain why the transfer of mtDNA genes to the nucleus, a process that has played out over the course of evolution, remains incomplete. Activating nuclear genes that promote mitochondrial biogenesis, while downregulating mtDNA and transcription factors that promote mtDNA gene expression, would predictably shift mitochondrial function from respiration and towards other non-respiratory activities such as gluconeogenesis. Retaining key respiratory chain subunit genes on the mtDNA, therefore, could help facilitate this high degree flexibility.

Lactate produced during exercise appears to have initiated the liver changes we observed. This is suggested by the apparent correlation between PGC-1 α expression and MCT2 protein. It is further supported by the fact that by the end of the training period, blood lactate levels actually decreased during exercise. We believe this represents evidence of an enhanced Cori Cycle, which would contribute to endurance by increasing the liver's ability to maintain glucose homeostasis under conditions of sustained exertion.

Also, we selected a treadmill speed of 18 m/min because this speed reportedly corresponds to the lactate threshold of untrained C57BL/6 mice (Billat *et al.*, 2005). By the end of the 6 week training period, the 18 m/min speed was clearly below the lactate threshold of our EX mice, which would correspond at most to a “moderate”-intensity human exercise regimen.

Our data do not establish a role for lactate in mediating exercise’s brain effects, but the paucity of brain changes we observed perhaps provides indirect support for this possibility. Certainly, if lactate entering the brain from the blood can modify the brain bioenergetics infrastructure, then an inability to deliver exercise-generated lactate to the brain would blunt exercise’s brain effects. The enhanced ability of the liver to clear exercise-generated lactate, a consequence of training, would function in this capacity.

Indeed, the only brain change we observed with exercise was a reduction in TNF- α expression. This suggests that exercise may reduce brain inflammation set points, and that this effect occurs independently of lactate. Interestingly, we did not detect an increase in brain BDNF expression, a change that has been reported in other mouse exercise studies (Chen & Russo-Neustadt, 2009; Liu *et al.*, 2009; Rasmussen *et al.*, 2009). On this point there is a fair amount of equipoise, though, as other treadmill exercise studies have found brain BDNF does not increase or even decreases (Aguiar *et al.*, 2007, 2008; Wu *et al.*, 2011). We wonder whether differences among these studies are due to methodological factors such as the age of the mice, the brain regions analyzed, or the exercise protocol that was used.

Similarly, while one study did report exercise increased mouse brain and liver COX activity (Navarro *et al.*, 2004), we did not observe a COX activity change in either tissue. We suspect this may also reflect methodological factors, as the mice in the positive study were older than the mice we used. Certainly, it might be expected that the ability of exercise to reverse physiologic or biochemical declines is greater than its ability to enhance already optimally functioning bioenergetic systems. Finally, Steiner *et al.* recently reported exercise did increase PGC-1 α expression in mouse brain, an effect we did not observe in our mice, but the intensity of the exercise protocol in that study (one hour treadmill sessions at 25 m/min and a 5% incline, 6 days a week for 8 weeks) was greater than in our study (Steiner *et al.*, 2011) .

On other points our molecular findings and literature data are essentially consistent. A prior study using male C57BL/6 mice found that after a single session of high intensity treadmill exercise, liver PGC-1 α expression increased (Hoene *et al.*, 2009). Hepatic ATP concentrations significantly decrease while AMP increases immediately after exercise, which would be expected to activate AMPK (Camacho *et al.*, 2006). We postulate this is why AMPK Thr172 phosphorylation, which is often used to assess AMPK's activation status (Hawley *et al.*, 1996), occurred in our mice.

It is unclear to us why CREB phosphorylation was reduced in the livers of our exercised mice. To our knowledge, no previous studies have investigated the effect of exercise training on liver CREB expression, although one study did find that under fasting conditions CREB regulates hepatic gluconeogenesis through effects on PGC-1 α (Herzig *et al.*, 2001).

Exercise is known to minimize weight gain, and over the course of our 6 week study exercise did associate with weight stabilization. Exercise is also said to enhance insulin sensitivity, but our HOMA-IR calculation showed no evidence of increased insulin sensitivity. We feel it is possible that an exercise-induced increase in gluconeogenesis capacity may have confounded the HOMA-IR calculation. If the livers of the exercised mice were more efficient at producing glucose, a compensatory increase in insulin secretion could have been triggered. Such a change would tend to obscure shifts in insulin sensitivity.

This study was limited by a relatively small sample size. Our results do show changes in the expression, level, or post-translational modification of several liver and brain proteins, but the power of our study to detect changes in either tissue was no doubt limited. Future studies with larger sample sizes could more sensitively define how exercise impacts non-muscle bioenergetics, and better reveal the mechanisms that mediate exercise-induced molecular changes. Despite this limitation, our study does have translational implications as it suggests that even a moderate intensity exercise program may enhance liver gluconeogenesis and reduce brain inflammation.

In conclusion, our results demonstrate that training C57BL/6 mice at moderate exercise intensities enhances the liver's ability to import lactate. This effect is associated with and potentially mediated by an increase in liver MCT2. Moderate exercise shifts the liver's bioenergetic profile in ways that should promote gluconeogenesis, some aspects of mitochondrial biogenesis, but not oxidative phosphorylation. These effects are possibly lactate-mediated. Liver lactate import appears to be favored over brain lactate import, so exercise-mediated brain effects may be robust only when lactate production reliably

exceeds the lactate threshold. Further research is needed to investigate whether exercise training above the lactate threshold increases brain lactate delivery and alters energy-sensitive pathways and proteins in that tissue.

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Author contributions

L.E contributed to project conception, experimental design, data collection, data analysis, data interpretation, and drafting the manuscript. J.L. contributed to experimental design and data collection. J.M.B. contributed to project conception and data analysis. R.H.S. contributed to project conception, experimental design, data analysis, data interpretation, drafting the manuscript, and revising the manuscript for intellectual content.

Table 1. Brain and liver COX and CS activities.

	COX (sec ⁻¹ /mg protein ± SEM)		CS (nmol/min/mg protein ± SEM)		COX/CS (± SEM)	
	CT	EX	CT	EX	CT	EX
Liver	1.11 ± 0.07	1.25 ± 0.07	207.87 ± 5.38	185.17 ± 11.70	0.0054 ± 0.00041	0.0070 ± 0.00080
Brain	1.35 ± 0.03	1.28 ± 0.03	999.79 ± 33.74	988.53 ± 24.50	0.0014 ± 0.00003	0.0013 ± 0.00004

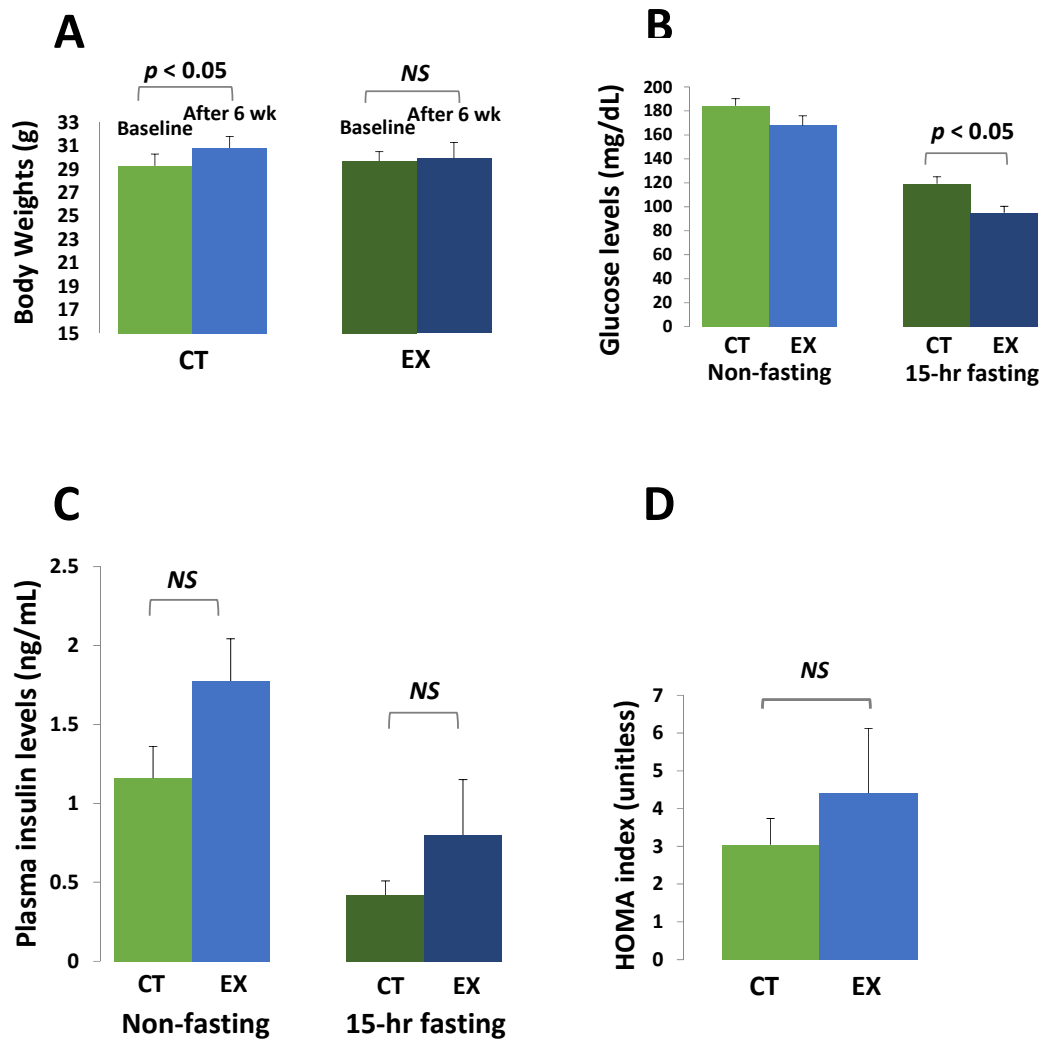


Figure 1. Effects of treadmill exercise training on weight, glucose, and insulin. (For figure legend, see next page)

Figure 1. Effects of treadmill exercise training on weight, glucose, and insulin. (A) CT mice gained significantly more weight while the EX mice maintained their weight over the 6 weeks. **(B)** A trend toward lower plasma glucose level without fasting was seen in the EX mice after 3 weeks of training ($p = 0.06$). After 3.5 weeks of training, fasting blood glucose levels were significantly lower in the EX mice. **(C)** After 6 weeks of training, inter-group differences were not observed in the non-fasting insulin level and the 15 hour fasting insulin level. **(D)** The HOMA-IR value was unchanged by 6 weeks of moderate intensity treadmill training. CT, control group; EX, exercise group; HOMA, homeostasis model assessment; NS, non-significant.

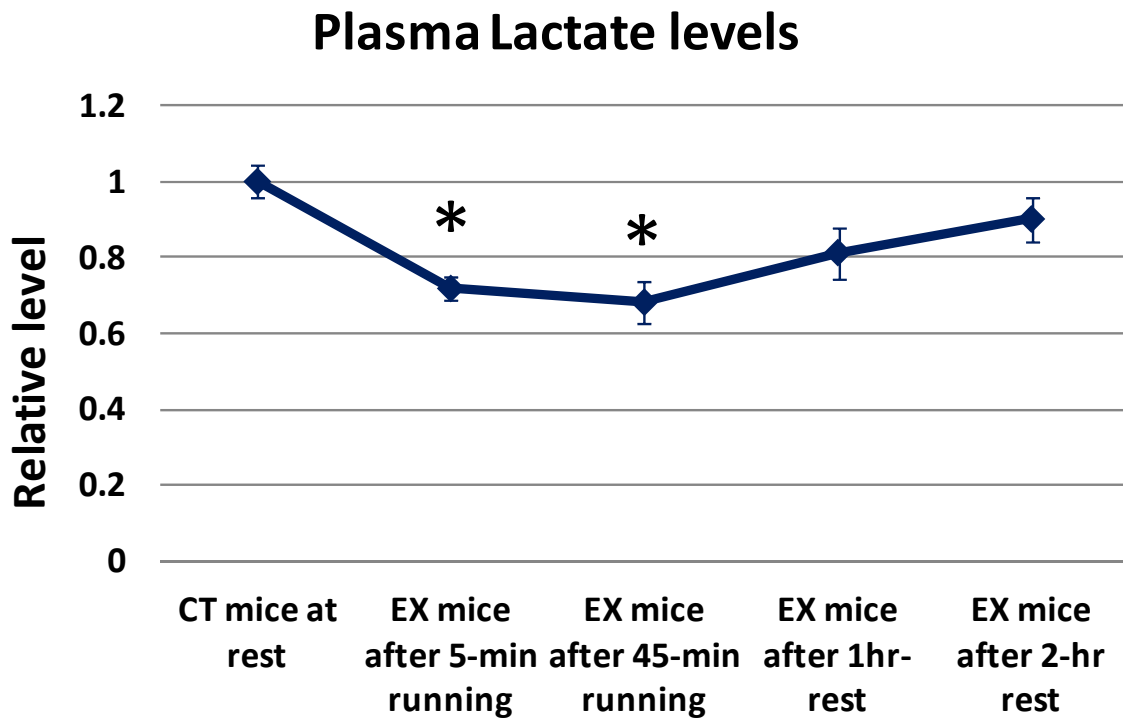


Figure 2. Plasma lactate levels during exercise. At the end of the 6 weeks, plasma lactate levels declined in EX mice during treadmill running. Plasma lactate levels appeared to gradually recover over the first 2 hours of the post-exercise period. $*p < 0.05$ relative to the at-rest lactate level. CT, control group; EX, exercise group.

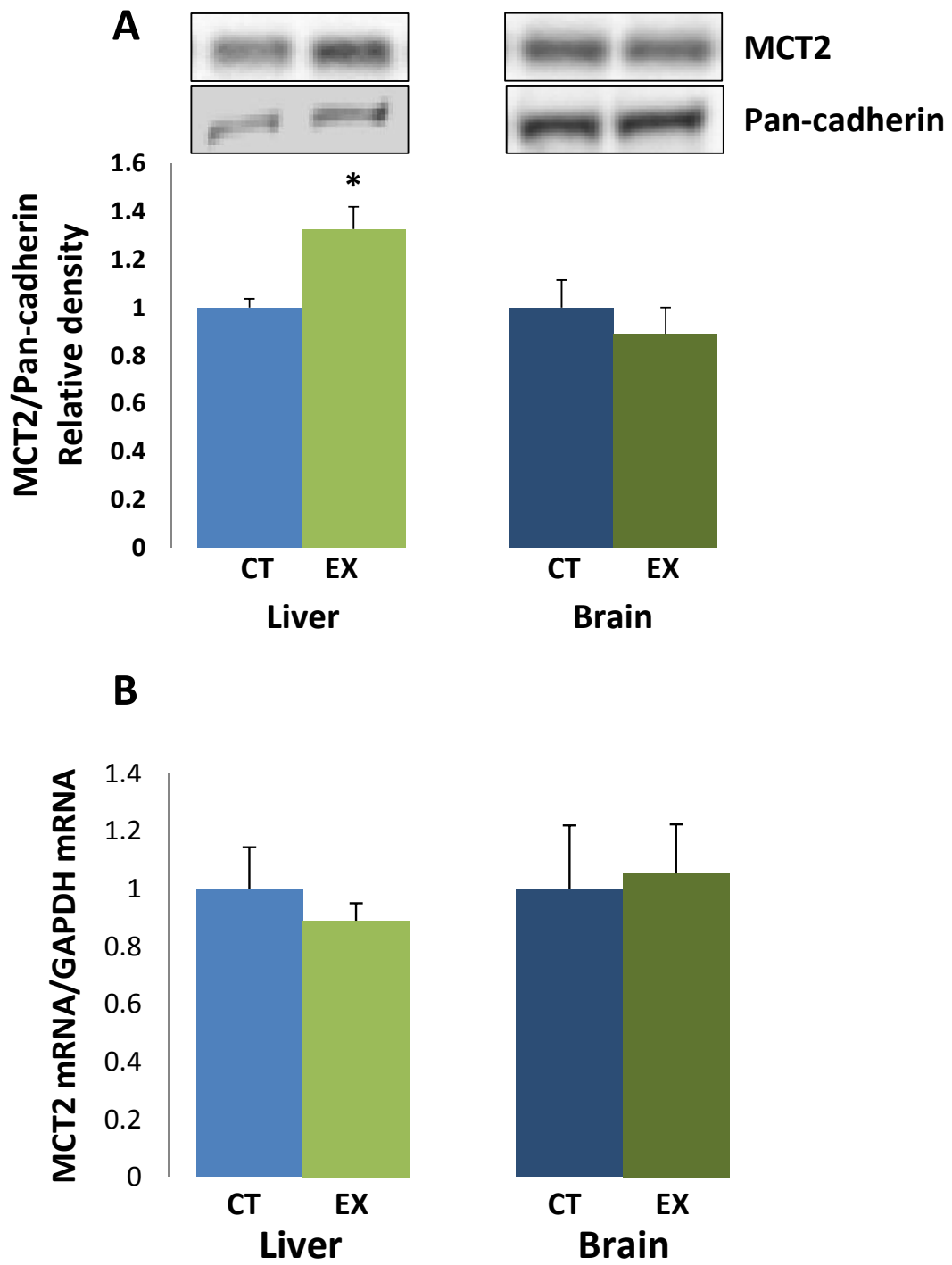


Figure 3. Effect of exercise on MCT2. (For figure legend, see next page).

Figure 3. Effect of exercise on MCT2. (A) MCT2 protein increased in the EX mouse livers, but brain MCT2 protein levels were unchanged. (B) No changes in MCT2 mRNA were seen in liver or brain. * $p < 0.05$. CT, control group; EX, exercise group; MCT2, monocarboxylate transporter 2.

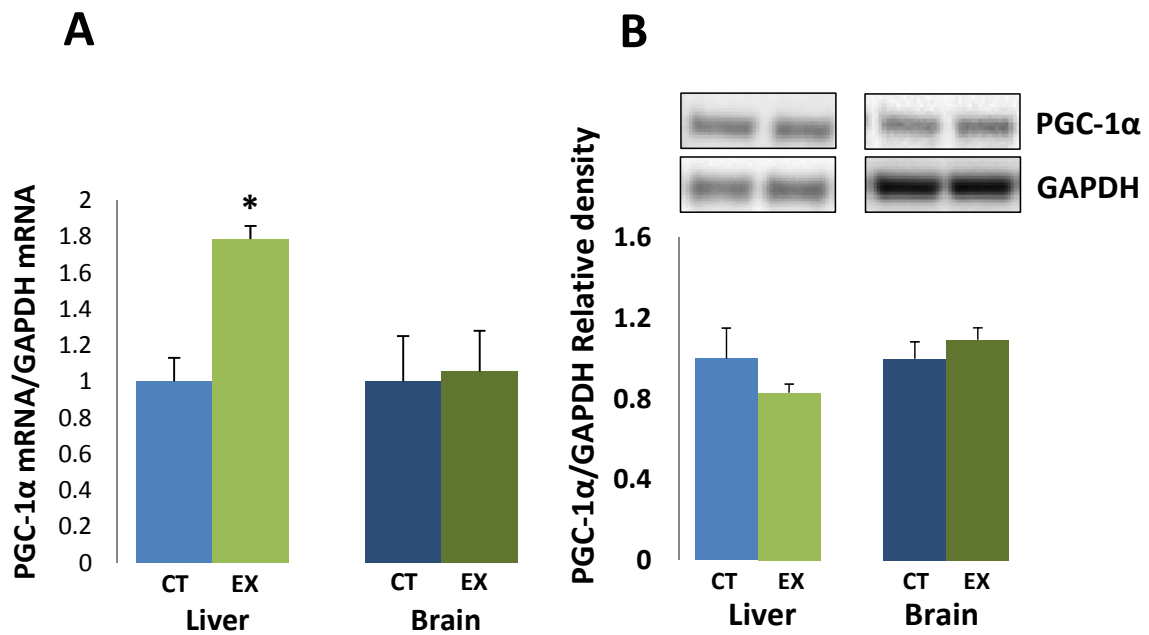


Figure 4. Effect of exercise on PGC-1 α . (A) PGC-1 α mRNA was increased in the EX group livers, but exercise training did not alter brain PGC-1 α mRNA. (B) Exercise did not affect PGC-1 α protein levels in either tissue. * $p < 0.05$. CT, control group; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGC-1 α , peroxisomal proliferator-activated receptor-gamma coactivator 1 alpha.

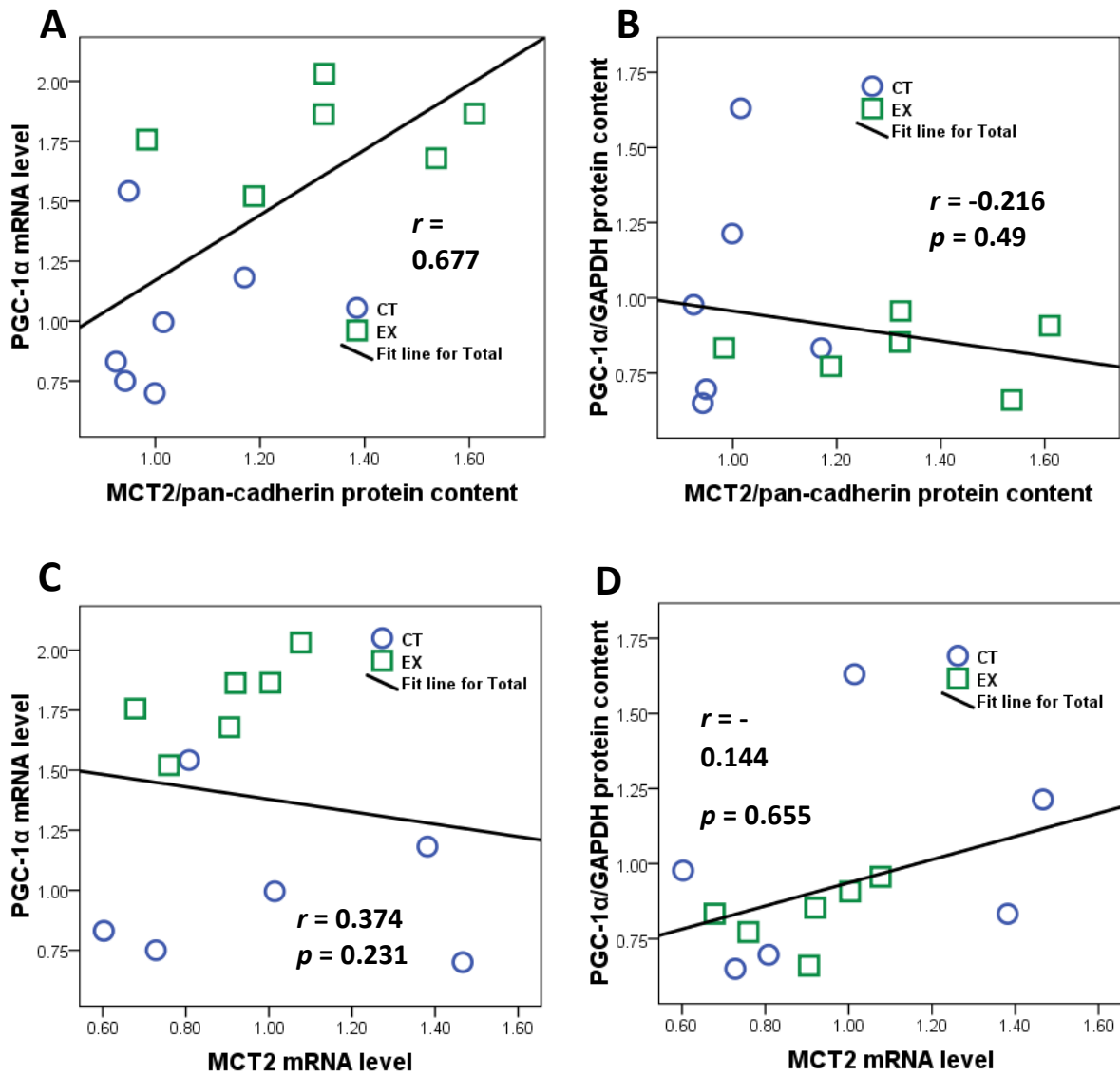


Figure 5. MCT2 and PGC-1 α relationships. (For figure legend, see next page).

Figure 5. MCT2 and PGC-1 α relationships. (A) MCT2 protein and PGC-1 α mRNA levels were positively correlated. (B-D) No correlation was seen between MCT2 protein levels and PGC-1 α protein levels, between MCT2 mRNA levels and PGC-1 α mRNA levels, or between MCT2 mRNA levels and PGC-1 α protein levels. CT, control group; EX, exercise group; MCT2, monocarboxylate transporter 2; PGC-1 α , peroxisomal proliferator-activated receptor-gamma coactivator 1 alpha.

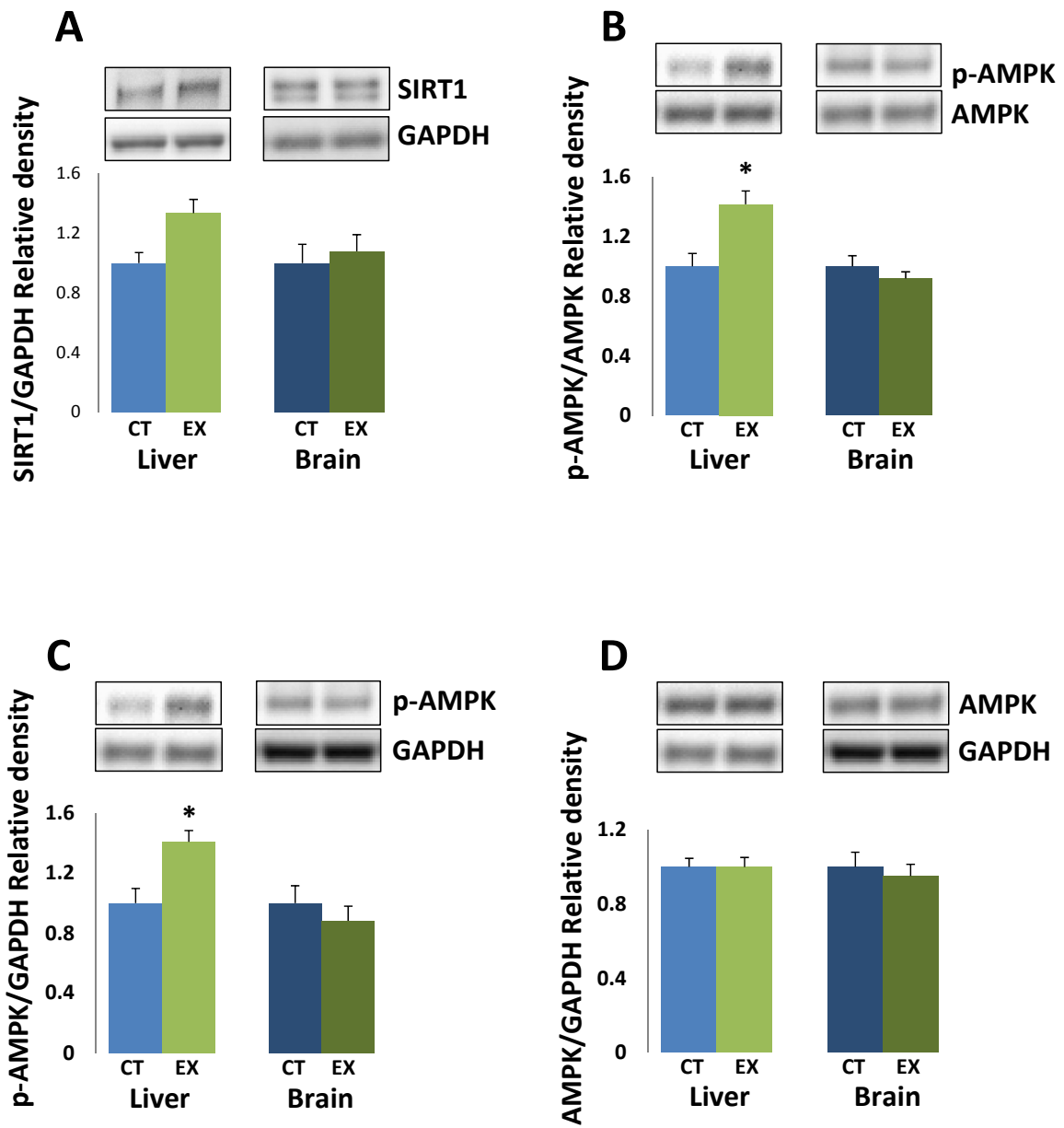


Figure 6. Effect of exercise on SIRT1 and AMPK. (For figure legend, see next page).

Figure 6. Effect of exercise on SIRT1 and AMPK. (A) SIRT1 protein was increased in the EX group livers, but exercise training did not alter the brain SIRT1 protein level. (B-D) AMPK Thr172 phosphorylation was increased in the EX mice livers. This increase was observed when corrected for both total AMPK and GAPDH. Exercise training did not alter brain AMPK phosphorylation and liver or brain AMPK protein levels. $*p < 0.05$. AMPK, AMP-activated protein kinase; CT, control group; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SIRT1, NAD-dependent deacetylase sirtuin-1.

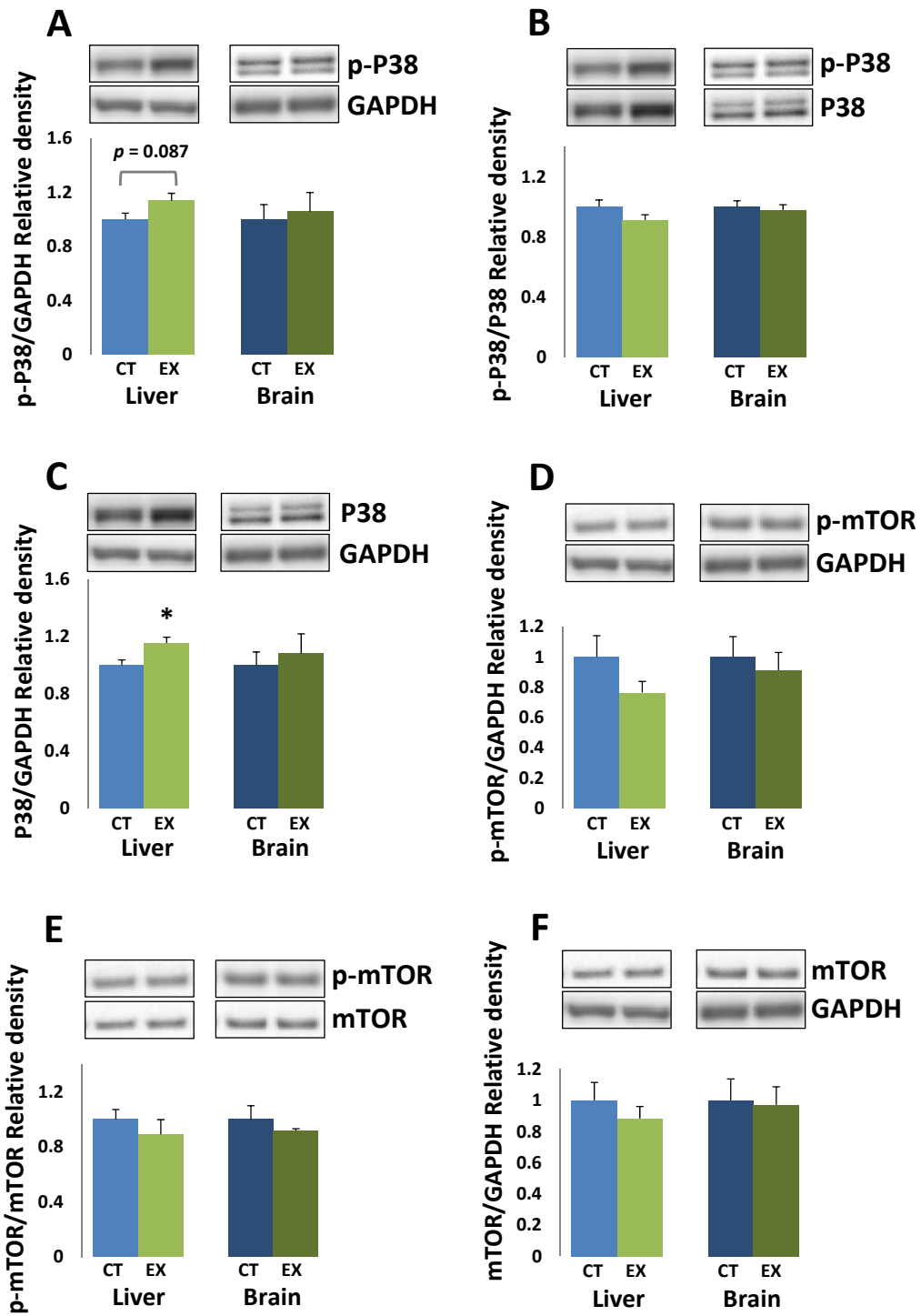


Figure 7. Effect of exercise on p38 and mTOR. (For figure legend, see next page).

Figure 7. Effect of exercise on p38 and mTOR. (A-C) When normalized for GAPDH, liver p38 phosphorylation trended higher in the EX mice, but no inter-group difference was suggested when phospho-p38 was normalized to total p38. Total p38 was significantly increased in EX mouse livers. No inter-group difference was observed in brain phospho-p38 or total p38. **(D-F)** Brain and liver phospho-mTOR and total mTOR were comparable between groups. $*p < 0.05$. CT, control group; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin.

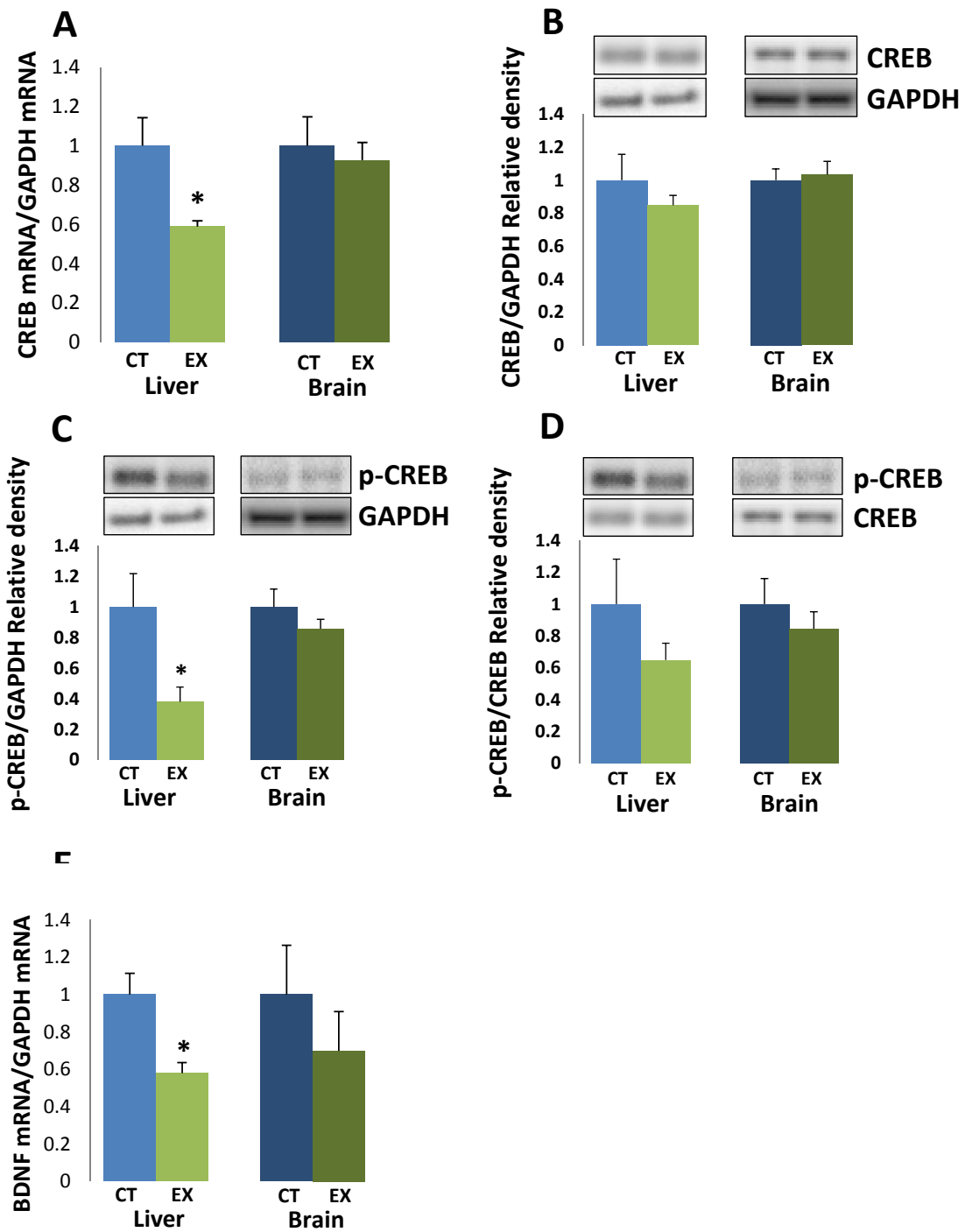


Figure 8. Effect of exercise on CREB and BDNF. (For figure legend, see next page).

Figure 8. Effect of exercise on CREB and BDNF. (A) Exercise training decreased CREB mRNA expression in the liver but did not alter its expression in the brain. (B-D) When normalized to GAPDH, CREB total protein was equivalent between groups while phospho-CREB (at Ser133) was significantly lower in the livers of EX mice. There was no difference in the phospho-CREB/CREB ratio. No changes in brain total or phospho-CREB protein were observed. (E) Although exercise did not alter brain BDNF mRNA expression, liver BDNF mRNA expression was reduced in the EX mice. * $p < 0.05$. BDNF, brain-derived neurotrophic factor; CT, control group; CREB, cAMP-response element binding protein; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

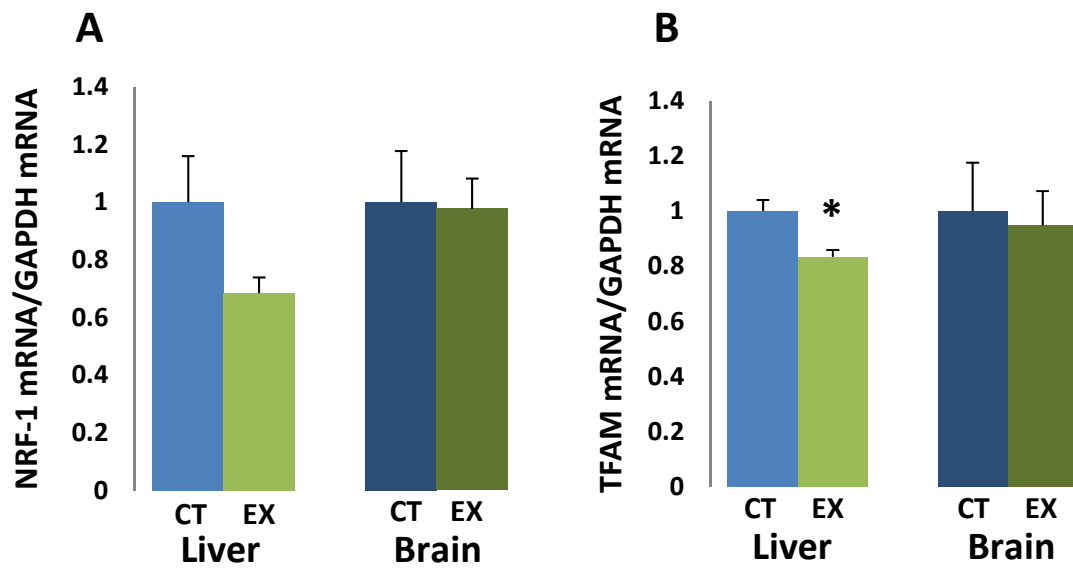


Figure 9. NRF-1 and TFAM expression. (A) Exercise training did not alter liver or brain NRF-1 mRNA levels. (B) TFAM mRNA expression was lower in the EX group livers, but unchanged in brain. * $p < 0.05$. CT, control group; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NRF-1, nuclear respiratory factor 1; TFAM, mitochondrial transcription factor A.

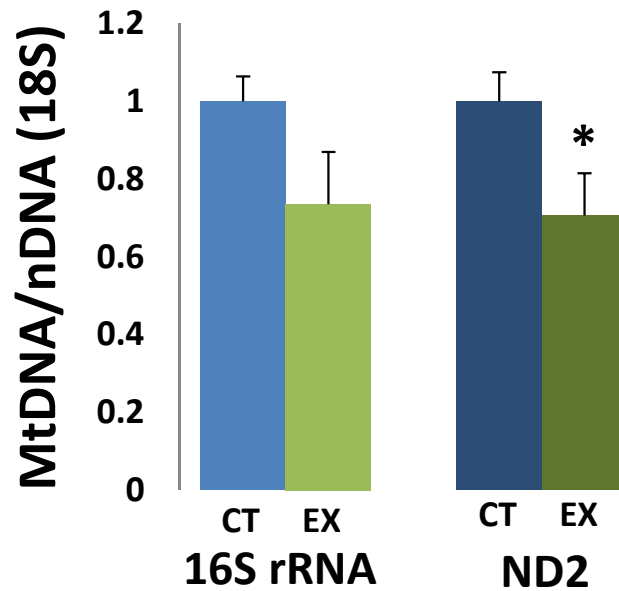


Figure 10. qPCR analysis of liver mtDNA content. Total DNA was extracted from liver tissue and mtDNA was analyzed by real-time quantitative PCR using primers targeting the 18S RNA nuclear gene and the ND2 or 16S RNA mtDNA genes. The 16S:18S ratio trended lower in the EX mice ($p = 0.1$), while the ND2:18S ratio was at the significance cut-off in the EX mice. $*p = 0.05$. CT, control group; EX, exercise group; mtDNA, mitochondrial DNA; ND2, NADH dehydrogenase subunit 2; nDNA, nuclear DNA; rRNA, ribosomal RNA.

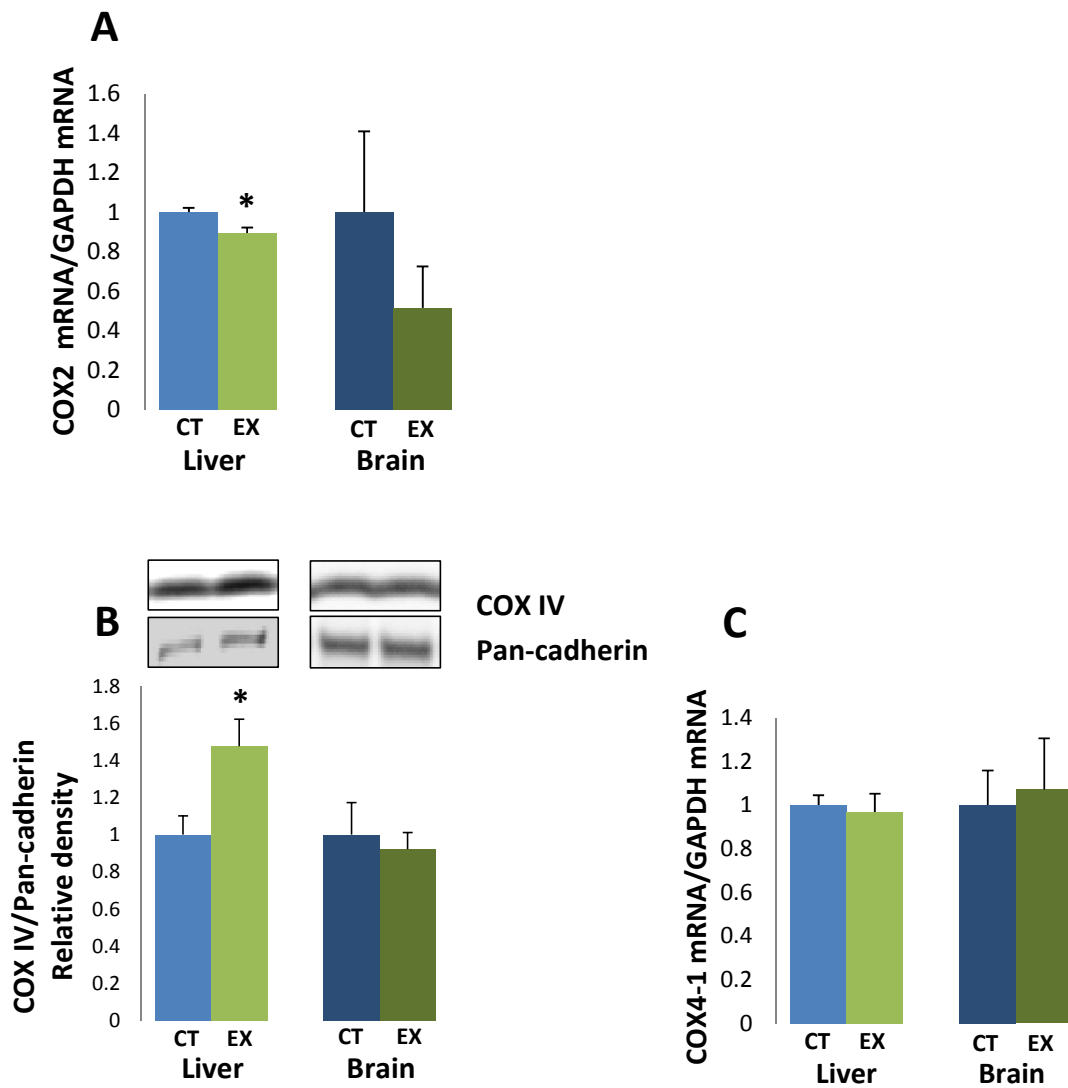


Figure 11. Effect of exercise on COX2 and COX4I1. (For figure legend, see next page).

Figure 11. Effect of exercise on COX2 and COX4I1. (A) Liver COX2 mRNA levels were decreased in the EX group, but were unchanged in the brain. (B-C) COX4I1 protein increased in the livers of EX mice, while liver COX4I1 mRNA expression did not change. Exercise did not alter brain COX4I1 expression or protein levels. * $p < 0.05$. CT, control group; COX2, cytochrome *c* oxidase subunit 2; COX4, cytochrome *c* oxidase subunit 4; EX, exercise group.

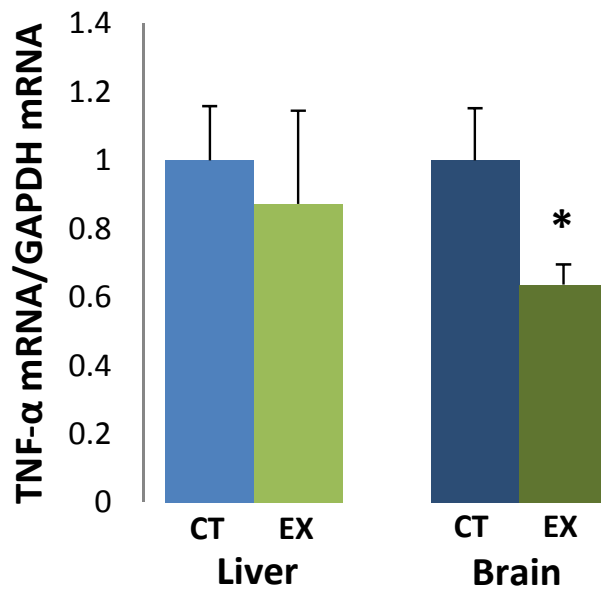


Figure 12. Effect of exercise on TNF- α expression. TNF- α mRNA expression was comparable in the livers but lower in the brains of EX mice. $*p < 0.05$. CT, control group; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF- α , tumor necrosis factor alpha.

Chapter III

Lactate Administration Reproduces Specific Brain and Liver Exercise- Related Changes

Lezi E, Jianghua Lu, J. Eva Selfridge, Jeffrey M. Burns, Russell H. Swerdlow

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Abstract

The effects of exercise are not limited to muscle and its ability to mitigate some chronic diseases is under study. A more complete understanding of how exercise impacts non-muscle tissues might facilitate design of clinical trials and exercise mimetics. Here, we focused on lactate's ability to mediate changes in liver and brain bioenergetic-associated parameters. In one group of experiments, C57BL/6 mice underwent seven weeks of treadmill exercise sessions at intensities intended to exceed the lactate threshold. Over time the mice dramatically increased their lactate threshold, and to ensure plasma lactate accumulated during the final week the mice were run to exhaustion. In liver, mRNA levels of gluconeogenesis-promoting genes increased. PGC-1 α expression increased, PGC-1 β expression decreased, and overall gene expression changes favored respiratory chain down-regulation. In brain, PGC-1 α and PGC-1 β were unchanged but PRC expression and mtDNA copy number increased. Brain TNF α expression fell, and VEGF-A expression rose. In another group of experiments, exogenously administered lactate was found to reproduce some but not all of these observed liver and brain changes. Our data suggest that lactate, an exercise byproduct, could mediate some of exercise's liver and brain effects, and that lactate itself can act as a partial exercise mimetic.

Introduction

Bioenergetic function plays a role in various chronic diseases including cardiovascular disease, diabetes, and some neurodegenerative disorders. In these instances bioenergetic function is thought to constitute a viable therapeutic target (Fontan-Lozano *et al.*, 2008; Ren *et al.*, 2010; Duncan, 2011; Swerdlow, 2011). Exercise, a non-pharmacologic intervention, affects cell and tissue bioenergetics. While exercise's effects on muscle bioenergetics are particularly robust these effects are not muscle-limited and, at least to a minor degree, occur in other tissues. It was recently postulated physical exercise might potentially delay or mitigate age-related central nervous system diseases such as Alzheimer's disease (AD), perhaps through effects on brain bioenergetics (Rockwood & Middleton, 2007).

In muscle, exercise facilitates mitochondrial biogenesis (Hood *et al.*, 2006), and this confers some benefits of endurance training. Exercise may also affect brain mitochondrial biogenesis. One study found exercise increases brain peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) mRNA levels (Steiner *et al.*, 2011). In several tissues PGC-1 α acts as a master regulator of mitochondrial biogenesis and cell energy metabolism. It binds and activates nuclear respiratory factor 1 (NRF-1), which in turn induces the expression of mitochondrial transcription factor A (TFAM) (Scarpulla, 2008b; Vina *et al.*, 2009). TFAM enables the replication, maintenance, and transcription of mitochondrial DNA (mtDNA). Under some conditions exercise has been shown to increase brain mtDNA copy number (Bayod *et al.*, 2011; Marosi *et al.*, 2012; Zhang *et al.*, 2012b; Zhang *et al.*, 2012c).

Exercise has long been thought to primarily modify brain molecular physiology by increasing amounts of brain derived neurotrophic factor (BDNF) (Stranahan *et al.*, 2009), but other factors may also mediate the non-muscle effects of exercise, or perhaps lie upstream of BDNF changes. Lactate, which is generated and released by exercising muscle, in particular appears to affect the brain. Blood lactate accesses the brain via endothelial monocarboxylate transporters (MCTs) (Pierre & Pellerin, 2005). Lactate imported from the blood to the brain is used to generate energy (Quistorff *et al.*, 2008; Gallagher *et al.*, 2009; van Hall *et al.*, 2009; Boumezbeur *et al.*, 2010; Wyss *et al.*, 2011), protects ischemic neurons (Berthet *et al.*, 2009), and facilitates memory formation (Newman *et al.*, 2011; Suzuki *et al.*, 2011). For these reasons and others, we considered whether lactate itself might reproduce and perhaps mediate exercise-associated changes in brain bioenergetic infrastructures.

Materials and Methods

Animals

The animal work described in this study was approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Whenever possible, efforts were made to minimize animal discomfort. Fifty-four C57BL/6 male mice were included in these studies. Our mice were obtained from the Jackson Laboratory, and were 4 months old when they reached our vivarium. All mice were maintained on an ad libitum diet, and were housed 4 per cage on a 12:12 hour light:dark schedule. After a 1 week accommodation period, the mice were randomly placed into 4

groups, a sedentary group (SED, $n = 11$), an exercise group (EX, $n = 12$), a lactate treatment group (LAC, $n = 16$), and a lactate-free vehicle group (VEH, $n = 15$).

Exercise training

EX mice were exercised for 7 weeks, 2 sessions per day, on a six-lane treadmill designed for mice (Columbus Instruments, Columbus, OH). The back of each treadmill lane contained an electrified grid, which delivered a shock stimulus to stationary mice (0.2 mA, 200 ms pulses, 1 Hz). For the first 3 weeks each session consisted of a 3 minute warm-up at 15 m/min followed by 42 minutes at 18 m/min. This speed approximates the lactate threshold for untrained C57BL/6 mice (Billat *et al.*, 2005). During weeks 4, 5, and 6 the treadmill speed was progressively increased to 20 m/min, 22 m/min, and 25 m/min respectively. In order to ensure that at least at the end of the training period exercise intensity exceeded the lactate threshold, during the last week (week 7) the mice did not run for a specified time interval at a specific speed, but instead ran to exhaustion. Mice were defined as exhausted if, for the third time, they remained on the shock grid for greater than 5 continuous seconds without attempting to run. During these 7 weeks running sessions were performed 5 days per week.

The SED mice served as the control group for the EX mice. SED mice did not receive any exercise training. To minimize potential confounding factors such as differences in sound and light exposure, during the EX mice training sessions SED mice were placed in the same room as the EX mice.

At the conclusion of the 7-week training period, EX mice were sacrificed by decapitation 1 hour after the last session, and SED mice were also decapitated at

approximately the same time on the same day. Liver and brain tissue were immediately frozen in liquid nitrogen, and saved at -80°C for subsequent analysis.

Lactate treatment

LAC mice received 2g/kg of sodium lactate dissolved in phosphate buffered saline (PBS). The lactate solution concentration was 200 mg/mL (~18 mM), and the pH was 7.4. The controls for the LAC group, the VEH group, were administered PBS at a volume that approximated the average volume administered to the LAC mice. Injections were performed intraperitoneally, once a day, for 14 consecutive days. 1 hour after the final injection mice were sacrificed by decapitation, liver and brain tissue were immediately frozen in liquid nitrogen, and these tissues were saved at -80°C for subsequent analysis.

Lactate, glucose, and insulin level determination

Plasma lactate levels were assayed using a commercial L-lactate assay kit (Eton Bioscience Inc., San Diego, CA). In order to minimize day-to-day assay variation, EX mouse values were normalized to the mean lactate level of the SED mice assayed on the same day. For the VEH and LAC groups, values were normalized to the mean lactate level of VEH mice assayed on the same day.

Blood glucose levels were measured using a One-Touch Ultra Blood Glucose Monitoring System (LifeScan, Milpitas, CA). Plasma samples were also prepared from tail vein blood that was collected in heparinized micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA). Plasma insulin levels were measured using an insulin

enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (American Laboratory Products Company, Salem, NH). Blood glucose and plasma insulin levels were measured after a 16 hour fast (fasting blood glucose and fasting plasma insulin). Values for the homeostasis model assessment of insulin resistance (HOMA-IR) were calculated as the product of fasting blood glucose level (mM) and plasma insulin level (microunits/ml) divided by 22.5 (Matthews *et al.*, 1985).

Quantitative real-time, reverse-transcription PCR

Total RNA was prepared from frozen brain and liver tissue using the TRI Reagent (Life Technologies, Grand Island, NY). Reverse transcription was performed on total RNA (1 μ g) using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, 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-1 α , PGC-1 β , PGC-1 related co-activator (PRC), NRF-1, TFAM, phosphoenolpyruvate carboxykinase 1 (PCK1), pyruvate dehydrogenase kinase 4 (PDK4), vascular endothelial growth factor A (VEGF-A), hypoxia-inducible factor alpha (HIF-1 α), and tumor necrosis factor alpha (TNF- α). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene. qPCR amplifications were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were quantified using the StepOnePlus Software v2.1 based on the *comparative $\Delta\Delta CT$* method.

MtDNA copy number determination

To quantify liver and brain mtDNA, total DNA was extracted using a phenol-chloroform based method as previously described (Guo *et al.*, 2009). Briefly, frozen mouse liver and brain (~15 mg) were homogenized by 10 strokes of a Dounce homogenizer in lysis buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, and 0.1 % SDS). After adding proteinase K, lysate tubes were incubated at 55 °C for 3 hours. Lysate solutions were vortexed vigorously and centrifuged at 8,000 g for 15 minutes. Supernatants were mixed with phenol/chloroform/isoamyl alcohol (25:4:1) (Sigma-Aldrich, St. Louis, MO), and centrifuged again at 8,000 g for 15 minutes. The supernatants were mixed with an equal volume of chloroform and centrifuged at 8,000 g for 15 minutes. The resulting supernatants were mixed with sodium acetate and isopropanol, stored overnight at -20 °C to facilitate DNA precipitation, and centrifuged at 8000 g for 15 minutes to pellet the DNA.

DNA pellets were then washed with 70% ethanol, air dried, and dissolved in nuclease-free water. DNA concentration and purity were determined spectrophotometrically by determining the absorbance at 260 nm and the 260 nm/280 nm ratio. Each real-time PCR reaction was performed using 10 ng of DNA as the template. We used TaqMan Gene Expression Assays (Applied Biosystems) to quantify the amounts of two target genes present on mtDNA, NADH dehydrogenase subunit 2 (ND2) and the 16S ribosomal RNA (rRNA), as well as the amount of a nuclear target gene, the 18S rRNA. The relative mtDNA to nuclear DNA copy number ratio was determined using the comparative $\Delta\Delta CT$ method, in which ND2: 18S rRNA and 16S rRNA: 18S rRNA ratios were calculated.

Immunoblotting

Nuclear and cytoplasmic protein extracts from brain 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, Rockford, IL). Cytoplasmic and nuclear HIF-1 α protein levels (primary antibody: 1:500 dilution; Ab113642; Abcam, Cambridge, MA) were analyzed by immunoblotting. GAPDH (1:2000 dilution; 2118; Cell Signaling Technology, Danvers, MA) and histone deacetylase 1 (1:2000 dilution; PA1-860; Thermo Scientific) were used as loading controls for the cytoplasmic and nuclear fractions, respectively. Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; Cell Signaling Technology) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Statistical analysis

Data were summarized by means and standard errors. Mean values were compared by two-way, unpaired *t*-tests or two-way, paired *t*-tests using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation analysis was used to test inter-parameter relationships. *p*-values less than 0.05 were considered statistically significant.

Results

Effect of treadmill training and lactate injection on plasma lactate levels

Treadmill training profoundly changed the exercise-related plasma lactate accumulation threshold (Fig. 1A). At the beginning of the first week of training, immediately after completing a treadmill session at 18 m/min plasma lactate levels were higher in the EX mice than they were in the SED mice. After one week of training at 18 m/min, though, exercise at 18 m/min no longer increased the plasma lactate levels and at the end of weeks 2 and 3 post-exercise lactate levels were actually lower in the EX mice than they were in the SED mice. Increasing the treadmill speed to 20 m/min, 22 m/min, and 25 m/min in weeks 4, 5, and 6 eventually eliminated this post-exercise lactate reduction, but did not increase the EX mouse post-exercise lactate level above the SED mouse lactate level. Only after running EX mice to exhaustion during week 7 did we again manage to exceed the lactate accumulation threshold and increase plasma lactate levels.

In the LAC mice, 2 g/kg of intraperitoneal lactate rapidly increased the plasma lactate level (Fig. 1B). Lactate levels peaked approximately 15 minutes after injection, remained significantly elevated over the pre-injection baseline for at least 1 hour, and returned to the baseline level by 3 hours.

Effect of treadmill training and lactate injections on weight, glucose, and insulin

At the beginning of the study body weights were comparable between the SED and EX groups, as well as between the VEH and LAC groups. At the end of the 7 week treadmill training period the EX mice did not increase their mean weight, while the SED mice did (SED baseline 28.36 ± 0.37 grams, increasing to 31.04 ± 0.55 grams; $p < 0.05$; Fig. 2A). After 14 days of lactate injections, LAC mice lost ~4% of their starting body

weight (LAC baseline 28.17 ± 0.55 grams, decreasing to 27.19 ± 0.61 grams; $p < 0.001$; Fig. 2A), while the VEH group maintained its weight.

Over the course of the study fasting blood glucose levels did not change in the EX mice, while SED mouse blood glucose levels increased. At the end of the 7 weeks, the SED fasting blood glucose was 111 ± 6.4 mg/dL, and in the EX mice it was 94.8 ± 4.61 mg/dL ($p = 0.05$; Fig. 2B). In the LAC mice, blood glucose levels rose over the course of the study. At the end of the 2 week injection period, the LAC mouse mean fasting blood glucose level rose from 91.25 ± 3.15 mg/dL to 110.31 ± 4.82 ($p < 0.05$; Fig. 2B).

Fasting plasma insulin levels were higher in the EX group at the end of the 7-week training period than they were in the SED group. The final plasma insulin level in the SED group was 0.33 ± 0.06 ng/mL, and in the EX group it was 0.68 ± 0.11 ng/mL ($p < 0.05$; Fig. 2C). At the end of the 2-week injection period, fasting plasma insulin levels were lower in the LAC group than they were in the VEH group. The final plasma insulin level in the LAC group was 0.24 ± 0.03 ng/mL, and in the VEH group it was 0.37 ± 0.05 ng/mL ($p < 0.05$; Fig. 2C). The HOMA-IR calculation, interestingly, at face value suggested that 7 weeks of intensive exercise training actually decreased insulin sensitivity. In the SED mice the HOMA-IR value was 2.25 ± 0.34 , and in the EX mice it was 3.94 ± 0.64 ($p < 0.05$; Fig. 2D). HOMA-IR values trended lower in the LAC group when compared to those in the VEH group (1.61 ± 0.22 in the LAC group, 2.2 ± 0.27 in the VEH group), but this difference was not significant ($p = 0.1$; Fig. 2D).

Effect of treadmill training and lactate injections on liver bioenergetics-related gene expression and mtDNA levels

Exercise and lactate injection enhanced the liver gluconeogenesis infrastructure. PCK1 expression was ~30% more in the EX group than it was in the SED group ($p < 0.05$). In the LAC versus VEH group comparison, PCK1 expression trended higher in the LAC group but this inter-group difference was not significant ($p = 0.07$) (Fig 3). PDK4 expression was ~120% higher in the EX group than it was in the SED group ($p < 0.05$), and ~50% higher in the LAC group than it was in the VEH group ($p < 0.05$) (Fig. 3).

The co-transcriptional activator PGC-1 α also influences hepatic gluconeogenesis (Herzig *et al.*, 2001). Relative to their respective control groups, PGC-1 α mRNA levels were increased ~150% in EX mouse livers ($p < 0.001$), and ~75% in LAC mouse livers ($p < 0.001$) (Fig. 4). Exercise and lactate, though, had the opposite effect on PGC-1 β , another member of the PGC-1 co-transcription factor family. Liver PGC-1 β mRNA expression decreased by ~35% in the EX mice ($p < 0.05$), and by ~50% in the LAC mice ($p < 0.001$) (Fig. 4). mRNA levels of PRC, a third member of the PGC-1 family (Scarpulla, 2008b), were comparable between the EX and SED groups as well as between the LAC and VEH groups (Fig. 4).

Both PGC-1 α and PGC-1 β mediate some co-transcriptional effects through the NRF-1 transcription factor (Lin *et al.*, 2002a; Lin *et al.*, 2003). Liver NRF-1 mRNA levels were ~15% lower in EX mice than they were in SED mice ($p < 0.05$), and ~15% lower in LAC mice than they were in VEH mice ($p < 0.05$) (Fig. 4). TFAM mRNA levels were ~20% lower in EX mouse livers than they were in SED mouse livers ($p < 0.001$), but liver TFAM expression was equivalent between the LAC and VEH groups (Fig. 4).

mtDNA levels were assessed by determining mtDNA to nDNA copy number ratios. Liver 16S rRNA/18S rRNA and ND2/18S rRNA ratios were comparable between the EX and SED groups (Fig. 5). For the VEH and LAC groups, no differences in either the 16S rRNA/18S rRNA or ND2/18S rRNA ratios were observed, although the ND2/18S rRNA ratio arguably trended lower in the LAC group ($p = 0.08$, Fig. 5).

Effect of treadmill training and lactate injections on brain bioenergetics-related gene expression and mtDNA levels

We did not find significant inter-group differences in brain PGC-1 α , PGC-1 β , NRF-1, or TFAM mRNA levels. We saw an equivalent expression of these genes between the SED and EX groups, and between the LAC and VEH groups. Expression levels of PRC, though, increased by ~25% in the EX mouse brains compared to the SED mouse brains ($p < 0.05$), and by ~30% in the LAC mouse brains compared to the VEH mouse brains ($p < 0.05$) (Fig. 6). As brain is not typically considered to be a gluconeogenic organ, we did not assess PCK1 and PDK4 expression.

The brain 16S rRNA/18S rRNA ratio was ~20% higher in the EX mice than it was in the SED mice ($p < 0.05$), and the ND2/18S rRNA ratio was ~15% higher in the EX mice than it was in the SED mice ($p < 0.05$) (Fig. 7). Brain mtDNA levels between the VEH and LAC groups were comparable.

We assessed the effects of supra-lactate threshold exercise and exogenous lactate treatment on the brain's expression of TNF- α , an inflammation mediator, and VEGF-A, an angiogenic/neurogenic growth factor (Jin *et al.*, 2002). Brain TNF- α mRNA levels were ~30% lower in EX mouse brains than they were in SED mouse brains ($p < 0.05$), but were comparable between VEH and LAC brains (Fig. 8). VEGF-A expression was

~20% higher in EX brains than it was in SED brains ($p < 0.05$), and ~15% higher in LAC brains than it was in VEH brains ($p < 0.05$) (Fig. 8). Despite this VEGF increase, mRNA and protein levels of HIF-1 α , a protein that activates VEGF transcription (Pugh & Ratcliffe, 2003), were equivalent between the EX and SED brains and between the LAC and VEH brains (data not shown).

We examined the relationship between brain VEGF-A and PRC mRNA levels. When data were combined from all 4 groups, a strong positive correlation between VEGF-A and PRC mRNA levels was observed ($r = 0.665$, $p < 0.001$) (Fig. 9).

Discussion

Our current study shows supra-lactate threshold exercise and lactate itself in some ways similarly alter brain and liver bioenergetic infrastructures. Overlapping changes involve the expression of genes that monitor, respond to, and modify cell bioenergetic states. Our data suggest that in the liver, exercise-generated lactate enhances gluconeogenic infrastructure while reducing expression levels of some proteins that maintain respiration-associated infrastructures. In the brain, exercise-generated lactate enhances PRC and VEGF expression and does not down-regulate respiration-related infrastructures. In general, in the liver and brain, lactate itself to some extent functions as an exercise mimetic. These findings have clinical and clinical trial-design implications.

In this study hepatic gluconeogenesis data come from mice exercised above the lactate threshold. Our current findings are consistent with a previous study in which mice exercised below the lactate threshold (E *et al.*, 2013a). The liver plays a central role in

the Cori Cycle, in which exercise-generated lactate is removed from the blood and used to generate glucose. It is not surprising, then, that the expression of two gluconeogenic genes, PCK1 and PDK4, increased in the livers of EX and LAC mice and that mRNA levels of PGC-1 α , which promotes hepatic gluconeogenic gene expression (Herzig *et al.*, 2001), also increased.

PGC-1 α is the most studied PGC-1 family member; the effects of exercise on PGC-1 β and PRC are not well known. In general, PGC-1 β drives gluconeogenic gene expression less robustly than PGC-1 α , but is a stronger activator of NRF-1 and inducer of respiration-relevant gene expression (Lin *et al.*, 2002a; Lin *et al.*, 2003). PGC-1 β , therefore, likely supports cell respiration to a greater extent than PGC-1 α .

The liver, of course, helps avoid hypoglycemia. To counter hypoglycemia under conditions of increased glucose utilization, a state that exercise may induce, the liver imports lactate from the blood, converts it to glucose, and releases glucose into the blood. An increase in hepatic respiration could theoretically compete with this role. Avoiding this competition could perhaps explain why exercise associates with increased liver PGC-1 α expression but decreased PGC-1 β expression.

We also explored the ability of exercise and lactate to modify the expression of bioenergetics-associated genes in the brain. Lactate certainly appears to play a critical role in brain energy metabolism (van Hall *et al.*, 2009; Boumezbeur *et al.*, 2010; Wyss *et al.*, 2011), and while most brain lactate is regionally generated by astrocytes in response to local metabolic activity, externally generated lactate accesses the brain via MCTs (Pierre & Pellerin, 2005). In this study, we found supra-lactate threshold exercise and intraperitoneal lactate injections both increase brain VEGF-A mRNA expression. This

suggests exercise-generated lactate could at least partly mediate exercise's ability to increase brain VEGF-A mRNA levels. VEGF-A expression is also elevated within tumor cells, which frequently overproduce lactate (Siemeister *et al.*, 1998; Walenta & Mueller-Klieser, 2004). The possibility that tumor-generated lactate might contribute to increased tumor VEGF-A expression seems worth considering.

VEGF is believed to play a critical role in exercise-induced neurogenesis (Fabel *et al.*, 2003a; Tang *et al.*, 2010; Latimer *et al.*, 2011). In general, this signal protein binds specific cell membrane tyrosine kinase receptors (VEGFRs), and this binding stimulates angiogenesis (Cross *et al.*, 2003). Exercise induces VEGF expression in mouse and rat brains, where it drives dentate gyrus, cerebellum, and hippocampus angiogenesis (Black *et al.*, 1990; Fabel *et al.*, 2003a; Pereira *et al.*, 2007). Angiogenesis in general is activated by hypoxia, which initiates HIF-1 α nuclear translocation, and HIF-1 complex formation activates VEGF transcription (Pugh & Ratcliffe, 2003). While one study found intense exercise reduced brain mitochondrial O₂ (Secher *et al.*, 2008), because HIF-1 α mRNA and protein levels did not change we cannot implicate hypoxia as the cause of our observed brain VEGF increase.

PGC-1 α , interestingly, increases VEGF expression via estrogen-related receptor alpha (ERR- α) co-activation (Arany *et al.*, 2008). While PGC-1 α expression was not increased in either EX or LAC mouse brains, we did not assess PGC-1 α protein levels or activation states. We therefore cannot conclude PGC-1 α did not mediate VEGF expression.

mRNA levels of PRC, another PGC-1 family member, increased in both EX and LAC mouse brains. PRC also interacts with ERR- α to induce mitochondrion-related gene

transcription, and in general supports mitochondrial ATP production (Vercauteren *et al.*, 2009; Mirebeau-Prunier *et al.*, 2010). Although it is not currently known whether PRC specifically stimulates VEGF expression, we observed a positive correlation between mouse brain PRC and VEGF-A mRNA expression. This correlation suggests PRC may play a role in mediating exercise and lactate-associated VEGF expression changes.

Unlike hepatocytes, which use lactate to support gluconeogenesis, neurons mostly use lactate to support respiration (Pellerin & Magistretti, 1994; Erlichman *et al.*, 2008; Gallagher *et al.*, 2009; Wyss *et al.*, 2011). While down-regulating respiration infrastructure in the liver may have physiologic advantages, it is difficult to envision situations in which down-regulating respiration infrastructure would benefit the brain. Perhaps for this reason brain PGC-1 β , NRF-1, and TFAM expression did not decline with either supra-lactate threshold exercise or the administration of exogenous lactate.

Our EX mice failed to increase brain PGC-1 α mRNA expression, although Steiner *et al.* previously reported exercise can increase brain PGC-1 α expression (Steiner *et al.*, 2011). In that study, mice exercised on a treadmill 1 hour per day, at 25 m/min and a 5% incline, for 8 weeks. Differences between our exercise protocol and that of Steiner *et al.* may account for this inter-study discrepancy. Our lactate injection data, though, suggest exercise-generated lactate did not drive the brain PGC-1 α mRNA increase observed by Steiner *et al.*

Similarly, we could not prove that lactate mediates an exercise-associated increase in brain mtDNA. While mtDNA content was higher in EX mice than it was in SED mice, mtDNA levels between LAC and VEH mice were equivalent. Although our study joins several others that also found exercise increases brain mtDNA copy number

(Bayod *et al.*, 2011; Steiner *et al.*, 2011; Marosi *et al.*, 2012; Zhang *et al.*, 2012b; Zhang *et al.*, 2012c), what causes this phenomenon remains unclear. In addition to arguing that lactate is not solely responsible, our data further suggest PGC-1 α expression changes are not a prerequisite.

As we found in a study of mice exercised below the lactate accumulation threshold (E *et al.*, 2013a), mice exercised above the lactate accumulation threshold have lower brain TNF- α expression. LAC mice did not show reduced brain TNF- α mRNA, which suggests lactate does not account for this change. The exercise-related mechanism that drives reduced brain TNF- α expression remains unknown.

In this study, exercise training profoundly enhanced lactate thresholds and exceeding this threshold was not, from an exercise protocol perspective, straightforward. This suggests that over the long term, exercising human subjects with chronic illnesses above the lactate threshold will be impractical. If it turns out that intensive exercise benefits the brain more than mild or moderate exercise, exercise mimetics will likely be needed.

It is worth considering whether lactate itself could serve as a clinically useful exercise mimetic. Over the two-week lactate treatment period mild weight loss occurred; it is unclear whether this reflects an adverse or exercise mimetic-like effect. Lactate, it is reported, may stimulate beta cell insulin secretion, suppress glycolysis, and cause insulin resistance (Meats *et al.*, 1989; Choi *et al.*, 2002). Although lactate treatment in our study increased blood glucose measurements, plasma insulin levels decreased and HOMA-IR values certainly did not rise.

In contrast to our lactate treatment HOMA-IR data, even though exercise is known to reduce insulin resistance our EX mice had elevated HOMA-IR values. This was driven by increased fasting insulin levels. Other investigators report that in mice exercise training increases islet insulin secretion (Huang *et al.*, 2011), although the underlying basis of this phenomenon is unclear. Also, the HOMA-IR value primarily provides an estimate of liver insulin resistance (Bonora *et al.*, 2000). For this reason and others we do not believe exercise truly increased overall insulin resistance in our EX mouse group.

For our two experimental groups, the exercised mice and the lactate-injected mice, we did not attempt to exactly match the pharmacokinetic parameters of brain and liver lactate exposure. Maximum levels and daily exposure durations were almost certainly different between these groups, and the number of days of increased lactate exposure definitely differed. This was partly a consequence of feasibility limitations, since accomplishing this would have required us to repeatedly sample mouse plasma lactate levels while the mice were running on the treadmill. Our goal, though, was simply to test whether administering lactate itself induces some of the same bioenergetic infrastructure and bioenergetic-related changes that exercise induces. Our data argue it does.

We did not specifically test whether lactate affects liver and brain bioenergetic pathway flux dynamics in different or similar ways, although our molecular-based data predict differences should outweigh similarities. Unlike the liver, the brain is not considered a gluconeogenic organ. Neurons do not express glucose-6-phosphatase, the enzyme that mediates the final step of gluconeogenesis (Bell *et al.*, 1993; Lehninger *et al.*, 2005). Although astrocytes do express glucose-6-phosphatase, astrocytes

dephosphorylate glucose at much lower rates than they phosphorylate glucose. This contrasts with liver-derived observations (Bhattacharya & Datta, 1993; Gotoh *et al.*, 2000; Ghosh *et al.*, 2005). Further, astrocytes express the MCT4 monocarboxylate transporter isoform, which favors lactate export over import, while neurons express MCT2 transporters that favor lactate import (Pierre & Pellerin, 2005). Overall, bioenergetic cross-talk between neurons and astrocytes seems designed to favor lactate production by astrocytes and lactate consumption by neurons. For these reasons, we suspect that while liver lactate exposure increases liver gluconeogenesis, increasing brain lactate levels should cause no or little increase in brain gluconeogenesis. This prediction, though, remains experimentally unconfirmed.

In conclusion, our results are consistent with the view that during exercise, muscle-generated lactate accounts for at least some exercise-associated brain and liver bioenergetic infrastructure and bioenergetic-associated adaptations. These adaptations are tissue-specific, are defined by each tissue's bioenergetic-related needs and obligations, and based on these needs and obligations are ultimately predictable. Lactate enhances hepatic gluconeogenesis while probably limiting hepatocyte respiration. Although lactate probably does not account for all major exercise-related, bioenergetics-associated brain changes, it does appear that it is able to induce brain VEGF expression and through this may drive brain angiogenesis and neurogenesis. For this reason, exercise mimetics that reproduce lactate's brain effects are worth developing for, and testing in, conditions with perturbed brain energy metabolism.

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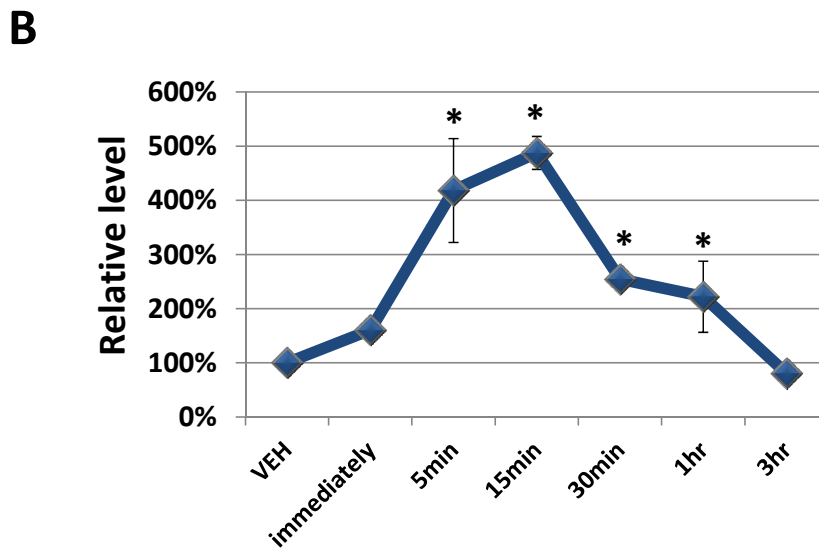
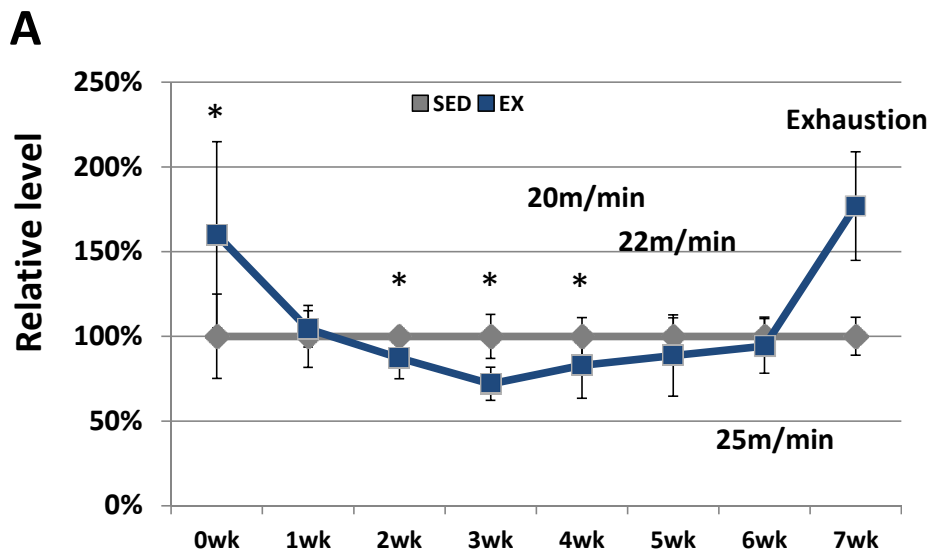


Figure 1. Plasma lactate levels. (For figure legend, see next page).

Figure 1. Plasma lactate levels. *A*, Treadmill exercise at 18 m/min initially elevated plasma lactate levels, but over time the exercise intensity needed to boost plasma lactate concentrations increased. Measurements were performed immediately after the completion of an exercise session. $*P < 0.05$ relative to the lactate levels of the SED mice. *B*, In LAC mice, plasma lactate levels peaked approximately 15 minutes after an intraperitoneal injection, remained significantly elevated for at least 1 hour, and returned to baseline by 3 hours. $*P < 0.05$ relative to the lactate levels of the VEH mice. EX, exercise group; SED, sedentary group; VEH, vehicle group.

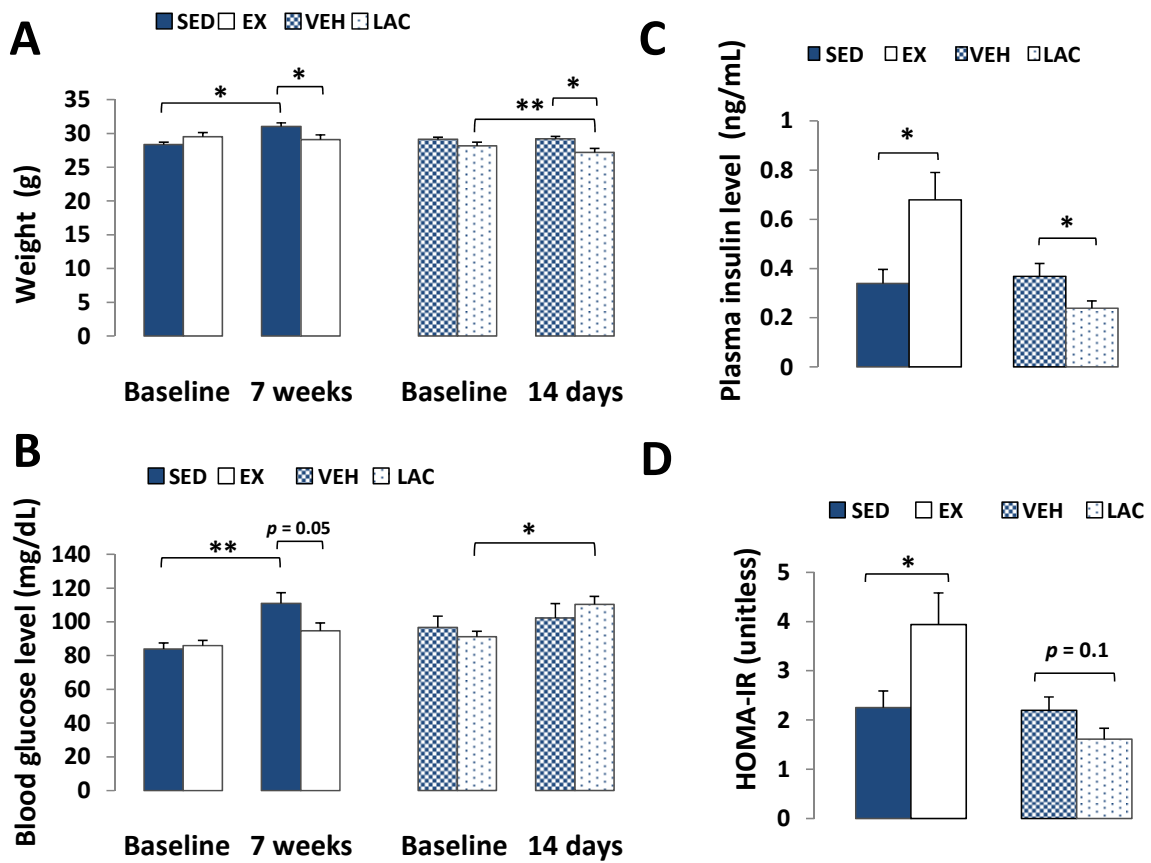


Figure 2. Effect of treadmill training and lactate injection on weight, fasting glucose, and fasting insulin. (For figure legend, see next page).

Figure 2. Effect of treadmill training and lactate injection on weight, fasting glucose, and fasting insulin. **A**, Over 7 weeks SED mice gained weight while EX mice did not. Over 2 weeks LAC mice lost ~4% of their starting body weight, while the VEH group maintained its weight. **B**, Over 7 weeks fasting blood glucose levels did not change in the EX mice but increased in the SED mice. After 2 weeks LAC mouse blood glucose levels were higher than at the start of the experiment. **C**, at the end of the 7-week training period, fasting plasma insulin levels were higher in the EX group than in the SED group; at the end of the 2-week injection period, fasting plasma insulin levels were lower in the LAC group than in the VEH group. **D**, The HOMA-IR value increased in the EX group. It was not significantly altered by lactate injection. * $P < 0.05$, ** $p < 0.001$. EX, exercise group; HOMA-IR, homeostasis model assessment of insulin resistance; LAC, lactate group; SED, sedentary group; VEH, vehicle group.

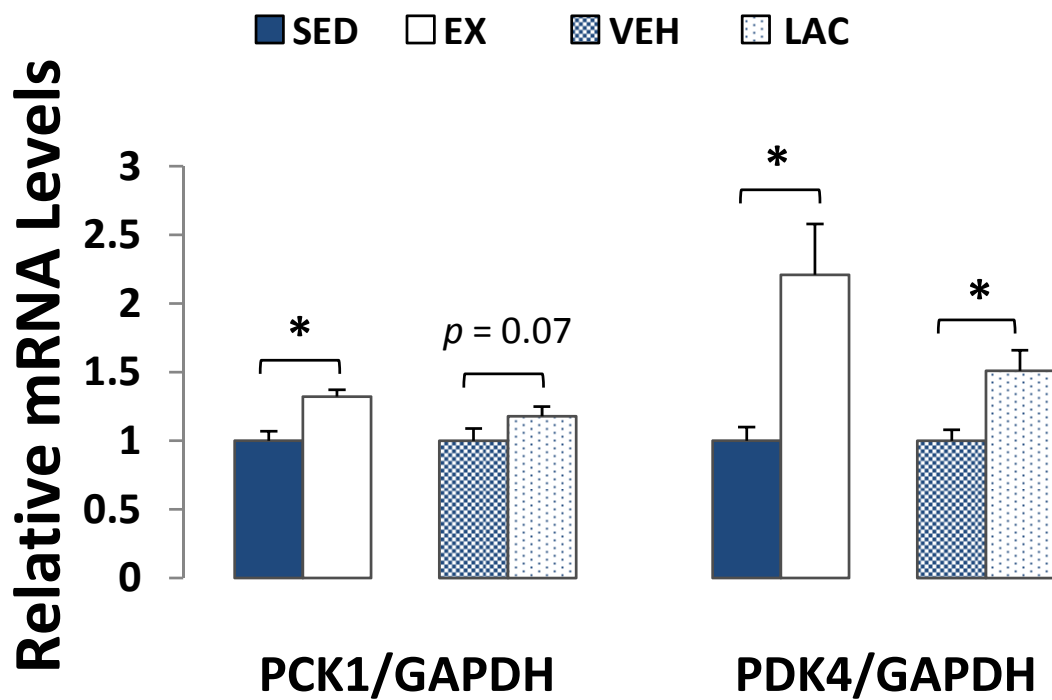


Figure 3. Effect of treadmill training and lactate injection on liver gluconeogenesis.

Compared to the SED mice, PCK1 and PDK4 mRNA levels were higher in the EX mouse livers. Compared to the VEH mice, PCK1 expression trended higher in the LAC group livers, while PDK4 expression increased in the LAC group livers. * $P < 0.05$. EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAC, lactate group; PCK1, phosphoenolpyruvate carboxykinase 1; PDK4, pyruvate dehydrogenase kinase 4; SED, sedentary group; VEH, vehicle group.

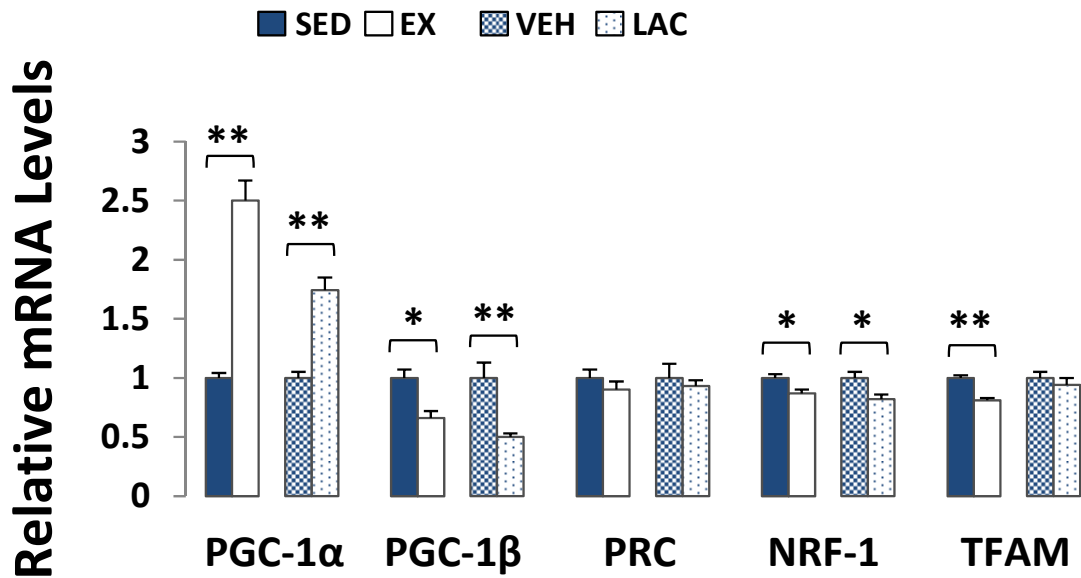


Figure 4. Effect of treadmill training and lactate injection on genes that regulate liver mitochondrial biogenesis. Relative to their respective control groups, EX and LAC group PGC-1 α mRNA levels increased whereas PGC-1 β mRNA levels decreased. PRC mRNA levels remained comparable. NRF-1 mRNA levels were lower in EX and LAC group livers. TFAM mRNA levels were lower in the EX group than they were in the SED group, and comparable between the LAC and VEH groups. * $P < 0.05$, ** $p < 0.001$. EX, exercise group; LAC, lactate group; NRF-1, nuclear respiratory factor 1; PGC-1, peroxisomal proliferator-activated receptor-gamma coactivator 1; PRC, PGC-1 related co-activator; SED, sedentary group; TFAM, mitochondrial transcription factor A; VEH, vehicle group.

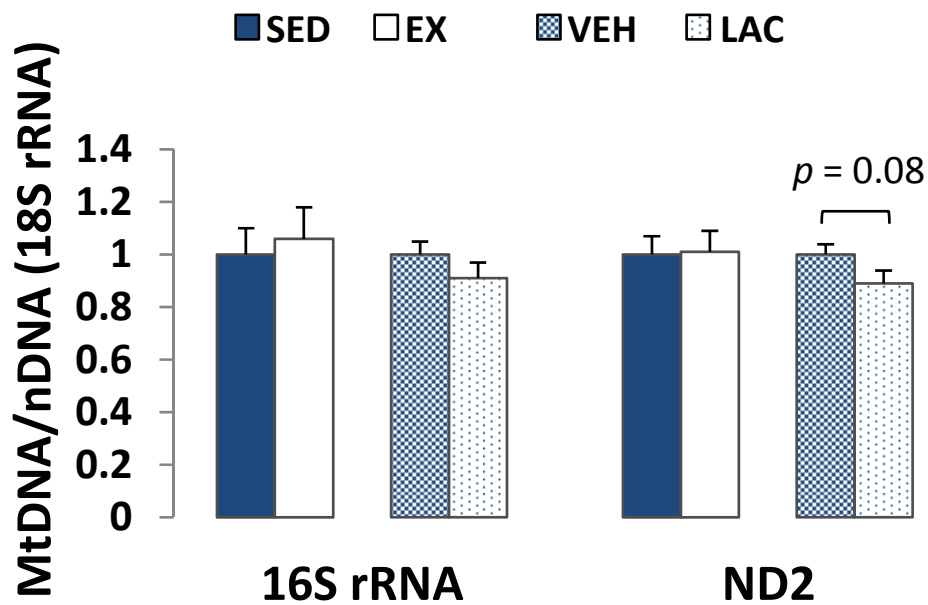


Figure 5. Effect of treadmill training and lactate injection on liver mtDNA copy number. In liver, 16S rRNA/18S rRNA and ND2/18S rRNA ratios were comparable between the EX and SED groups. The 16S rRNA/18S rRNA ratio was comparable between the VEH and LAC groups. Compared to the VEH group, the LAC group ND2/18S rRNA ratio trended lower but these values were not statistically different. EX, exercise group; LAC, lactate group; mtDNA, mitochondrial DNA; ND2, NADH dehydrogenase subunit 2; nDNA, nuclear DNA; rRNA, ribosomal RNA; SED, sedentary group; VEH, vehicle group.

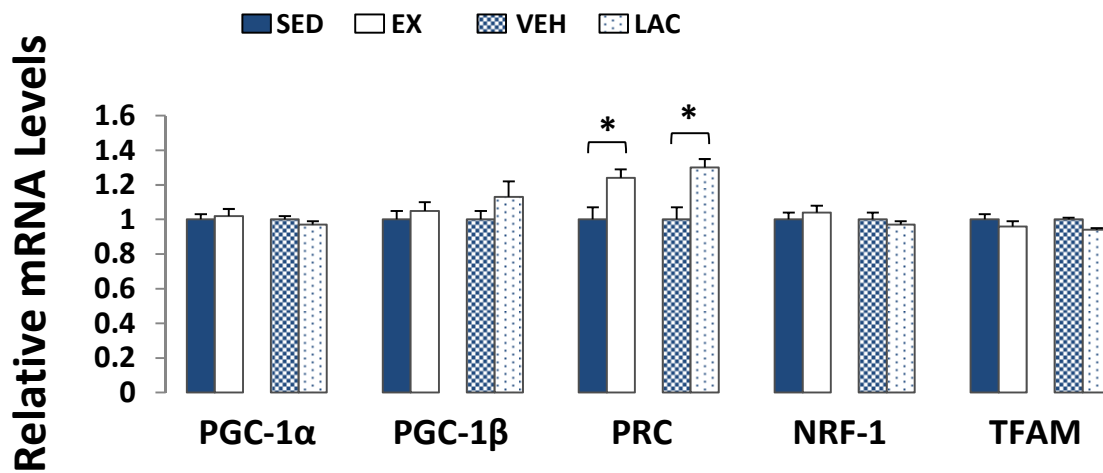


Figure 6. Effect of treadmill training and lactate injection on genes that regulate brain mitochondrial biogenesis. Compared to their respective control groups, brain PRC expression increased in the EX and LAC groups. Exercise and lactate injection did not alter brain PGC-1 α , PGC-1 β , NRF-1, or TFAM mRNA levels. * $P < 0.05$. EX, exercise group; LAC, lactate group; NRF-1, nuclear respiratory factor 1; PGC-1, peroxisomal proliferator-activated receptor-gamma coactivator 1; PRC, PGC-1 related co-activator; SED, sedentary group; TFAM, mitochondrial transcription factor A; VEH, vehicle group.

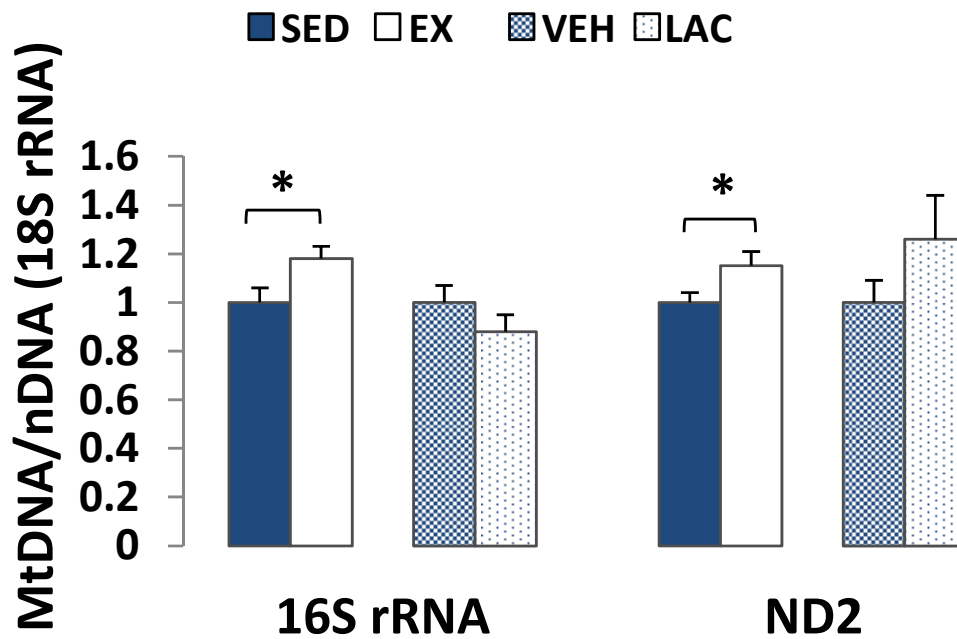


Figure 7. Effect of treadmill training and lactate injection on brain mtDNA copy number. 16S rRNA/18S rRNA and ND2/18S rRNA ratios were higher in EX brains than they were in SED brains. VEH and LAC brain mtDNA levels were comparable. $*P < 0.05$. EX, exercise group; LAC, lactate group; mtDNA, mitochondrial DNA; ND2, NADH dehydrogenase subunit 2; nDNA, nuclear DNA; rRNA, ribosomal RNA; SED, sedentary group; VEH, vehicle group.

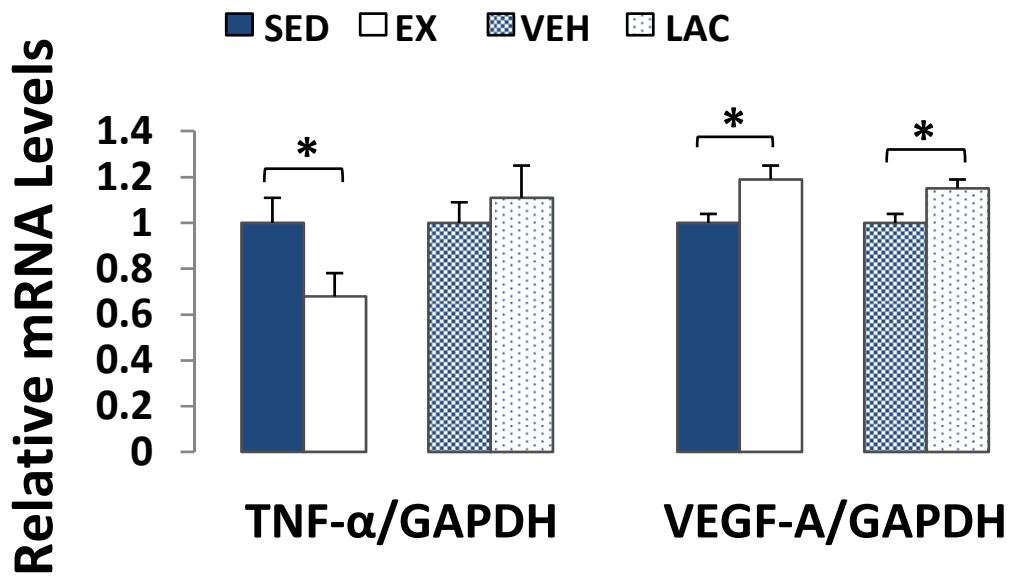


Figure 8. Effect of treadmill training and lactate injection on brain TNF- α and VEGF-A expression. TNF- α mRNA levels were lower in EX brains than they were in SED brains, but were comparable between VEH and LAC brains. Brain VEGF-A expression was higher in EX brains than it was in SED brains, and was higher in LAC brains than it was in VEH brains. * $P < 0.05$. EX, exercise group; LAC, lactate group; SED, sedentary group; TNF- α , tumor necrosis factor alpha; VEGF-A, vascular endothelial growth factor A; VEH, vehicle group.

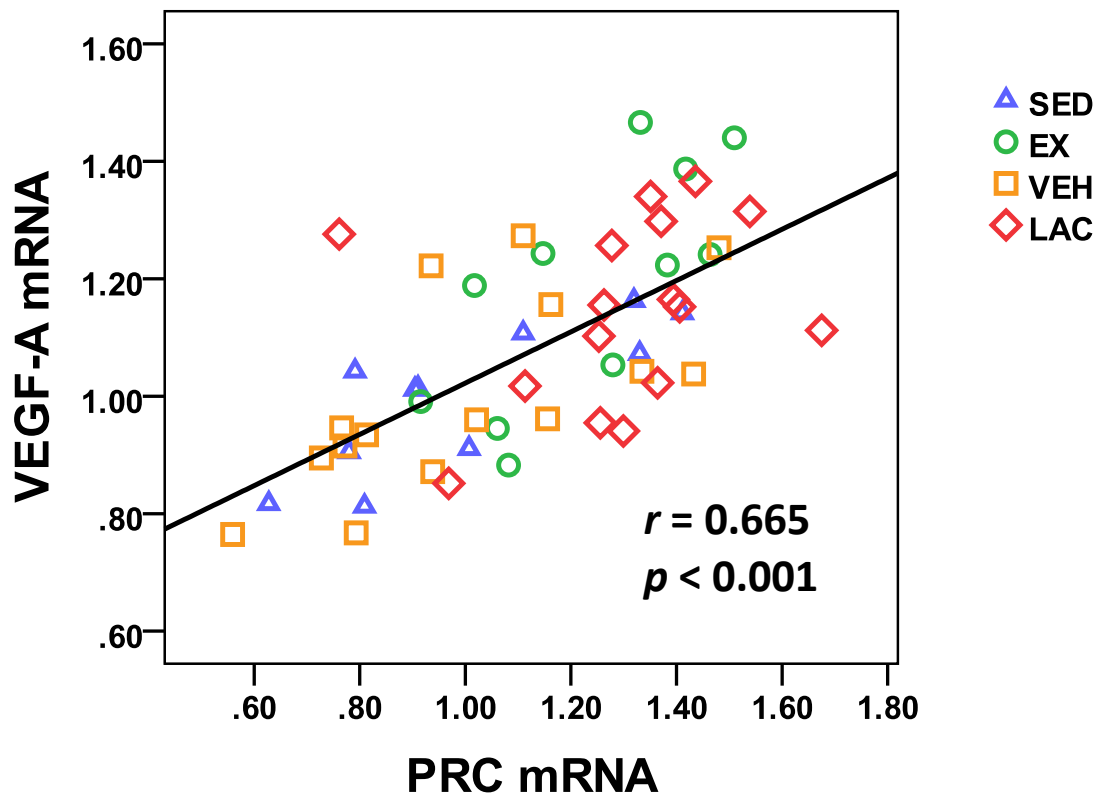


Figure 9. Relationship between brain VEGF-A and PRC mRNA levels. When data from all 4 groups were combined, a positive correlation between brain VEGF-A and PRC mRNA levels was observed. EX, exercise group; LAC, lactate group; PRC, peroxisomal proliferator-activated receptor-gamma co-activator 1 related co-activator; SED, sedentary group; VEGF-A, vascular endothelial growth factor A; VEH, vehicle group.

Chapter IV

Supra-lactate Threshold Exercise Training Induces Brain Mitochondrial Biogenesis in Aged Mice: Is there a Connection between Brain Mitochondrial Bioenergetics, Inflammation, and Neurogenesis?

(Manuscript in preparation)

Abstract

Intensive exercise training increases brain mitochondrial bioenergetics in young adult mice, while few studies have examined its effects on aging models. In this study, 19-month old C57BL/6 mice were subjected to supra-lactate threshold intensive treadmill training for 8 weeks. We primarily aimed to test whether the training induces brain mitochondrial biogenesis in aged mice. Meanwhile, we assessed the expression of neurogenesis-related factors, and central/systemic inflammatory factors to better understand the networking between exercise-induced benefits on brain. We found that our exercise training significantly increased brain peroxisomal proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1 α), citrate synthase (CS), and mitochondrial DNA (mtDNA) content, suggesting mitochondrial biogenesis was induced. This was accompanied by a slight increase in brain vascular endothelial growth factor A (VEGF-A) expression, and a strong positive correlation between brain PGC-1 related co-activator (PRC) and VEGF-A expression. Expression of doublecortin (DCX), brain-derived neurotrophic factor (BDNF), tumor necrosis factor alpha (TNF- α), and C-C motif chemokine 11 (CCL11) in the brain was not altered by exercise training. No clear associations were detected between mtDNA content and neurogenesis-related factors or central/systemic inflammatory factors. Plasma CCL11 negatively correlated with brain DCX expression, supporting the view that systemic inflammation has a strong suppressive effects on neurogenesis, while our intensive exercise training protocol did not alter plasma CCL11 levels in aged mice. Considering these and our previously published findings, we conclude that supra-lactate threshold exercise training induces brain mitochondrial biogenesis in both young and aged mice. Future mechanistic study will be

needed to unravel the relationships between brain mitochondrial biogenesis, neurogenesis, and inflammation with exercise training.

Introduction

Endurance exercise training enhances mitochondrial biogenesis, and improves bioenergetic metabolism and insulin resistance in muscle. This has been well established in young, aging, healthy, and diseased models (Lira *et al.*, 2010). How exercise training influences brain mitochondrial function and bioenergetics is relatively understudied.

A few studies have reported that long-term intensive exercise training induces brain mitochondrial biogenesis in healthy young adult rodents (Steiner *et al.*, 2011; E *et al.*, 2013b). Prolonged exercise training seems able to partially prevent aging-associated decline in the activities of brain mitochondrial electron transport chain (ETC) enzymes (Boveris & Navarro, 2008), while little is known regarding whether and how exercise training modulates brain mitochondrial biogenesis and bioenergetic metabolism in aged mice.

Considerable evidence has shown that exercise training induces neurogenesis in both young and aged mice (Fabel *et al.*, 2003a; van Praag *et al.*, 2005). Similar findings are also reported with older human subjects that long-term exercise increases brain volume, especially the hippocampus volume (Colcombe *et al.*, 2006; Erickson *et al.*, 2011). The mechanisms of exercise inducing neurogenesis are not clearly understood. The involvement of neurotrophic factors, specifically brain derived neurotrophic factor (BDNF), has been suggested (Rothman *et al.*, 2012), yet it is unknown what is mediating the exercise-induced increase of BDNF in the brain.

A recently published study has reported that increased plasma level of a member of C-C motif chemokine ligands (CCL), CCL11, has a strong suppressive effect on neurogenesis in mouse hippocampus, and the plasma level of this chemokine increases with age in both rodents and humans (Villeda *et al.*, 2011). Besides systemic

inflammation, local inflammatory response in the brain induced by microglia activation has also been shown detrimental for neurogenesis in adult brain (Ekdahl *et al.*, 2003). Mitochondria are one of the primary targets of inflammatory injury. Cytokines, such as TNF- α , have been shown to damage mitochondria through suppression of enzymes on mitochondrial ETC (Stadler *et al.*, 1992; Samavati *et al.*, 2008). Conversely, mitochondria-generated reactive oxygen species themselves also contribute to inflammatory response (Mukhopadhyay *et al.*, 2012), which may result in a vicious cycle between mitochondrial dysfunction and increased inflammation.

The relationships between brain mitochondrial function, inflammation, and neurogenesis under the condition of exercise training remain unknown. We wonder whether there is a role for exercise-mediated mitochondrial protection as a partial mechanism for the increase of neurogenesis associated with decreased inflammation.

The primary purpose of this study was to evaluate the effects of supra-lactate threshold intensive exercise training on brain mitochondrial biogenesis in aged mice. In addition, we tested the expression of neurogenesis-related factors, and systemic/brain levels of inflammatory cytokine/chemokine in these exercise trained and untrained aged mice. The relations between mitochondrial bioenergetics, inflammation, and neurogenesis were analyzed to better understand the connections between exercise-induced benefits on brain.

Methods

Animals

The animal work described in this study was approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Whenever possible, efforts were made to minimize animal discomfort. Twenty-four C57BL/6 male mice were included in these studies. Our mice were obtained from the National Institute of Aging, and were 18 months old when they reached our vivarium. All mice were maintained on an ad libitum diet, and were housed 2 per cage on a 12:12 hour light: dark schedule. After a one-month accommodation period, the mice were randomly placed into two groups, a control group (CT, $n = 12$), and an exercise group (EX, $n = 12$).

Exercise training

EX mice were exercised for 8 weeks, 5 days per week, 2 sessions per day, on a six-lane treadmill designed for mice (Columbus Instruments, Columbus, OH). The back of each treadmill lane contained an electrified grid, which delivered a shock stimulus (0.2 mA, 200 ms pulses, 1 Hz) to the mice who refused to run. The treadmill was set at 5° incline.

The EX mice were subjected to an incremental exercise training protocol in order to ensure that exercise intensity exceeded the lactate threshold. For the first week each session consisted of a 10 minute warm-up at 15 m/min followed by 30 minutes at 18 m/min. This speed approximates the lactate threshold for untrained C57BL/6 mice (Billat *et al.*, 2005). During the following 7 weeks, treadmill speed was progressively increased every week, based on the blood lactate levels measured immediately after a running session. Specifically, for weeks 2, 3, 4, 5, 6, 7, and 8, the treadmill speed was set to 21 m/min, 22 m/min, 23 m/min, 24 m/min, 25 m/min, 25 m/min, and 26 m/min, respectively.

Starting from the second week, warm-up was reduced to 5 min at 15 m/min, and the duration of each running session was adjusted every week in order to keep the same workload (running distance) everyday throughout the study. For example, the workload for each session during the first week was $10 \text{ min} \times 15 \text{ m/min} + 30 \text{ min} \times 18 \text{ m/min} = 690 \text{ m}$. During the sixth week, running time at 25 m/min was calculated as $[690 \text{ m} - (5 \text{ min} \times 15 \text{ m/min})] \div 25 \text{ m/min} = \sim 25 \text{ min}$.

The CT mice did not receive any exercise training. To minimize potential confounding factors such as differences in sound and light exposure, during the EX mice training sessions CT mice were placed in the same room as the EX mice.

At the conclusion of the 8-week training period, EX mice were sacrificed by decapitation one hour after the last session, and CT mice were also decapitated at approximately the same time on the same day. Brain tissue were immediately harvested and frozen in liquid nitrogen, and saved at -80°C for subsequent analysis.

Lactate, glucose, and insulin level determination

Blood lactate levels were measured using a Lactate Scout Analyzer (Senslab, Leipzig, Germany). Blood glucose levels were measured using a One-Touch Ultra Blood Glucose Monitoring System (LifeScan, Milpitas, CA). Plasma samples were also prepared from tail vein blood that was collected in heparinized micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA). Plasma insulin levels were measured using an insulin enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (American Laboratory Products Company, Salem, NH). Blood glucose levels were measured during a period of unrestricted food access (non-

fasting) and after a 17 hour fast (fasting), and plasma insulin levels were measured after a 17 hour fast. Values for the homeostasis model assessment of insulin resistance (HOMA-IR) were calculated as the product of fasting blood glucose level (mM) and plasma insulin level (microunits/ml) divided by 22.5 (Matthews *et al.*, 1985).

Quantitative real-time, reverse-transcription PCR

Total RNA was prepared from hippocampus of left hemisphere preserved in RNAlater® Solution (Life Technologies), 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 peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), PGC-1 β , PGC-1 related co-activator (PRC), nuclear respiratory factor 1 (NRF-1), NRF-2, mitochondrial transcription factor A (TFAM), cytochrome *c* oxidase subunit 2 (COX2), COX4, citrate synthase (CS), doublecortin (DCX), vascular endothelial growth factor A (VEGF-A), brain-derived neurotrophic factor (BDNF), TNF- α , and CCL11.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene. qPCR amplifications were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were quantified using the StepOnePlus Software v2.1 based on the *comparative $\Delta\Delta CT$* method.

MtDNA copy number determination

To quantify mtDNA, total DNA was extracted from cortex of left hemisphere using a phenol-chloroform based method as previously described (Guo *et al.*, 2009). DNA concentration and purity were determined spectrophotometrically by determining the absorbance at 260 nm and the 260 nm/280 nm ratio. Each real-time PCR reaction was performed using 10 ng of DNA as the template. We used TaqMan Gene Expression Assays (Applied Biosystems) to quantify the amounts of three target genes present on mtDNA, NADH dehydrogenase subunit 1 (ND1), cytochrome *c* oxidase subunit 2 (COX2) and ATP synthase F0 subunit 6 (ATP6), as well as the amount of a nuclear target gene, the 18S rRNA. The relative mtDNA to nuclear DNA copy number ratio was determined using the comparative $\Delta\Delta CT$ method, in which ND1:18S rRNA, COX2:18S rRNA, and ATP6:18S rRNA ratios were calculated.

Immunoblotting

Nuclear and cytoplasmic protein extracts from brain cortex of left hemisphere 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). 10 μ g of nuclear protein extracts or 40 μ g of cytoplasmic protein extracts was loaded per lane, and subjected to electrophoresis on a 4-15 % Criterion TM TGX TM Precast Gel (Bio-Rad Laboratories). The proteins were then transferred to a nitrocellulose membrane using iBlot TM (Invitrogen, Carlsbad, CA). The membrane was blocked with phosphate buffered saline (PBS) containing 0.1 % Tween-20 and 5 % non-fat dry milk (Bio-Rad

Laboratories) for 1 hour at room temperature, and subsequently incubated with the primary antibody (diluted in the blocking solution) overnight at 4 °C. The membrane was then rinsed in PBS with 0.1 % Tween and incubated with the horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG # 7074 or anti-mouse IgG # 7076; 1:2000 dilution; Cell Signaling Technology, Beverly, MA) for 1 hour at room temperature. After rinsing the membrane with PBS with 0.1 % Tween, the protein bands were detected with a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). In order to quantify protein band intensity, densitometry was performed using a ChemiDoc XRS with Quantity One software (Bio-Rad).

Several proteins of bioenergetic pathways were analyzed. Primary antibodies purchased from Cell Signaling Technology included antibodies to Akt (1:1000 dilution; #5373), phospho-Ser473 Akt (1:1000 dilution; #4060), glycogen synthase kinase 3 beta (GSK3 β) (1:1000 dilution; #9315), phospho-Ser9 GSK3 β (1:1000 dilution; #9322), 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), forkhead box O3a (FOXO3a) (1:500 dilution; #2497), 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 (1:500 dilution; #ab96600). Antibody to NRF1 (1:200 dilution; #sc-33771) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 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 (1:

2000 dilution; #A21348) was purchased from Life Technologies. GAPDH and HDAC1 were used as internal loading controls for cytoplasmic and nuclear fractions, respectively.

Plasma CCL11 levels

Non-fasting plasma samples were used to evaluate plasma CCL11 levels with a commercially available Mouse CCL11/Eotaxin Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. Plasma CCL11 levels were measured in both CT and EX groups at baseline and at the end of 8-week study.

Statistical analysis

Data were summarized by means and standard errors. Mean values were compared by independent samples *t*-tests or paired *t*-tests using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation analysis was used to test inter-parameter relationships. *p*-values less than 0.05 were considered statistically significant.

Results

Effects of treadmill training on blood lactate levels

In order to ensure that mice were running above the lactate threshold, blood lactate levels in EX group were measured every week immediately after a running session, and the treadmill speed was increased based on the measured levels. As shown in Fig. 1, blood lactate levels in CT group at rest were measured at week 1, 5, and 6, and the mean values ranged from 2.1 ~ 2.7 mM. Post-running blood lactate levels in EX group

ranged from 3.8 ~7.7 mM, and were always significantly higher than the resting levels in CT group.

Effects of treadmill training on body weight, glucose and insulin

At the beginning of the study, body weights were comparable between CT and EX groups. After 8 weeks, the weights in EX group were ~5 % lower than they were in CT group ($p < 0.05$, Fig. 2A). Paired t test analysis also showed EX mice lost ~2 % of their starting body weight ($p < 0.05$, Fig. 2A), while CT group maintained its weight.

Non-fasting blood glucose levels were comparable between CT and EX groups at the beginning of the study, and after 8 weeks they trended lower in EX group compared with the levels in CT group, or compared with its own levels at baseline, but the differences were not significant (Fig. 2B). No inter- or intra-group differences were detected in 17-hour fasting blood glucose levels as well as 17-hour fasting plasma insulin levels (Fig. 2C-D). HOMA-IR values in CT group were trending higher after sedentary 8 weeks when compared with the values at baseline, but the difference was not statistically significant (Fig. 2E). 8-week treadmill training did not alter HOMA-IR values.

Effects of treadmill training on brain mitochondrial biogenesis

The PGC-1 family, a group of transcriptional co-activators, plays a central role in a regulatory network governing the transcriptional control of mitochondrial biogenesis and respiratory function (Scarpulla, 2011). Three members have been identified: PGC-1 α , PGC-1 β and PRC. These co-activators target NRF-1 and NRF-2 which in turn regulate the transcriptional activity of TFAM (Scarpulla, 2008b; Vina *et al.*, 2009), and TFAM initiates the replication and transcription of mitochondrial DNA (mtDNA).

Our results showed that mRNA levels of PGC-1 α , PGC-1 β , PRC, NRF1, and NRF2 were all comparable between CT and EX brains (Fig. 3A). TFAM mRNA expression was decreased in EX group compared to CT group (~10 %, $p < 0.05$, Fig. 3A). mRNA levels of complex IV subunit 2 and 4 were comparable between groups (Fig. 3A). CS mRNA levels were ~30 % higher in EX brains when compared to CT brains ($p < 0.05$, Fig. 3A).

While brain PGC-1 α mRNA levels were comparable between groups, nuclear PGC-1 α protein levels were significantly higher in EX brains than they were in CT brains (~50 % increase, $p < 0.05$, Fig. 3B). No inter-group differences were detected in nuclear PGC-1 β or NRF1 protein levels. TFAM protein levels were also comparable between groups. COX4 protein levels trended higher in EX brains but the inter-group difference did not reach statistical significance (~35 % increase, $p = 0.27$, Fig. 3B). Protein levels of CS was comparable between CT and EX brains (Fig. 3B), despite the significant increase in the mRNA levels.

To further quantify mitochondrial biogenesis in the brain, relative brain mitochondrial DNA (mtDNA) copy numbers were assessed by quantitative real-time PCR. The ratios of ATP6/18S rRNA, COX2/18S rRNA and ND1/18S rRNA were all significantly higher in EX brains than they were in CT brains (40~50 % increase, $p < 0.05$, Fig. 3C).

Effects of treadmill training on bioenergetic metabolism related proteins in brain

Expression or phosphorylation levels of several bioenergetics-related proteins were analyzed by immunoblotting (Fig. 4). Cytoplasmic Akt phosphorylation at Ser 473, after correction for total Akt protein levels, was comparable between CT and EX groups.

Despite the unchanged levels of Akt phosphorylation, one of its downstream substrates, cytoplasmic GSK3 β phosphorylation at Ser 9, after correction for total GSK3 β protein levels, trended higher in EX brains, but the difference was not significant (~20 % increase, $p = 0.09$, Fig. 4A). When corrected for total mTOR protein levels, cytoplasmic mTOR phosphorylation at Ser 2448 in EX brains trended higher compared to CT brains (~25 % increase, $p = 0.1$, Fig. 4A). When corrected for internal loading control GAPDH, compared to CT group, cytoplasmic mTOR phosphorylation at Ser 2448 was significantly increased in EX group (~70 % increase, $p < 0.05$, Fig. 4A), and total mTOR protein levels were also higher in EX group (~40 % increase, $p < 0.05$, Fig. 4A). We also assessed the subcellular localization of FOXO1 as well as FOXO3a. Ratio of FOXO1 protein level in the nuclear fraction over cytoplasmic fraction trended lower in EX group when compared to CT group (~20 % decrease, $p = 0.2$, Fig. 4B). Ratio of FOXO3a protein level in the nuclear fraction over cytoplasmic fraction was significantly lower in EX brains than they were in CT brains (~20 % decrease, $p < 0.05$, Fig. 4C).

Effects of treadmill training on neurogenesis related factors and their associations with brain mitochondrial biogenesis

mRNA levels of three neurogenesis related factors, DCX, VEGF-A, and BDNF were evaluated (Fig. 5A). DCX is a microtubule-associated protein expressed specifically in young, immature neurons in hippocampus, and quantification of DCX-expressing cells allows for an accurate measurement of adult neurogenesis (Couillard-Despres *et al.*, 2005). VEGF, an angiogenic factor, and BDNF, a neurotrophin, have both been implicated in exercise-induced neurogenesis in rodents. (Jin *et al.*, 2002; Fabel *et al.*, 2003a; Rossi *et al.*, 2006). In the present study, due to technical issue, evaluation of

DCX-expressing cells in dentate gyrus was not conducted. Alternatively, mRNA expression of DCX in the hippocampus was measured; it was comparable between CT and EX groups (Fig. 5A). We found an insignificant increase of VEGF-A mRNA expression in EX brains when compared to CT brains (~12 % increase, $p = 0.09$, Fig. 5A). No inter-group difference was observed in BDNF mRNA expression (Fig. 5A).

Next, we tested the relations between the expression of neurogenesis-related factors and mitochondrial biogenesis. When combining the data from CT and EX groups, there was a slight insignificant trend for a positive correlation between brain DCX mRNA levels and ATP6, COX2, or ND1 copy number, respectively (DCX & ATP6: $r = 0.22$, $p = 0.3$; DCX & COX2: $r = 0.18$, $p = 0.4$; DCX & ND1: $r = 0.23$, $p = 0.27$; Fig. 5B-D). When analyzed by group separately, in CT group, significant positive correlations were detected between brain DCX mRNA levels and mtDNA content (DCX & ATP6: $r = 0.694$, $p < 0.05$; DCX & COX2: $r = 0.648$, $p < 0.05$; DCX & ND1: $r = 0.635$, $p < 0.05$; Fig. 5B-D). Interestingly, no correlations were observed between DCX mRNA levels and mtDNA content in EX group (Fig. 5B-D).

There was no significant correlations between brain VEGF-A mRNA levels and mtDNA copy numbers when combining the data from both CT and EX groups, or analyzed by group separately (Fig. 5E-G). However, a strong positive correlation was detected between brain VEGF-A mRNA expression and brain PRC mRNA expression when combining the data from CT and EX groups ($r = 0.614$, $p < 0.05$, Fig. 5H). When analyzed by group separately, the strong positive correlation remained in both groups (CT: $r = 0.751$, $p < 0.05$; EX: $r = 0.873$, $p < 0.001$; Fig. 5H). These results are consistent with what we previously reported in young adult mice (E *et al.*, 2013a).

Effects of treadmill training on brain and systemic inflammatory factors and their associations with brain mitochondrial biogenesis

Exercise training has been shown to decrease brain TNF- α expression in young adult mice (E *et al.*, 2013a). In the present study, we did not observe changes in TNF- α expression in aged mice subjected to 8-week treadmill training (Fig. 6A). No inter-group difference was seen in brain CCL11 mRNA levels as well (Fig. 6A).

We also tested the relations between brain TNF- α mRNA levels and mtDNA copy numbers, but did not observe significant correlations (data not shown). Similarly, plasma CCL11 levels were not correlated with mtDNA copy numbers (data not shown).

As shown in Fig. 6B, plasma CCL11 levels were comparable between CT and EX groups at baseline, and also comparable at completion of 8-week study. In both CT and EX groups, significant increases in plasma CCL11 levels were observed after completion of 8-week study, when compared to their respective levels at baseline (CT: ~30% higher after 8 weeks compared to baseline, $p < 0.001$; EX: ~40% higher after 8 weeks compared to baseline, $p < 0.05$; Fig. 6B).

Although we did not observe inter-group difference in brain DCX mRNA levels or plasma CCL11 levels, Pearson correlation analysis detected a moderate negative association between these two parameters when combining the data from CT and EX groups ($r = -0.415$, $p < 0.05$, Fig. 6C). When analyzed by group separately, in CT group, the strong negative correlation remained between brain DCX mRNA levels and plasma CCL11 levels ($r = -0.666$, $p < 0.05$, Fig. 6C), while no significant relation was detected in EX group ($r = -0.263$, $p = 0.4$, Fig. 6C). Loss of significance in the correlation in EX group was likely due to one outlier that falls in the leftmost part of Fig. 6C.

Discussion

Main findings of this study are that 8-week supra-lactate threshold treadmill training induces brain mitochondrial biogenesis and a slight increase of hippocampal VEGF-A expression in aged mice. A strong positive correlation exists between hippocampal PRC expression and VEGF-A expression. Also, plasma CCL11 levels are negatively associated with expression of hippocampal DCX in both trained and untrained aged mice, supporting the view that systemic inflammation has suppressive effects on neurogenesis.

Scientific evidence regarding the effects of exercise training of different duration, frequencies and intensities is needed to establish the rationale for clinically prescribing exercise for an individual or a certain population. In the present study, we are particularly interested in high-intensity exercise training above the lactate threshold. A previous study has shown that supra-lactate threshold treadmill training induces mitochondrial biogenesis and angiogenic/neurogenic factor expression, and drives reduction of inflammatory mediators in the brains of young adult mice (E *et al.*, 2013a). On the other hand, exercise training below lactate threshold does not alter expression of brain mitochondrial biogenesis components in mice of same strain and same age (E *et al.*, 2013a).

Over the 8 weeks of training, we did not observe any adverse effects in EX group during or after a running session. Our incremental exercise protocol seemed important in adapting aged mice to such a high-intensity training program. We did observe a slight decrease in body weights after 8 weeks of training, but the training certainly did not alter blood glucose or plasma insulin levels in these aged mice. It is possible that running

above lactate threshold provides liver with more substrate, lactate, for gluconeogenesis to maintain blood glucose levels.

While several studies have demonstrated exercise training increases brain mitochondrial biogenesis in young rodents (Steiner *et al.*, 2011; E *et al.*, 2013a), to our knowledge, few have studied whether exercise training modifies brain mitochondrial function in healthy aged mice/rats, especially from the perspective of mitochondrial biogenesis. Brain complex I and IV activities decrease upon aging, and regular moderate treadmill exercise for 24 to 50 weeks was able to prevent the age-associated decline in the activities of these enzymes in mice (Boveris & Navarro, 2008). Safdar and colleagues studied the effects of exercise training in mtDNA mutator mice displaying symptoms of accelerated aging, elevated mtDNA point mutations, and a systemic reduction in mitochondrial function, evident as early as 6-month of age. After moderate-intensity treadmill training for 5 months, the decline in mitochondrial function was prevented in the brains of these mtDNA mutator mice, but no difference in brain PGC-1 α expression was observed (Safdar *et al.*, 2011). In another study, 12-month old rats were subjected to daily 30-minute treadmill exercise at 18 m/min for 15 weeks, and brain PGC-1 α and NRF1 protein levels were found increased but mtDNA copy number or any other mitochondrial protein levels were not evaluated (Marosi *et al.*, 2012).

With mtDNA copy number as the most commonly used biochemical marker for mitochondrial biogenesis (Medeiros, 2008), we have demonstrated that intensive exercise training that exceeds lactate threshold significantly increases brain mtDNA copy number as well as PGC-1 α expression. Our results have important implication for preventing or treating aging-related neurodegenerative diseases, especially the most common form of

Alzheimer's disease (AD), sporadic late-onset AD, that often occurs after age of 65. Numerous studies provide evidence that mitochondrial enzyme deficiency, reduced mitochondrial biogenesis, and increased mtDNA damage in the AD brains may play a central role in the pathogenesis and progression of the disease (Readnower *et al.*, 2011; Selfridge *et al.*, 2013; Swerdlow *et al.*, 2013). Regular intensive exercise training may predispose aging individuals to healthier brain mitochondria that may be beneficial for preventing the onset of such diseases.

Although brain Akt phosphorylation was not increased in EX mouse brains, several downstream targets of Akt were likely activated. mTOR is one of them. Akt phosphorylates mTOR at Ser 2448, which leads to mTOR activation promoting protein synthesis and cell proliferation (Nave *et al.*, 1999; Reynolds *et al.*, 2002). mTOR is also known to control mitochondrial oxidative function through a Yin Yang 1 - PGC-1 α transcriptional complex (Cunningham *et al.*, 2007). Interestingly, inhibition of mTOR by rapamycin decreased expression of the genes encoding PGC-1 α and cytochrome *c*, but an Akt inhibitor did not, suggesting mTOR positively controls mitochondrial biogenesis-related gene expression in an Akt-independent manner (Cunningham *et al.*, 2007). Significant increase of nuclear PGC-1 α protein levels in EX brains were possibly mediated by elevated activity of mTOR.

GSK3 β is a ubiquitously expressed serine/threonine kinase that phosphorylates and inactivates glycogen synthase. The activity of this kinase can be inhibited by Akt-mediated phosphorylation at Ser 9 (Cole, 2012). GSK3 β has been implicated in the development of AD, because it induces tau hyperphosphorylation, the major component of neurofibrillary tangles that are involved in the disease pathogenesis (Lee *et al.*, 2005).

Transgenic mice overexpressing GSK3 β develop tau hyperphosphorylation and other neurological defects (Lucas *et al.*, 2001), while treatment of transgenic mice overexpressing mutant tau with a GSK3 β inhibitor (lithium) reduces the number of tangles in their brains (Noble *et al.*, 2005). In the present study, GSK3 β was slightly more phosphorylated at Ser 9 in EX brains when compared to sedentary mice, indicating our treadmill training protocol may decrease GSK3 β activity in aged mouse brain. Our results along with previous evidence showing treadmill exercise inhibits GSK3 β activation in a transgenic mouse model of AD (Liu *et al.*, 2013) further support the potential of aerobic exercise training in preventing AD development and/or progression.

Exercise-induced neurogenesis in mouse hippocampus has been reported previously in both young and older rodents (Fabel *et al.*, 2003a; van Praag *et al.*, 2005). Human subject studies have also evidenced that long-term moderate-to-intensive exercise (60 – 75% heart rate reserve) significantly increases hippocampal volume accompanied by improved memory function in older adults over 55 years old (Colcombe *et al.*, 2006; Erickson *et al.*, 2011). The most commonly applied method for evaluation of neurogenesis in rodents is using an exogenous cell tracer, 5'-bromo-2'-deoxyuridine (BrdU) in combination with endogenous neuronal markers, such as neuronal nucleus protein (NeuN), or quantification of DCX-expressing cells in dentate gyrus of the hippocampus with immunofluorescence technique (Wojtowicz & Kee, 2006). As mentioned before, DCX can be used to identify young, immature neurons specifically. In this study, due to technical issues, we did not quantify DCX-expressing neurons but alternatively estimated the mRNA expression of DCX in the hippocampus; however, it was not changed by 8-week exercise training.

Impairment of hippocampal neurogenesis has been suggested to be involved in neurodegenerative diseases with cognitive dysfunction, such as AD and Parkinson's disease (PD) (Winner *et al.*, 2011). It is also significantly associated with aging (Couillard-Despres *et al.*, 2011). All these conditions are associated with brain mitochondrial impairment, including dysfunction of mitochondrial ETC enzymes, mtDNA mutations, and decreased mitochondrial biogenesis as mentioned above. Whether mitochondrial impairment plays a mechanistic role in the reduction of neurogenesis remains relatively unstudied. Dysfunctioning mitochondria may impair neuronal progenitor cell differentiation, as it requires energy produced by mitochondria for growth of neuronal processes, cytoskeletal remodeling, and organelle transport (Voloboueva & Giffard, 2011). Here, we indirectly tested the relation between brain mitochondrial function and neurogenesis by correlating brain mtDNA content and neurogenesis-related factors in treadmill trained and untrained aged mice. When CT and EX groups were combined, we saw a trend for a positive association between DCX mRNA levels and mtDNA copy numbers. When we analyzed the relations in CT or EX group separately, significantly strong correlations were detected between brain DCX mRNA levels and mtDNA copy numbers in CT group but not in EX group. The discordant results might be explained by the possibility that decreased brain mitochondrial biogenesis would impair the process of neurogenesis, while exercise-induced mitochondrial biogenesis may not directly improve neurogenesis, implying that other mediators, such as inflammation, may be involved in governing neurogenesis, which are discussed below. In addition, small sample size may also result in false positive or false negative results in correlation analysis. One thing to be pointed out here is that

these mRNA levels were measured with RNA extracted from hippocampi while mtDNA copy numbers were evaluated using DNA extracted from cortices. We need to take this technical factor into account when conclude about the relations between brain mitochondrial biogenesis and the expression of neurogenic factors in hippocampus.

VEGF-A, an angiogenic factor, has been implicated as essential in exercise-induced neurogenesis (Jin *et al.*, 2002; Fabel *et al.*, 2003a; Font *et al.*, 2010). Our data show that the ratio of nuclear to cytoplasmic protein levels of FOXO3a, known to repress VEGF-A expression (Karadedou *et al.*, 2012), was significantly decreased, and VEGF-A expression levels trended higher in EX brains. Our previous study has shown that brain VEGF-A expression induced by exercise training is likely mediated by circulating lactate which is released by exercising muscles (E *et al.*, 2013a).

While significant association between VEGF-A expression and mtDNA content was not detected in the present study, a strong positive correlation has been found between brain VEGF-A and PRC mRNA levels independent of exercise training. The results are consistent with what we found previously in young adult mice (E *et al.*, 2013a). This strong positive correlation seen in both young and aged mouse brain suggests that PRC may play a role in mediating VEGF expression changes, and further implies an association between brain bioenergetic metabolism and the process of neurogenesis.

Inflammation may be involved in aging or neurodegenerative disease-related reduction of neurogenesis. A previous study has shown that lipopolysaccharide-induced inflammation mediated by microglia activation in rat hippocampus strongly impairs hippocampal neurogenesis (Ekdahl *et al.*, 2003). In another study, it was reported that plasma CCL11 levels increased with age in both rodents and human subjects, and

increased plasma CCL11 level was identified as associated with reduction of hippocampal neurogenesis seen in aged mice (Villeda *et al.*, 2011). Injecting CCL11 intraperitoneally significantly decreases DCX-positive cells in the dentate gyrus of healthy young mice, and this decrease could be rescued by neutralizing CCL11 within the dentate gyrus by injecting CCL11-specific antibody to the area (Villeda *et al.*, 2011).

In the present study, we found that after 8 weeks, the plasma levels of CCL11 were increased in both CT and EX groups when compared to their respective levels at baseline. Also, plasma CCL11 levels (after 8-week study) are negatively correlated with DCX mRNA in the hippocampus. Our data support the notion that systemic CCL11 may exert suppressive effects on hippocampal neurogenesis.

Whether or not exercise training is able to reduce systemic CCL11 levels, which may potentially contribute to increased neurogenesis, was tested in the present study. However, we did not observe 8-week exercise training lowering plasma CCL11 levels as we expected, even though numerous studies have shown that long-term exercise training decreases the levels of pro-inflammatory cytokines in the blood of older human subjects (Woods *et al.*, 2012). Future research with longer period (6-12 months) of exercise training may be able to address the effects of exercise in modifying plasma CCL11 levels.

Inflammation down-regulates neurogenesis, and multiple factors may be mediating it. Mitochondria can be one of them. Mitochondria play an important role in pro-inflammatory signaling, and vice versa, pro-inflammatory mediators may also alter mitochondrial function, resulting in a vicious cycle. Little is known whether mitochondrial protection is able to attenuate inflammation-induced decrease of neurogenesis. Although we found that intensive exercise training significantly increased

brain mitochondrial biogenesis in the aged mice, no clear correlations between brain mtDNA content and brain TNF- α , or CCL11 mRNA levels were detected. This is probably because in these aged mouse brains, expression of TNF- α and CCL11 was not altered by 8-week intensive exercise training, though similar training protocol reduced TNF- α expression in brains of young adult mice (E *et al.*, 2013a). Also, no correlation between plasma CCL11 levels and brain mtDNA content was observed, suggesting the brain mitochondrial biogenesis induced by exercise training was unlikely mediated by the modification of inflammatory factors in the blood.

In the present study, 19-month old C57BL/6 mice were able to tolerate supra-lactate threshold treadmill exercise training over the 8 weeks, with the exception of four EX mice that often reached exhaustion before completing a running session. However, the data from these mice did not deviate from the others in the EX group, even though their exercise workloads (running distance) were less. Future studies may be needed to investigate the minimum exercise workload/intensity that is sufficient to induce brain mitochondrial biogenesis, which could provide more clinically relevant information for designing individualized exercise protocol for older adults.

In summary, we have demonstrated that supra-lactate threshold exercise training increases brain mitochondrial biogenesis in aged mice. The event is accompanied by a slight increase of brain expression of VEGF-A. While mitochondrial biogenesis is not clearly associated with neurogenesis or inflammatory factors in the brains of aged mice, a transcription factor and a co-activator that play important roles in regulating bioenergetic network (PRC; FOXO3a) are likely influencing a brain angiogenesis/neurogenesis mediator (VEGF-A). Meantime, our data support the view that systemic inflammation

has a strong suppressive effect on hippocampal neurogenesis, though intensive exercise training does not affect the aging-associated increase of plasma chemokine level. Nonetheless, together with our previously published study (E *et al.*, 2013a), we conclude that supra-lactate threshold exercise training induces brain mitochondrial biogenesis in both young and aged mice, which has important therapeutic implications for neurological disorders with perturbed brain bioenergetics, such as neurodegenerative diseases. Mechanistic studies will be needed to identify the possible causal relationships between brain mitochondrial biogenesis, neurogenesis, and inflammation, under the condition of exercise.

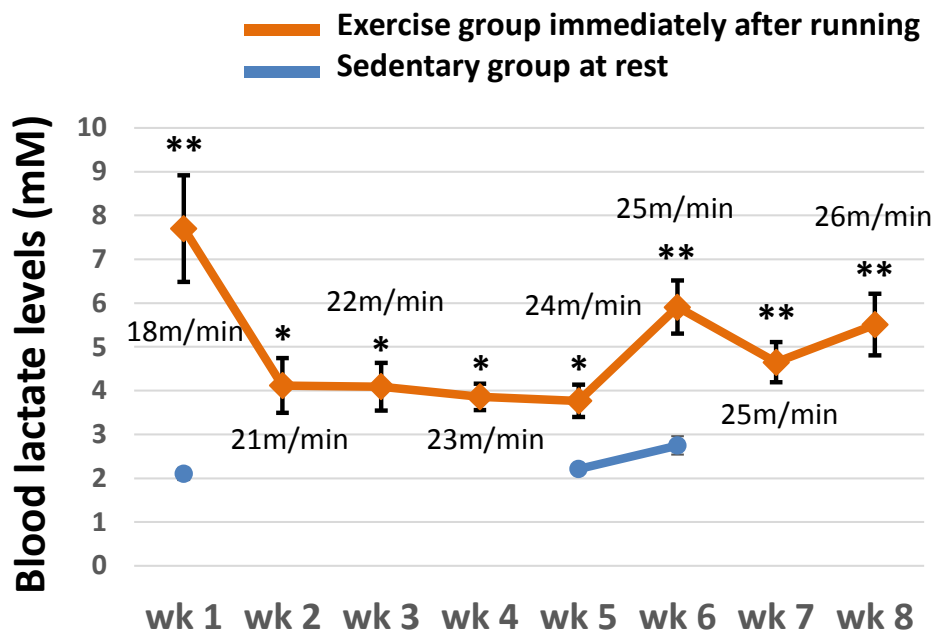


Figure 1. Blood lactate levels. Post-running blood lactate levels in EX group were measured immediately after a running session every week. Blood lactate levels in CT group at rest were measured at weeks 1, 5, and 6. Post-running blood lactate levels in EX group were always significantly higher than they were in CT group at rest. The treadmill speed for each week is indicated. CT, control group; EX, exercise group. For weeks 1-4, the blood lactate levels in EX group were statistically compared to the levels in CT group from week 1; for week 5, the blood lactate levels in EX group were statistically compared to the levels in CT group from week 5; for weeks 6-8, the blood lactate levels in EX group were statistically compared to the levels in CT group from week 6. * $p < 0.05$, compared with CT group; ** $p < 0.001$, compared with CT group.

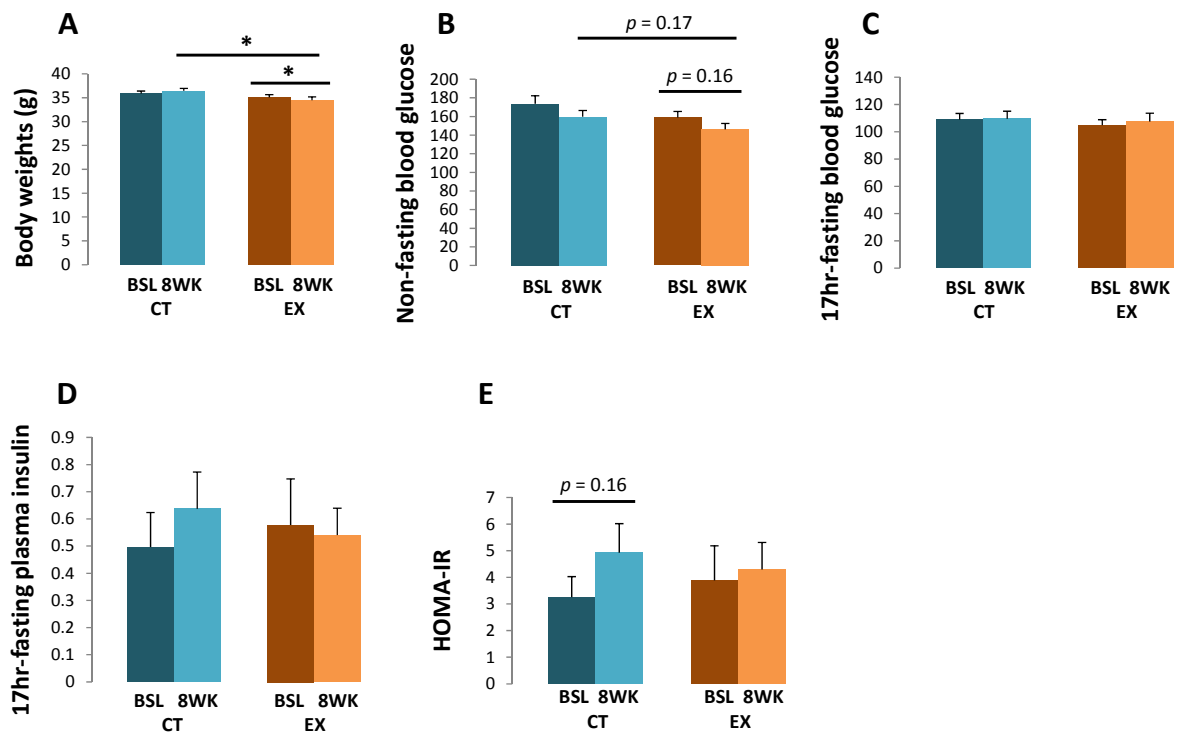


Figure 2. Effects of treadmill training on body weights, blood glucose levels, plasma insulin levels, and HOMA-IR. (For figure legend, see next page).

Figure 2. Effects of treadmill training on body weights, blood glucose levels, plasma insulin levels, and HOMA-IR. (A) 8-week exercise training significantly decreased the weights in EX group when compared to CT group at the end of the study. Paired *t* test analysis also showed a statistically significant decrease in EX group weights after 8-week exercise training when compared to baseline. **(B)** Non-fasting blood glucose level trended lower in EX group than that in CT group after 8-week training, but the difference was not significant. **(C)** No inter- or intra-group differences were detected in 17-hour fasting blood glucose levels. **(D)** No inter- or intra-group differences were detected in 17-hour fasting plasma insulin levels as well. **(E)** HOMA-IR values in CT group were trending higher after sedentary 8 weeks when compared with the values at baseline, but the difference was not significant. No intergroup difference was detected in HOMA-IR values. * $p < 0.05$. BSL, baseline; CT, control group; EX, exercise group; HOMA-IR, homeostasis model assessment of insulin resistance.

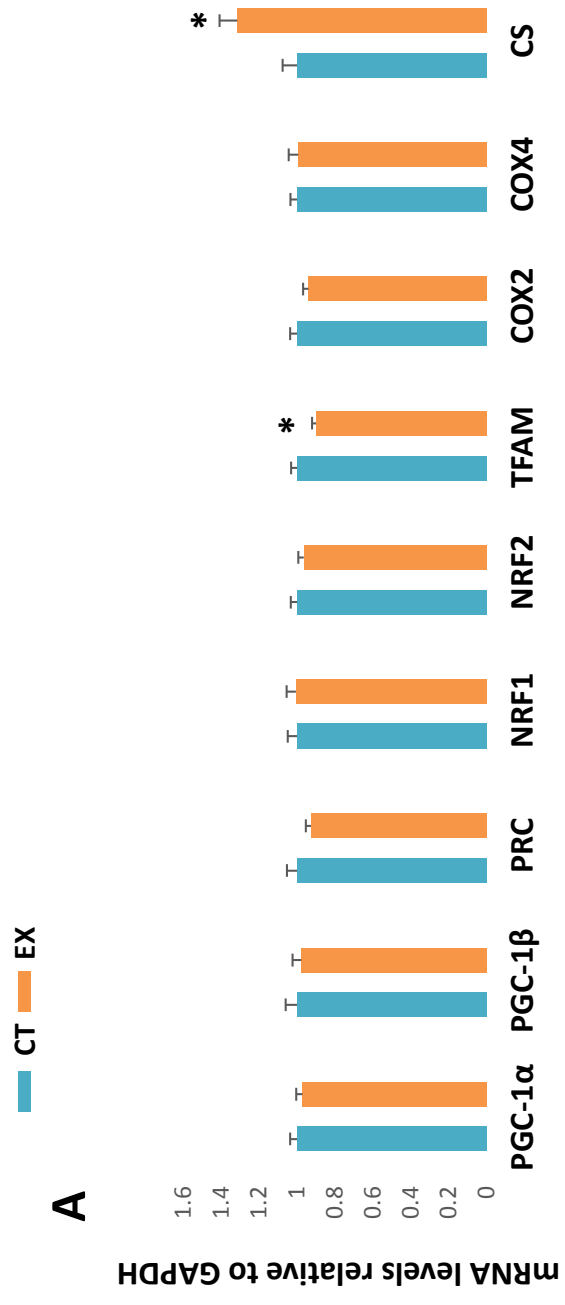


Figure 3: Effects of treadmill training on mitochondrial biogenesis in aged mouse brain (continued).

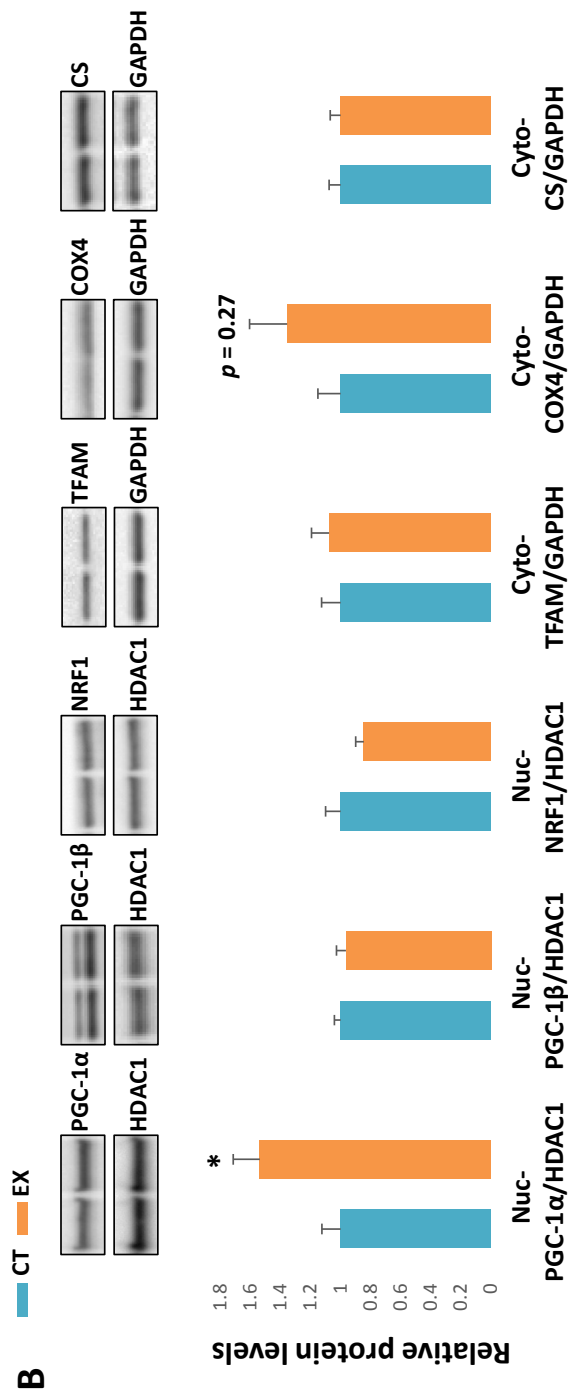


Figure 3: Effects of treadmill training on mitochondrial biogenesis in aged mouse brain (continued).

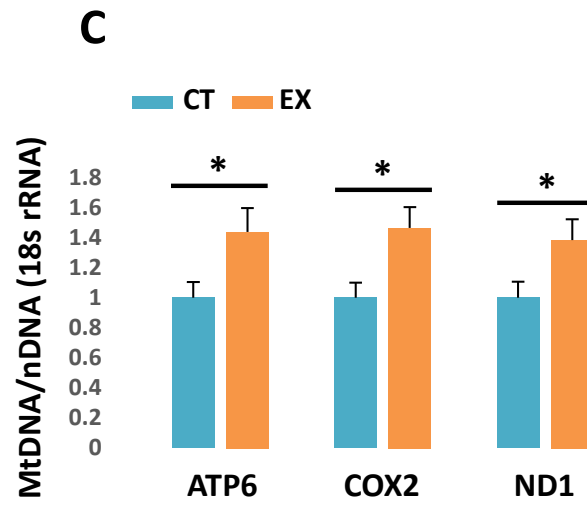


Figure 3: Effects of treadmill training on mitochondrial biogenesis in aged mouse brain (continued). (For figure legend, see next page).

Figure 3: Effects of treadmill training on mitochondrial biogenesis in aged mouse brain (continued). (A) mRNA levels of PGC-1 α , PGC-1 β , PRC, NRF1, and NRF2 were all comparable between CT and EX groups. TFAM mRNA expression was decreased in EX group. mRNA levels of complex IV subunit 2 (COX2) and subunit 4 (COX4) were comparable between groups. CS mRNA levels were significantly increased in EX brains when compared to CT group. (B) Protein levels of PGC-1 α in nucleus were significantly higher in EX brains than they were in CT brains, while no inter-group differences were detected in nuclear PGC-1 β and NRF1 protein levels. TFAM protein levels were also comparable between groups. COX4 protein levels trended higher in EX brains but the inter-group difference did not reach statistical significance. Protein expression of CS was comparable between CT and EX groups, despite the significant increase in its mRNA levels induced by treadmill training. GAPDH and HDAC1 served as an internal loading control for cytoplasmic and nuclear fraction, respectively. (C) Relative brain mitochondrial DNA (mtDNA) copy numbers were evaluated. ATP synthase F0 subunit 6 (ATP6)/18S rRNA, COX2/18S rRNA and NADH dehydrogenase subunit 1 (ND1)/18S rRNA ratios were significantly higher in EX brains than they were in CT brains. * $p < 0.05$, compared to CT group. ATP6, ATP synthase F0 subunit 6; COX2, cytochrome c oxidase subunit 2; COX4, cytochrome c oxidase subunit 4; CS, citrate synthase ; CT, control group; EX, exercise group; ND1, NADH dehydrogenase subunit 1; NRF, nuclear respiratory factor; PGC-1, peroxisomal proliferator-activated receptor-gamma co-activator 1; PRC, PGC-1 related co-activator; TFAM, mitochondrial transcription factor

A.

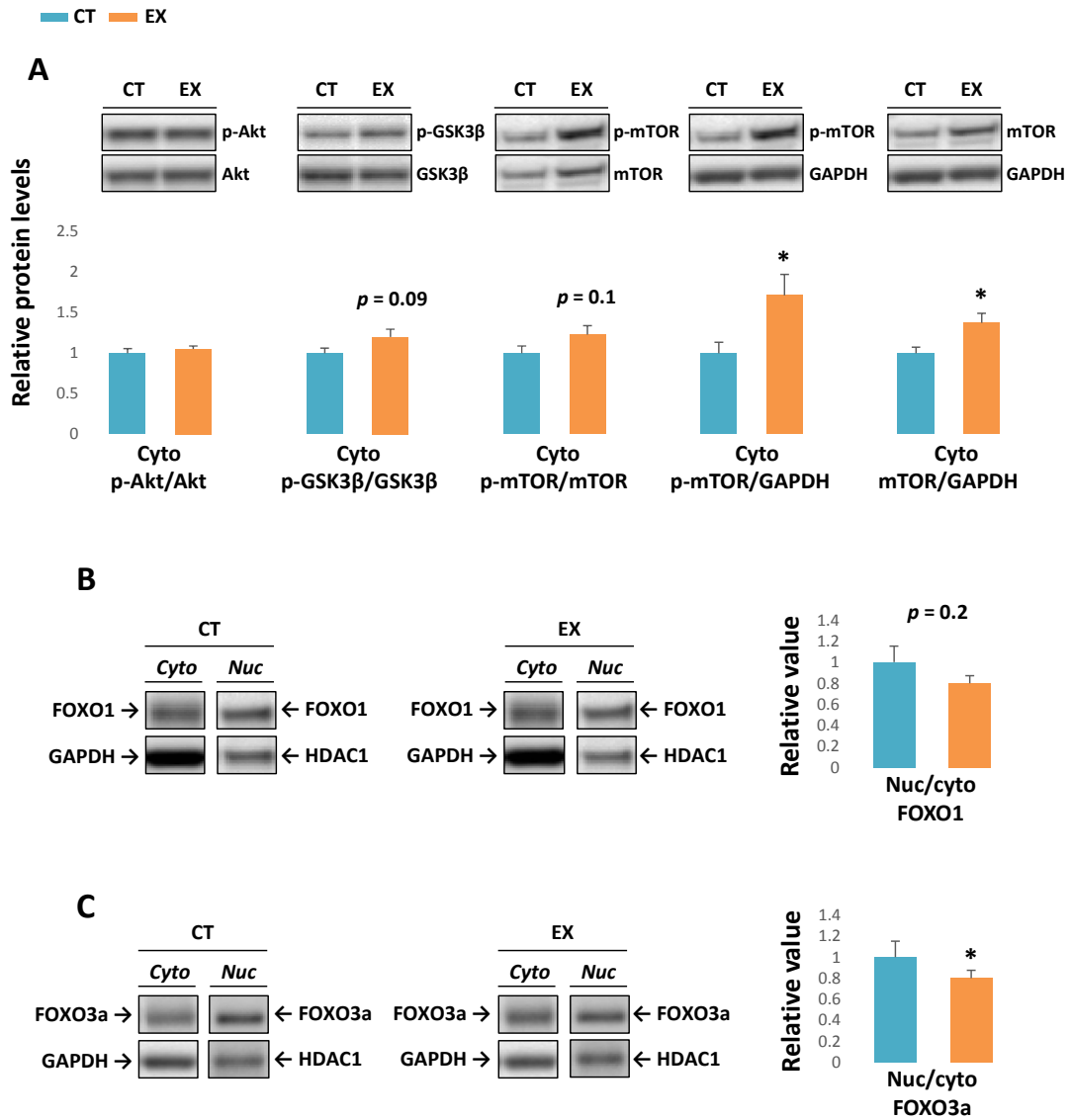


Figure 4. Effects of treadmill training on bioenergetic metabolism related protein levels in aged mouse brain. (For figure legend, see next page).

Figure 4. Effects of treadmill training on bioenergetic metabolism related protein levels in aged mouse brain. (A) Cytoplasmic Akt phosphorylation at Ser 473, corrected for total Akt protein levels, was comparable between CT and EX groups. Cytoplasmic GSK3 β phosphorylation at Ser 9, corrected for total GSK3 β protein levels, trended higher in EX brains than that in CT group, but the difference was not significant. When corrected for total mTOR protein levels, cytoplasmic mTOR phosphorylation at Ser 2448 in EX brains trended higher compared to CT brains. When corrected for internal loading control GAPDH, cytoplasmic mTOR phosphorylation at Ser 2448 was significantly increased in EX group, and total mTOR protein levels were also higher in EX group, compared to CT group. **(B)** The ratio of FOXO1 protein level in nuclear fraction over cytoplasmic fraction was calculated to evaluate its compartmentalization. The ratio trended lower in EX group. **(C)** The ratio of FOXO3a protein level in nuclear fraction over cytoplasmic fraction was significantly lower in EX group than it was in CT group. All values were ratioed to the mean values in CT group. Cyto: cytoplasmic; nuc: nuclear. * $p < 0.05$, compared to CT group. CT, control group; cyto, cytoplasmic; EX, exercise group; FOXO1, forkhead box protein O1; FOXO3a, forkhead box O3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK, glycogen synthase kinase; HDAC1, histone deacetylase 1; mTOR, mammalian target of rapamycin; nuc, nuclear.

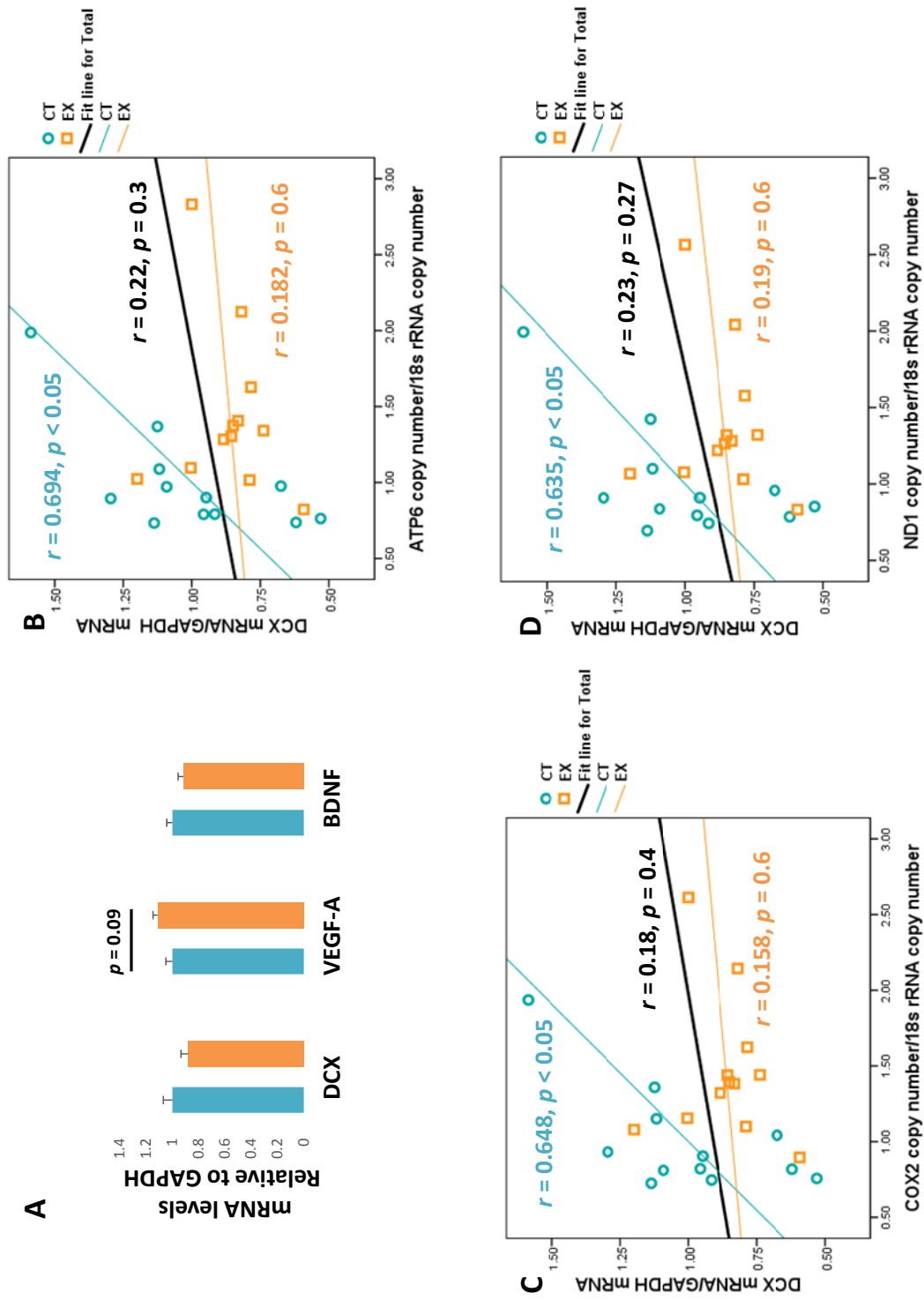


Figure 5: Effects of treadmill training on neurogenesis-related factors in aged mouse brain.

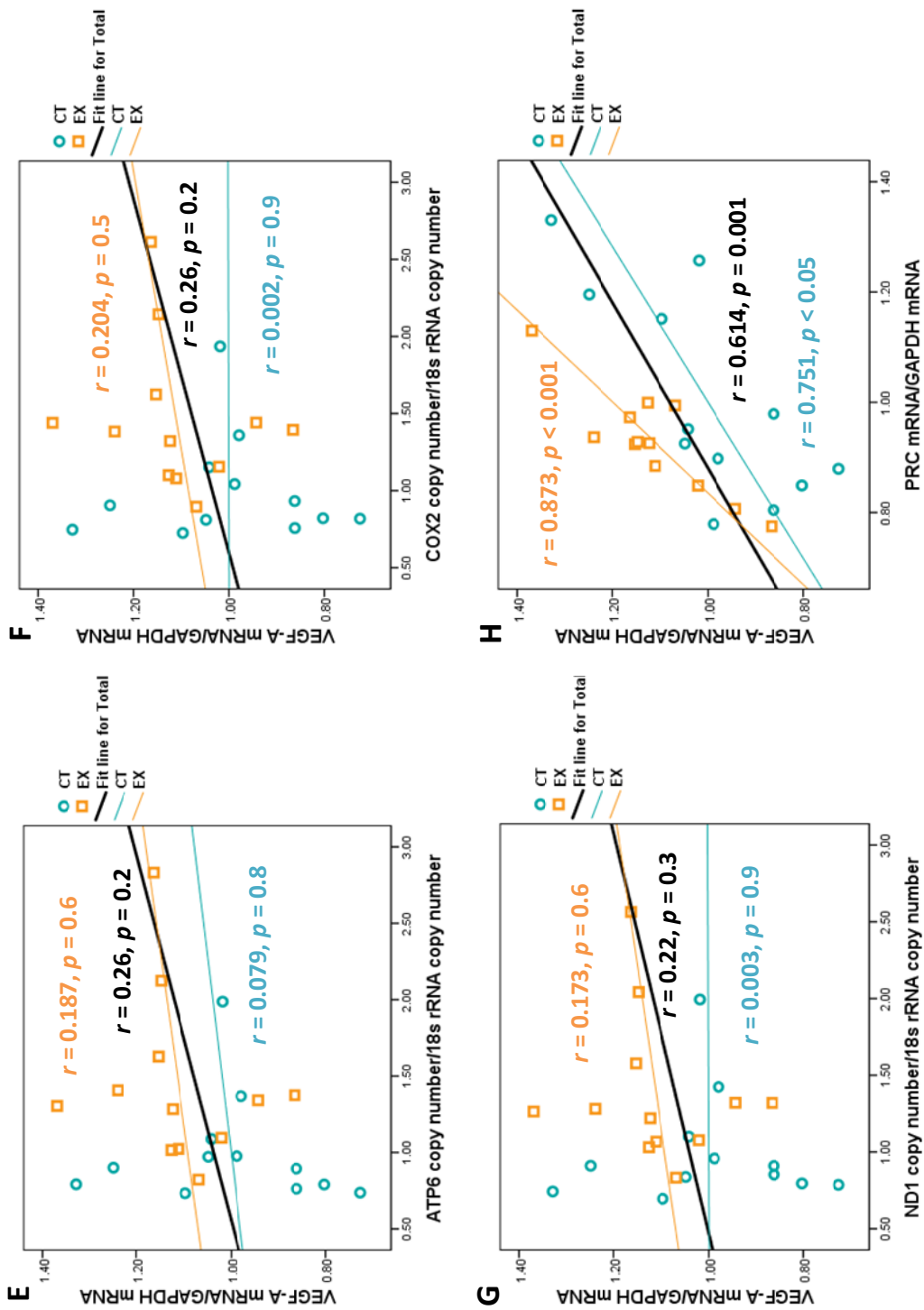


Figure 5: Effects of treadmill training on neurogenesis-related factors in aged mouse brain (continued). (For figure legend, see next page).

Figure 5: Effects of treadmill training on neurogenesis-related factors in aged mouse

brain. (A) mRNA levels for all genes are presented relative to the internal control GAPDH. DCX mRNA levels were comparable between CT and EX groups. VEGF-A mRNA levels were trending higher in EX group than they were in CT group, but the difference was not significant. BDNF mRNA levels were also comparable between groups. **(B-D)** shows the relations between brain DCX mRNA levels and mtDNA copy numbers. When combining the data from CT and EX groups, there was a slight trend for a positive correlation between brain DCX mRNA levels and ATP6, COX2, and ND1 copy numbers, respectively, but without statistical significance. When analyzed by group separately, in CT group, significant positive correlations were detected between brain DCX mRNA levels and ATP6, COX2, and ND1 copy numbers, respectively. No correlations were observed between DCX mRNA levels and mtDNA copy numbers in EX group. **(E-G)** shows the relations between brain VEGF-A mRNA levels and mtDNA copy numbers. No significant correlations were detected when combining the data from both CT and EX groups, or analyzed by group separately. **(H)** A strong positive correlation was detected between brain VEGF-A mRNA levels and brain PRC mRNA levels. When analyzed by group separately, the strong positive correlation was observed in both CT and EX groups. AT6, ATP synthase F0 subunit 6; BDNF, brain-derived neurotrophic factor; COX2, cytochrome c oxidase subunit 2; CT, control group; DCX, doublecortin; EX, exercise group; ND1, NADH dehydrogenase subunit 1; PRC, peroxisomal proliferator-activated receptor-gamma co-activator 1 related co-activator; VEGF-A, vascular endothelial growth factor A.

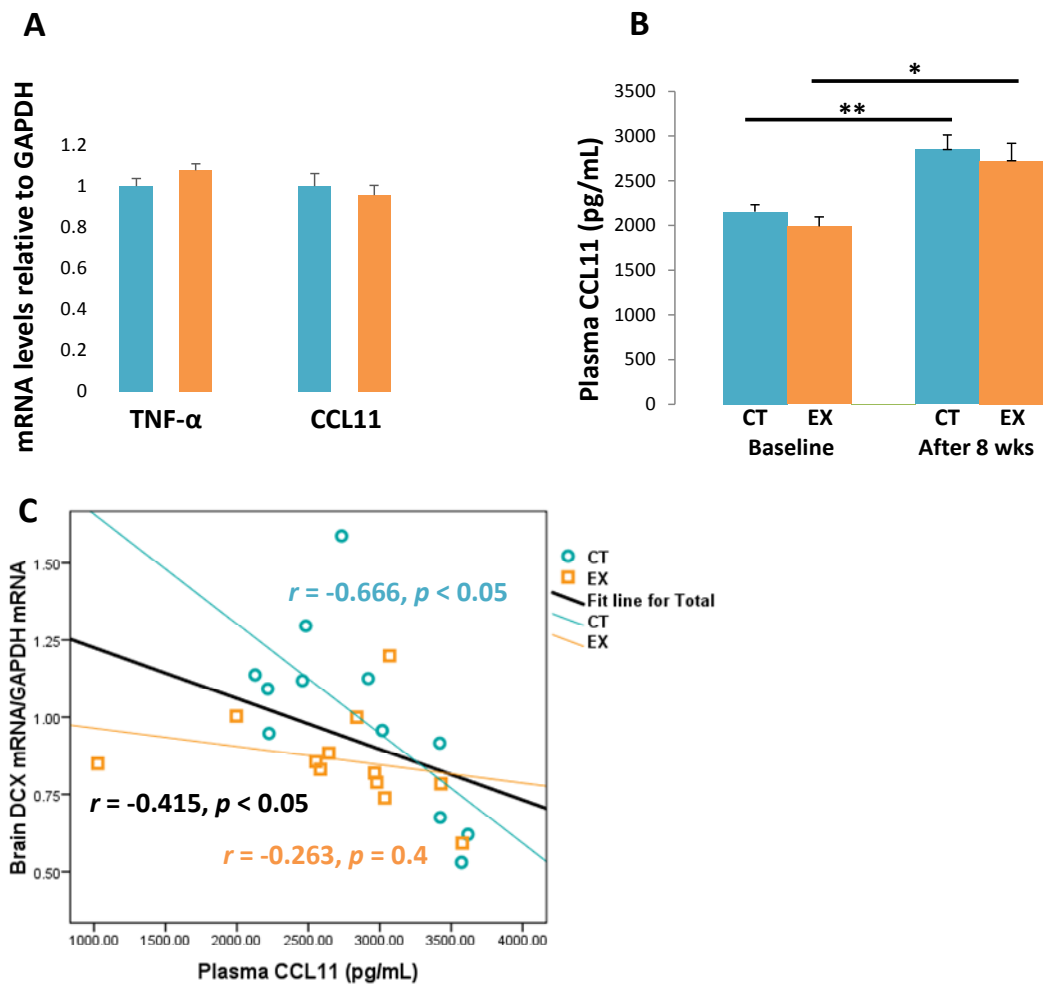


Figure 6: Effects of treadmill training on brain and systemic inflammatory factors in aged mice. (For figure legend, see next page).

Figure 6: Effects of treadmill training on brain and systemic inflammatory factors in aged mice. (A) TNF- α and CCL11 mRNA levels were comparable between CT brains and EX brains. (B) Plasma CCL11 levels were comparable between CT and EX groups at baseline and after completion of 8-week study. In both CT and EX groups, significant increases in plasma CCL11 levels were observed after completion of 8-week study, when compared to the levels at baseline. (C) The relation between brain DCX mRNA levels and plasma CCL11 levels is shown. When combining the data from CT and EX groups, a strong negative correlation between brain DCX mRNA levels and plasma CCL11 levels was detected. When analyzed by group separately, in CT group, brain DCX mRNA levels were strongly negatively related to plasma CCL11 levels, while no significant relation was detected in EX group. * $p < 0.05$; ** $p < 0.001$. CCL11, C-C motif chemokine 11; CT, control group; DCX, doublecortin; EX, exercise group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF- α , tumor necrosis factor alpha.

Chapter V

Lactate Treatment Modifies Bioenergetic Fluxes and Infrastructures in Human Neuronal Cells

(Manuscript in preparation)

Abstract

More than a metabolic waste, lactate has neuroprotective effects as an alternative energy source, and possibly mediates exercise-induced benefits for brain. Whether exogenous lactate modifies brain bioenergetic fluxes and infrastructures is however an unanswered question. In this study, we treated SH-SY5Y human neuronal cells with 10 mM or 20 mM lactate for 6 hours. Lactate treatment significantly decreased glycolysis flux and had a prolonged enhancement of mitochondrial respiration. Over time exogenous lactate gradually shifted the bioenergetic metabolism towards a more aerobic state. An increase of NAD⁺/NADH ratio was observed in lactate-treated cells. The changes in these fluxes were not accompanied by alteration of mitochondrial coupling efficiency or overall ATP production. Although we did not observe changes in mitochondrial DNA (mtDNA) copy number, expression of peroxisomal proliferator-activated receptor-gamma coactivator 1 beta (PGC-1 β), nuclear respiratory factor 1 (NRF-1), and cytochrome *c* oxidase subunit 1 (COX1) were significantly increased in lactate-treated cells, suggesting some components of mitochondrial biogenesis were up-regulated. We also found activities of COX and citrate synthase (CS) increased with lactate treatment. Expression of vascular endothelial growth factor A (VEGF-A), an angiogenic factor, was also raised. These lactate-induced expression changes were likely mediated by activation of AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (MAPK), and Akt signaling pathways to modify bioenergetic infrastructures. Additionally, lactate treatment decreased the activity of mammalian target of rapamycin (mTOR) but induced nuclear translocation of forkhead box protein O1 (FOXO1), suggesting a potential role for lactate in longevity. The results from this study provide novel insights into bioenergetics-based

pharmacological therapies for neurodegenerative diseases with perturbed brain
bioenergetic fluxes.

Introduction

Mitochondrial dysfunction and perturbed energy metabolism in different organs increase with age (Navarro *et al.*, 2004; Bratic & Larsson, 2013). These bioenergetic dysfunctions are observed in brains of some neurological disorders, such as Alzheimer's and Parkinson's diseases (Lin & Beal, 2006; Lezi & Swerdlow, 2012). Bioenergetic medicine has been proposed for treatment of these diseases; it refers to the manipulation of mitochondrial respiratory flux as well as bioenergetic pathways outside the mitochondria to positively affect health (Swerdlow, 2013).

Although lactate has long been considered a metabolic waste end product from anaerobic glycolysis, recent research supports the notion that lactate may be a potential therapeutic candidate in bioenergetic medicine. Previous studies have shown that exercise training shifts mouse liver mitochondrial function from respiration towards non-respiratory activities, such as gluconeogenesis to help maintain blood glucose levels (E *et al.*, 2013a). Systemic lactate administration induces same effects in mouse liver (E *et al.*, 2013a). In mouse brain, exercise training above lactate threshold and intraperitoneal lactate injection have similar effects in terms of increasing the expression of peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) related coactivator (PRC), which has a role in inducing the activities of mitochondrial respiration related enzymes (Mirebeau-Prunier *et al.*, 2010), as well as an angiogenic factor, vascular endothelial growth factor (VEGF) (E *et al.*, 2013a). These studies suggest exercise-generated lactate partially accounts for exercise-induced modification of bioenergetics in liver and brain, potentially mediated by lactate-induced bioenergetic flux changes (Swerdlow, 2013).

Here, we further study the possibility of lactate treatment as a strategy for bioenergetic flux manipulation, and investigate the underlying mechanisms using human

neuronal cells. In the present study, we report for the first time that lactate treatment increases mitochondrial respiratory fluxes while suppresses glycolysis rate in human neuronal cells. In addition, lactate modifies bioenergetic infrastructure to maintain the improved electron transfer via the mitochondrial electron transport chain (ETC), and it activates several signaling pathways that are involved in mitochondrial biogenesis as well as in longevity.

Methods

Cell culture

Undifferentiated SH-SY5Y 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). Cells were cultured to 60-80 % confluency in T75 culture flasks before use.

Lactate treatment

Cells were grown in SH-SY5Y regular growth medium, and medium was changed twice a day in order to minimize the effects by endogenously produced lactate. 24 hours after seeding the cells for the experiment, 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), for both control and treatment groups. After cells

have adapted to the condition with 5mM glucose and without FBS for overnight (18-20 hours), for treatment groups (LAC), medium was changed to DMEM-5 with either 10mM or 25 mM sodium lactate (#L7022, Sigma-Aldrich, St. Louis, MO). The concentrations of lactate were selected as those levels were seen in human blood and muscles after intensive exercise (Ohkuwa *et al.*, 1984; Cheetham *et al.*, 1986). For all experiments, the cells were pre-incubated with lactate for 6 hours, except for the mitochondrial respiration and glycolysis assays (detailed treatment procedures for these two assays are described in both 'Methods' and 'Results'). For the control group (CT), DMEM-5 without sodium lactate was added to the cell culture at the same time.

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-1 α , PGC-1 β , PGC-1 related co-activator (PRC), nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (TFAM), cytochrome *c* oxidase subunit 1 (COX1), COX4, pyruvate dehydrogenase alpha 1 (PDHA1), pyruvate dehydrogenase kinase 4 (PDK4), pyruvate carboxylase (PC), malic enzyme 1 (ME1), malic enzyme 2 (ME2), and vascular endothelial growth factor A (VEGF-A). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene. qPCR

amplifications were performed using an Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). Relative mRNA levels were quantified using the StepOnePlus Software v2.1 based on the *comparative* $\Delta\Delta CT$ method.

Mitochondrial DNA copy number determination

To quantify mitochondrial DNA (mtDNA), total DNA was extracted using a phenol-chloroform based method as previously described (Guo *et al.*, 2009). DNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 nm and the 260 nm/280 nm ratio. Each real-time PCR reaction was performed using 10 ng of DNA as the template. We used TaqMan Gene Expression Assays (Applied Biosystems) to quantify the amounts of two target genes present on mtDNA, NADH dehydrogenase subunit 2 (ND2) and the 16S ribosomal RNA (rRNA), as well as the amount of a nuclear target gene, the 18S rRNA. The relative mtDNA to nuclear DNA copy number ratio was determined using the comparative $\Delta\Delta CT$ method, in which ND2:18S rRNA and 16S rRNA:18S rRNA ratios were calculated.

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). 10 μg of nuclear protein extracts or 40 μg of cytoplasmic protein extracts was loaded per lane, and subjected to electrophoresis on a 4-15 % CriterionTM TGXTM Precast Gel (Bio-Rad Laboratories). The proteins were then

transferred to a nitrocellulose membrane using iBlot™ (Invitrogen, Carlsbad, CA). The membrane was blocked with phosphate buffered saline (PBS) containing 0.1 % Tween-20 and 5 % non-fat dry milk (Bio-Rad Laboratories) for 1 hour at room temperature, and subsequently incubated with the primary antibody (diluted in the blocking solution) overnight at 4 °C. The membrane was then rinsed in PBS with 0.1 % Tween and incubated with the horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG # 7074 or anti-mouse IgG # 7076; 1:2000 dilution; Cell Signaling Technology, Beverly, MA) for 1 hour at room temperature. After rinsing the membrane with PBS with 0.1 % Tween, the protein bands were detected with a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). In order to quantify protein band intensity, densitometry was performed using a ChemiDoc XRS with Quantity One software (Bio-Rad).

Several proteins of bioenergetic pathways were analyzed. Primary antibodies purchased from Cell Signaling Technology included antibodies to phospho-Thr172 AMPK (1:1000 dilution; #2531), AMPK (1:1000 dilution; #2603), phospho-Ser133 cAMP-response element binding protein (CREB) (1:500 dilution; #9198), p38 (1:1000 dilution; #9212), phospho-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), hypoxia-inducible factor 1-alpha (HIF1 α) (1:1000 dilution; #3716), and GAPDH (1:2000 dilution; #2118). Primary antibodies purchased from Abcam (Cambridge, MA) included antibodies to PGC-1 β (1:500 dilution; #ab61249), PDHA1 (1:500 dilution; #ab110330),

phosphor-Ser 293 PDHA1 (1:200 dilution; #ab92696), and citrate synthase (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.

Mitochondrial stress test and glycolysis stress test

Mitochondrial oxygen consumption rate and glycolytic rate of lactate-treated SH-SY5Y cells were measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) which gives real-time measurements in a 24-well plate. 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. Cells were treated with sodium lactate as above-mentioned in the 'Lactate treatment' section.

The assays were performed as previously described with minor modifications (Silva *et al.*, 2013). Briefly, for mitochondrial stress test, unbuffered DMEM (pH 7.4) containing 5 mM glucose and 1 mM glutamine was used as assay running medium. For LAC groups, the assay medium also contained either 10 mM or 25 mM sodium lactate. Oxygen consumption rate (OCR) was measured using a cycling protocol consisting of 3 min mixing, 2 min waiting, and 3 min reading in one cycle. Basal OCR at 5 mM glucose (near physiological concentration) was measured during the first four reading periods.

Next, we injected oligomycin at final concentration of 1 μM to measure the proton leak rate for the three reading periods. The proton ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was subsequently injected at final concentration of 0.3 μM to measure the maximal respiration for another three reading periods. At last, the complex I inhibitor rotenone and complex III inhibitor antimycin A were injected at final concentrations of 1 μM and 0.2 μM , respectively, to shut down the mitochondrial respiration, yielding non-mitochondrial OCR of cells. For quantitation, the area under the OCR curve was used. The area under the curve will be referred to as AUC in this study. The OCR AUC at basal respiration, post-oligomycin injection, and post-FCCP injection was calculated, and the non-mitochondrial OCR AUC value was subtracted.

For the glycolytic stress test, unbuffered DMEM (pH 7.4) containing 1 mM glutamine and no glucose was used as assay running medium. For LAC groups, the assay medium also contained either 10 mM or 25 mM sodium lactate. The Seahorse Analyzer measures extracellular acidification rate (ECAR) that can be used as an indirect indicator of glycolysis rate. Initially, ECAR under glucose deprivation was measured for the first three reading periods. Next, to measure basal glycolysis rate, we added glucose at final concentration of 25 mM. For evaluating glycolysis capacity, oligomycin was subsequently injected at final concentration of 1 μM . At last, we injected 2-deoxyglucose (2-DG) at final concentration of 100 mM to yield non-glycolysis ECAR. Basal glycolysis rate was calculated by post-glucose injection ECAR AUC subtracting non-glycolysis ECAR AUC, and glycolysis capacity was calculated by post-oligomycin injection ECAR AUC subtracting non-glycolysis ECAR AUC.

In addition to pre-treating cells with lactate, we also tested the acute effects of lactate treatment on SH-SY5Y mitochondrial respiration and glycolysis rate. Cells were seeded in a 24-well Seahorse V7 plate in regular SH-SY5Y growth medium. Unbuffered DMEM (pH 7.4) supplemented with 5 mM glucose and 1mM glutamine but without lactate was used as assay running medium for all groups. After 3 reading periods of basal respiration in the Seahorse XF24 Analyzer, lactate was injected at final concentration of 10 mM or 25 mM to the wells of LAC groups. For the control group, assay running medium of the same volume was injected. The fluxes were measured for five reading periods after the first injection. Oligomycin, FCCP, and rotenone plus antimycin were injected subsequently.

Cytochrome *c* oxidase and citrate synthase activity assays

We evaluated COX and citrate synthase (CS) Vmax activities of SH-SY5Y cells treated with 25 mM lactate for 6 hours. The cells were trypsinized and washed with ice-cold PBS twice, and pelleted at 500 x g for 3 minutes. The pellet was resuspended in ice-cold HBSS containing no magnesium and no calcium, at concentration of 30×10^6 cells/mL. COX and CS Vmax activities were determined as previously described (Ghosh *et al.*, 2007). COX Vmax activity was normalized to protein concentration, as well as to CS activity.

ATP and ADP/ATP ratio assays

5×10^4 cells per well were plated in 96-well plates, and were treated with 25 mM lactate for 6 hours as described above. ATP levels and ADP/ATP ratios were determined

using a EnzyLight™ ATP Assay Kit and ADP/ATP Ratio Assay Kit (Bioassay Systems, Hayward, CA), respectively, according to the manufacturer's instruction.

NAD⁺/NADH assay

Cells were seeded in 60 mm dishes at a density of 1.5×10^6 cells, and were treated with 25 mM lactate for 6 hours as described above. The NAD⁺/NADH levels were measured using a commercially available Fluorescent NAD⁺/NADH Detection Kit (Cell Technology Inc, Mountain View, CA). Briefly, cells were harvested, washed with ice-cold PBS, and divided into two different 1.5 mL tubes. Two separate extractions were prepared for measuring NAD⁺ and NADH levels individually, according the manufacturer's instruction. Readings were taken with excitation at 530 nm and emission at 590 nm. NAD⁺ to NADH ratio was calculated, and absolute NAD⁺ and NADH levels were also calculated individually, after normalization to protein content.

Statistical analysis

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

Results

Mitochondrial respiration and glycolysis rate of lactate treated SH-SY5Y cells

Mitochondrial stress test – 6-hour lactate pre-treatment

We first tested the effects of lactate treatment on mitochondrial respiratory flux in SH-SY5Y cells. The assays with Seahorse Extracellular Flux Analyzer demonstrated that 6-hr pre-treatment with both 10 mM and 25 mM lactate significantly increased the basal mitochondrial OCR AUC by ~25%, when compared with CT group ($p < 0.05$, Fig. 1A-B), and the increase was comparable between 10 mM LAC and 25 mM LAC groups. After injecting oligomycin to inhibit ATP synthase, while no significant difference was observed between CT and 10 mM LAC groups or between 10 mM LAC and 25 mM LAC groups, the proton leak associated OCR AUC was ~80% higher in 25 mM LAC group than that in CT group ($p < 0.05$, Fig. 1A, 1C). However, when we ratioed proton leak associated OCR AUC to basal respiration OCR AUC, it was comparable between groups ($p > 0.05$, Fig. 1A, 1D). The ATP production associated OCR AUC was calculated by subtracting proton leak OCR AUC from basal respiration OCR AUC. Then, we evaluated the percentage of ATP production associated OCR AUC in basal respiration OCR AUC, and no inter-group difference was seen ($p > 0.05$, Fig. 1A, 1E). The FCCP-induced maximal respiratory OCR AUC was ~40% higher in 10 mM LAC group and ~45% higher in 25 mM LAC group when either group was compared to the CT group ($p < 0.05$, Fig. 1A, 1F). To calculate maximal respiratory capacity, the maximal respiratory OCR AUC was ratioed to basal respiration OCR AUC, while they were comparable between groups ($p > 0.05$, Fig. 1A, 1G). Spare respiratory capacity, which is the extra mitochondrial capacity available in a cell to produce energy under conditions of increased work or stress, was calculated by subtracting basal respiration OCR AUC from maximal respiratory OCR AUC for each group. No significant inter-group differences (Fig. 1A,

1H). The results indicate that lactate treatment may increase mitochondrial oxygen consumption by enhancing overall mitochondrial electron transfer, but not necessarily increasing the coupling efficiency of oxidative phosphorylation (OXPHOS).

Glycolytic stress test – 6-hour lactate pre-treatment

Next, we evaluated the effects of lactate pre-treatment on glycolysis rate. Fig. 2A, 2B show that 6-hr lactate pre-treatment dramatically suppressed glycolysis rate in SH-SY5Y cells when high concentrations of glucose (25 mM) was given (~50% decrease in 10 mM LAC group and ~75% decrease in 25 mM LAC group, ANOVA $p < 0.001$, LSD post-hoc analysis $p < 0.001$ for both comparisons). Statistically significant difference was also detected between 10 mM LAC and 25 mM LAC group (LSD post-hoc analysis $p < 0.001$). The glycolysis capacity was not altered by lactate pre-treatment ($p > 0.05$, Fig. 2A, 2C). Glycolysis reserve was calculated by subtracting the glycolysis rate from glycolysis capacity. SH-SY5Y is a tumor cell line, and favors glycolysis over mitochondrial respiration, which is known as Warburg effect (Warburg, 1956). With plenty of glucose present in the medium, SH-SY5Y cells mostly rely on glycolytic ATP and have very low glycolysis reserve, but when given 10 mM and 25 mM lactate pre-treatment, glycolysis reserve was dramatically increased by ~290% and ~340%, respectively (LSD post-hoc analysis: $p < 0.05$, and $p < 0.001$, respectively, Fig. 2D). No difference in glycolysis reserve was seen between 10 mM and 25 mM LAC groups.

Mitochondrial stress test – acute lactate treatment

The results from acute lactate treatment showed that, in both 10 mM and 25 mM LAC group, OCR started to increase immediately after lactate injection (there are ~ 5 minutes between lactate injection and the next measuring point), and the increase was

similar between 10 mM and 25 mM LAC groups (Fig. 3A). Although one-way ANOVA analysis did not show statistical significance for the inter-group difference in post-lactate or assay medium injection OCR AUC ($p = 0.073$, Fig. 3B), we clearly observed that the OCR continued to increase throughout the 5 reading periods after lactate injection, while OCR was unchanged in CT group in which assay medium was injected instead. Similarly, statistical analyses did not detect inter-group differences in proton leak OCR AUC (Fig. 3C), while a trend towards an increasing was seen in maximal respiration OCR AUC with lactate injection (ANOVA analysis, $p = 0.15$, Fig. 3D). As shown in Fig. 3E, ECAR decreased immediately after lactate injection, and the effect was more obvious in 25 mM LAC group than that in 10 mM LAC group. Glycolysis ECAR AUC was not calculated for this experiment, because the experiment was not designed for testing glycolysis stress, and non-glycolytic ECAR was not measured for reference.

Prolonged effects of lactate pre-treatment

We also tested how long the lactate's effect on mitochondrial respiration and glycolysis rate would last. Cells in group LAC-1 and LAC-2 were both pre-treated with 25 mM lactate for 6 hours as described in 'Lactate treatment'. The plate was placed in the Seahorse Analyzer for measuring continuously for ~5.5 hours. In LAC-1 group, 25 mM lactate was present in the assay running medium throughout the flux assay. In LAC-2 group, at the end of 6-hour pre-treatment but before placement in the Seahorse Analyzer, lactate was washed out by rinsing the cells twice with assay running medium containing no lactate. Lactate was also absent in the running medium throughout the flux assay. 6-hour lactate pre-treatment significantly increased the mitochondrial respiration in both LAC-1 and LAC-2 group (Fig. 4A). After washing out the lactate, cells in LAC-2 group

maintained higher but sustained OCR when compared with untreated CT cells (Fig. 4A). On the other hand, the OCR of LAC-1 cells kept increasing throughout the 5.5-hour long flux assay, with lactate present in the assay running medium (Fig. 4A). The OCR AUC was ~100% higher in LAC-1 group and ~50% higher in LAC-2 group when compared with CT group ($p < 0.05$, Fig. 4B), and a significant difference was also observed between LAC-1 and LAC-2 groups ($p < 0.05$, Fig. 4B).

Fig. 4C shows that cells in LAC-2 group acted similarly to CT cells in terms of basal glycolysis rate. LAC-1 group had considerably lower glycolysis rate than LAC-2 and CT groups (glycolytic ECAR AUC was not calculated due to the lack of non-glycolytic ECAR as reference). Interestingly, ECAR was unchanged in CT group and was increasing in LAC-2 group during the first ~60 minutes of flux measuring, but then both started to decrease, while LAC-2 group in which there was 25 mM lactate present in the assay running medium had constantly decreasing ECAR throughout the assay. It is possible that in CT and LAC-2 groups, over the time, endogenously generated lactate through glycolysis was being exported continuously from cells and accumulated in the medium, and this accumulated lactate had similar glycolysis suppression effect observed in LAC-1 cells.

OCR to ECAR ratio can be used as an indicator for cellular preference for OXPHOS versus glycolysis when mitochondria are coupled for oxygen consumption; the higher the ratio is, the more aerobic the cells are (Zhang *et al.*, 2012a). According to the Seahorse Analyzer manufacturer, the OCR/ECAR ratios between 5 ~ 8 pmoles/mpH are indicative of normal cells or tumors that are non-invasive (Ferrick *et al.*, 2006). While OCR/ECAR ratios of untreated SH-SY5Y cells and LAC-2 group were maintained at ~3

and ~4.5 pmoles/μpH, respectively, OCR/ECAR in LAC-1 group was constantly increasing over the time, which suggests that SH-SY5Y neuronal cells get more and more aerobic when lactate is constantly present in the extracellular environment (Fig. 4D).

Energy and redox intermediates in lactate treated SH-SY5Y cells

Cells were either 1) untreated, 2) treated with 100 mM glycolysis inhibitor 2-deoxyglucose (2-DG), 3) treated with 25 mM lactate, or 4) treated with 100 mM 2-DG + 25 mM lactate, for 6 hours. There was no difference in ATP levels between CT and LAC groups, as well as between 2-DG and LAC + 2-DG groups (Fig. 5A), while the levels in both 2-DG and LAC + 2-DG groups were considerably lower than those in the CT group (~80 % decrease, $p < 0.001$, Fig. 5A). We also evaluated the effect of lactate treatment on ADP/ATP levels in SH-SY5Y cells. ADP/ATP ratio has been used as a way to differentiate the different modes of cell death and viability. In general, higher ADP/ATP ratios indicate apoptosis or necrosis in the cells. Also, detection of the cellular ADP/ATP ratio is particularly valuable because this ratio indicates the actual free energy of ATP hydrolysis available for cellular reactions (Berg *et al.*, 2009). ADP/ATP level was significantly increased in 2-DG group with glycolysis inhibited (~45% higher than CT group, $p < 0.001$, Fig. 5B), which was likely caused by reduction in ATP level (Fig. 5A). Lactate treatment lowered ADP/ATP level in SH-SY5Y cells when compared to CT group (~30% decrease, $p < 0.001$, Fig. 5B). Also, lactate treatment rescued the increase in ADP/ATP level induced by 2-DG (~60 % lower in LAC + 2-DG group than that in 2-DG group, $p < 0.001$, Fig. 5B), and ADP/ATP levels in LAC + 2-DG group were even lower than those in CT group (~30 % decrease, $p < 0.05$, Fig. 5B).

When lactate dehydrogenase catalyzes the conversion of lactate to pyruvate in cytoplasm, there is a concomitant conversion of NAD^+ to NADH. We speculated that lactate supplement increased mitochondrial respiration by it being converted to pyruvate which would participate in the TCA cycle. Therefore, we predicted that NADH levels would be increased in LAC group. To test this, we evaluated intracellular NAD^+ and NADH levels in SH-SY5Y cells treated with 25 mM lactate for 6 hours, and also calculated NAD^+ to NADH ratio to evaluate the redox state of the cells. However, our results showed that NAD^+/NADH ratio was significantly ~20 % higher in LAC group when compared to CT group ($p < 0.05$, Fig. 6A). This increase was likely induced by the decrease in intracellular NADH level (~30% decrease, $p < 0.05$, Fig. 6B) with unchanged NAD^+ level ($p > 0.05$, Fig. 6C).

COX and CS Vmax activities of lactate treated SH-SY5Y cells

With the increase in mitochondrial respiration, we considered whether the activity of COX, an important regulator of OXPHOS, would increase in lactate treated SH-SY5Y cells as well. Spectrophotometrically measured COX Vmax activities were ~30 % higher in the LAC group (25 mM lactate, 6-hr pre-treatment) when normalized to protein content ($p < 0.05$, Fig. 7A). CS is an enzyme marker for mitochondrial mass. Thus, CS activity was measured in lactate-treated cells. Lactate-treated cells had significantly higher CS activities when compared with untreated cells (~10% increase, $p < 0.05$ Fig. 7B). Despite the fact that LAC group had increased CS activity, when normalized to CS activity, COX Vmax activities were still ~15 % higher in the LAC group ($p < 0.05$, Fig. 7C), suggesting that the mitochondrial electron transfer on the ETC is possibly enhanced by lactate treatment.

Effects of lactate treatment on the expression of components involved in bioenergetics in SH-SY5Y cells

PGC-1 α is known to act as a master regulator of mitochondrial biogenesis and cellular energy metabolism, by binding and activating NRF-1 which in turn regulates the transcriptional activity of TFAM (Scarpulla, 2008b; Vina *et al.*, 2009). TFAM is a final effector essential for the replication, maintenance, and transcription of mtDNA. PGC-1 β , another member from PGC-1 family, also binds and coactivates NRF-1 (Lin *et al.*, 2002a). It has been shown to robustly induce the expression of mitochondrial respiration related genes such as cytochrome *c* and ATP synthase to a greater extent than PGC1- α (Lin *et al.*, 2003). PRC also belongs to PGC-1 family; it interacts with estrogen related receptor- α (ERR- α) to induce mitochondrion-related gene transcription, and in general supports mitochondrial ATP production (Vercauteren *et al.*, 2009; Mirebeau-Prunier *et al.*, 2010).

In lactate treated cells, PGC-1 α mRNA and protein levels were both unchanged. However, PGC-1 β expression was significantly increased in 25 mM LAC group at mRNA level (~35% increase, $p < 0.05$, Fig. 8A) and protein level (~65% increase, $p < 0.05$, Fig. 8B), while its mRNA level was not elevated in 10 mM LAC group. Interestingly, PRC mRNA levels were ~20% lower in both 10 mM and 25 mM LAC groups when compared with CT group ($p < 0.05$, Fig. 8A). NRF-1 mRNA level was unchanged with 10 mM lactate treatment but was ~55 % higher in 25 mM LAC group than those in CT or 10 mM LAC group ($p < 0.05$, Fig. 8A), though we did not observe inter-group difference at protein level (Fig. 8B). Despite the changes in PGC-1 β and NRF-1 expression in lactate treated SH-SY5Y cells, TFAM mRNA and protein levels were both unchanged in LAC groups (Fig. 8A, 8B). We also measured the expression

level of two subunits of COX, specifically, COX1, encoded by mtDNA, and COX4, encoded by nuclear DNA. The mRNA level of COX1 was unchanged in 10 mM LAC group but significantly increased in 25 mM LAC group when compared with CT or 10 mM LAC group (~60% increase, $p < 0.05$, Fig. 8A). Although, we could not see lactate treatment induced changes in COX4 mRNA or protein levels (Fig. 8A, 8B). We also evaluated the protein levels of CS, but did not observe inter-group difference (Fig. 8B). The CS Vmax activity increase appeared uncorrelated with its gene expression level. MtDNA copy numbers were also assayed; they were comparable between untreated cells and lactate treated cells (data not shown).

We then assessed the effect of lactate treatment on expression of VEGF-A, an angiogenic/neurogenic growth factor (Jin *et al.*, 2002), in SH-SY5Y cells. VEGF-A mRNA levels were comparable between CT group and 10 mM LAC group, but ~75 % higher in 25 mM LAC group than those in CT or 10 mM LAC groups ($p < 0.05$, Fig. 7C).

Effects of lactate treatment on energy metabolism sensitive proteins in SH-SY5Y cells

AMPK is a crucial sensor of the energy status of the cell, and it becomes activated when the AMP/ATP ratio is elevated. The activation of AMPK phosphorylates and activates PGC-1 α (Jager *et al.*, 2007). P38 MAPK, has also been shown to increase PGC-1 α activity (Puigserver *et al.*, 2001). While no changes in PGC-1 α at mRNA or protein levels were observed with lactate treatment, Fig. 9 shows that AMPK phosphorylation and p38 phosphorylation were significantly increased in SH-SY5Y cells treated with 25 mM lactate for 6 hours, when compared to CT cells (p-AMPK: ~35 % increase, $p < 0.05$; p-p38: ~40 % increase, $p < 0.05$). CREB is known to activate PGC-1 α (Herzig *et al.*,

2001) and induce PGC-1 β transcription through reactive oxygen species (ROS) (Ishii *et al.*, 2009), and phosphorylation of CREB at Ser 133 is required for CREB-mediated transcription (Johannessen *et al.*, 2004). In our study, nuclear CREB phosphorylation was ~150 % higher in LAC group than that in CT group ($p < 0.05$, Fig. 9).

Akt signaling regulates two key proteins that are involved in longevity: 1) mTOR, a protein kinase that regulates protein synthesis and cell proliferation (Reynolds *et al.*, 2002), and 2) FOXO1, a transcription factor playing important roles in energy metabolism (Furuyama *et al.*, 2003). Reduced mTOR activity or increased FOXO1 activity has been shown to increase mammalian lifespan (Daitoku & Fukamizu, 2007; Kenyon, 2010; Wilkinson *et al.*, 2012; Wu *et al.*, 2013). mTOR can be phosphorylated by Akt at Ser 2448, leading to its activation (Nave *et al.*, 1999; Reynolds *et al.*, 2002). On the other hand, FOXO1 activity is negatively controlled by phosphorylation through Akt signaling pathway: phosphorylated FOXO1 by Akt is translocated from the nucleus into the cytoplasm, resulting in reduced transcriptional activity, while reduced phosphorylation maintains active FOXO1 in nucleus (Furuyama *et al.*, 2003). We found that Akt phosphorylation was significantly reduced with lactate treatment (~40% decrease, $p < 0.05$, Fig. 9). Consistent with this decrease, the level of mTOR phosphorylation at Ser 2448 was lower in LAC group than in CT group (~10% decrease, $p < 0.05$, Fig. 9). FOXO1 protein levels were ~20 % lower in the cytoplasm but ~130 % higher in the nucleus of LAC cells, when compared to CT cells ($p < 0.05$, Fig. 9). The data suggest a potential role of lactate in promoting longevity.

Pyruvate decarboxylation and carboxylation in lactate treated SH-SY5Y cells

The pyruvate dehydrogenase complex (PDHC) is a nuclear-encoded mitochondrial multienzyme complex that catalyzes the decarboxylation of pyruvate to acetyl-CoA, providing the primary link between glycolysis and the TCA cycle. PDHA1 is a subunit of one of the enzyme components of PDHC, and plays a key role in the PDHC function. PDK4, an enzyme located in the mitochondrial matrix, specifically inhibits the PDHC by phosphorylating PDHA1 at Ser 293 (Sugden & Holness, 2003). We presumed that lactate supplement increased mitochondrial respiration by providing more pyruvate to participate in the TCA cycle, as mentioned above. However, PDHA1 mRNA levels were ~10 % and ~15 % lower in 10 mM and 25 mM LAC groups, respectively, than those in CT group, and PDK4 mRNA levels were ~15 % higher in 25 mM LAC group than those in CT or 10 mM LAC group ($p < 0.05$, Fig. 10A). More interestingly, immunoblotting analysis showed that PDHA1 phosphorylation at Ser 293 was actually reduced in SH-SY5Y cells treated with 25 mM lactate (~20% decrease, $p < 0.05$, Fig. 10B), suggesting PDHC in lactate-treated cells possibly had increased activity. The apparently discordant mRNA expression and protein phosphorylation levels may result from a negative feedback loop between the protein complex activity and its mRNA levels.

We also considered the possibility of converted pyruvate being carboxylated to either oxaloacetate or malate, catalyzed by pyruvate carboxylase (PC) or malic enzyme (ME), respectively, to subsequently participate in the TCA cycle. However, the mRNA levels of PC, as well as two isoforms of ME, ME1 (NADP⁺-dependent, cytoplasmic) and ME2 (NAD⁺-dependent, mitochondrial), were all comparable between groups (Fig. 10C).

Discussion

This is the first study to examine the effects of lactate on mitochondrial respiratory flux and glycolysis rate in human neuronal cells. Our data show that lactate supplement lowers glycolysis rate and increases mitochondrial oxygen consumption by improving the efficiency of electron transport on the mitochondrial ETC in SH-SY5Y cells. Lactate also triggers AMPK, Akt and p38 MAPK signaling pathways, which may have contributed to the increased expression of mitochondrial respiration-related genes as well as longevity factors.

Lactate has been suggested to serve as an alternative energy source in the brain when blood glucose level decreases and/or blood lactate level increases (Dienel, 2012). Lactate also has neuroprotective effects. Under ischemic conditions, lactate is able to protect against neuronal death (Berthet *et al.*, 2009), and it plays an important role in memory formation (Newman *et al.*, 2011; Suzuki *et al.*, 2011).

In the present study, we demonstrate that electron transport on the ETC is likely accelerated or improved by lactate. In SH-SY5Y cells pretreated with either 10 mM or 25 mM lactate, the basal mitochondrial respiration and maximal respiration induced by FCCP are considerably increased. In addition, the enzymatic activities of COX, the final electron transport chain enzyme, and CS are increased with lactate treatment. However, the ATP production associated OCR relative to basal respiration remained unchanged, suggesting that lactate treatment improves electron transfer on the ETC but probably does not alter the efficiency of OXPHOS coupling in SH-SY5Y cells.

While addressing the improved mitochondrial respiration, we found that glycolysis rate was reduced with lactate treatment in SH-SY5Y cells. When cellular ATP

concentrations are high, glycolysis is slowed due to the allosteric inhibition of phosphofructokinase (PFK), the rate-limiting enzyme of glycolysis, by ATP (Lehninger *et al.*, 2005). However, in lactate-treated SH-SY5Y cells, overall intracellular ATP levels were unchanged. The conformational transition between the low and high-affinity state of PFK is also regulated by intracellular H^+ : when pH falls, the affinity of PFK for its substrate, fructose-6-phosphate, decreases and hence the rate of glycolysis decreases (Trivedi & Danforth, 1966; Hames *et al.*, 2000). When cells are treated with exogenous lactate, lactate is co-transported into cells with H^+ via proton-linked monocarboxylate transporters, presumably decreasing the pH inside the cells. Thus, the glycolysis rate in lactate-treated cells is reduced probably due to the decrease of intracellular pH.

An intriguing finding in the present study is that, when cells are kept in lactate enriched environment, mitochondrial oxygen consumption and glycolysis rate keep increasing and decreasing, respectively, making the cells more aerobic over the time. When lactate is washed out, the effect of lactate on mitochondrial respiration seems sustained for at least several hours, while the glycolysis suppression effect of lactate disappears sooner. After lactate is taken out from the medium, cells may have reset the glycolysis to the original rate, because there is likely no more co-transportation of lactate and H^+ into cells. On the other hand, the prolonged effect of lactate on mitochondrial respiration indicates that: 1) the effect is not transient but sustainable; 2) lactate may not act only as a fuel; and 3) the mechanisms underlying lactate's prolonged effects on mitochondrial respiration might involve signaling transduction to alter the bioenergetic infrastructures and therefore induce expression of mitochondrial respiration-related components to enhance respiratory function.

In the lactate-treated cells, it seems the decrease in glycolysis rate precedes and may drive the increase of mitochondrial respiration. In Fig. 3A and 3E where we assessed the acute effects of lactate treatment, immediately after lactate injection, glycolysis rate dropped rapidly, while the increase in mitochondrial respiration was gradual. This might also explain why increased oxygen consumption of lactate treated SH-SY5Y cells was not accompanied by more ATP production: mitochondrial respiration is enhanced to compensate the decrease in glycolytic ATP production, resulting in unchanged overall ATP levels. Especially for cells with tumor-like features, such as SH-SY5Y cells, glycolytic ATP are the primary and major source for cell functioning even under aerobic condition, due to Warburg effect (Warburg, 1956).

Despite the unchanged ATP levels, lactate treatment does induce AMPK phosphorylation. AMPK phosphorylation is primarily considered to result from alterations in the intracellular AMP/ATP ratio under starvation, hypoxia or from increased ATP consumption (Hardie, 2007). Recent studies have shown that increased levels of intracellular reactive oxygen species (ROS) can stimulate AMPK activity, without a change in overall ATP levels (Toyoda *et al.*, 2004; Emerling *et al.*, 2009). In lactate-treated SH-SY5Y cells, increased oxygen consumption may be associated with more ROS production to some extent, and this might contribute to AMPK activation without a change in ATP levels.

Indeed, it is paradoxical that lactate increases mitochondrial oxygen consumption but decreases intracellular NADH levels. We expected to see NADH increase in the lactate-treated cells, assuming lactate was converted to pyruvate. The possible

explanation is that less NADH is generated from slowed glycolysis but more NADH is consumed as substrate for complex I (NADH dehydrogenase) on the ETC.

Previous human studies using nuclear magnetic resonance techniques have reported that peripherally infused C¹³-labeled lactate can be uptaken by brain and oxidized subsequently through TCA cycle (Gallagher *et al.*, 2009; van Hall *et al.*, 2009; Boumezbeur *et al.*, 2010). We speculated that lactate, as a fuel, would feed into the TCA cycle by being converted to pyruvate which is subsequently decarboxylated to acetyl-coA. In our lactate-treated SH-SY5Y cells, evaluation of PDHC and PDK at mRNA and protein levels could not provide a clear conclusion to this assumption. The pathway(s) in which lactate is used as a carbon source for TCA cycle remain to be determined.

With increased mitochondrial respiration and activities of mitochondrial enzymes, it is reasonable to predict that mitochondrial biogenesis occurs in lactate-treated SH-SY5Y cells. While TFAM expression and mtDNA copy number are unchanged with lactate treatment in the present study, some components of mitochondrial biogenesis pathway are definitely increased. 25 mM lactate pre-treatment increases expression of PGC-1 β at both mRNA and protein levels, as well as the mRNA levels of NRF-1 and COX1 in SH-SY5Y cells. Indeed, we did not observe a classic pattern of mitochondrial biogenesis with lactate treatment, but the increase in the expression of a PGC-1 family member other than PGC-1 α still has an important role in improving mitochondrial function. Unlike PGC-1 α , PGC-1 β and PRC are both poorly studied PGC-1 family members. PGC-1 β is the closest homolog of PGC-1 α and it shares extensive sequence identity. PRC has more limited homology. (Lin *et al.*, 2005). Both family members interact with NRF-1 which regulates TFAM transcriptional activity (Wu *et al.*, 1999;

Andersson & Scarpulla, 2001). Despite the functional similarities between the PGC-1 α and PGC-1 β , the latter is a stronger inducer of mitochondrial respiration-relevant gene expression and promotes a much higher level of coupled respiration than PGC-1 α , which may make the fine-tuning of mitochondrial function possible in response to specific metabolic needs (Lin *et al.*, 2003; St-Pierre *et al.*, 2003; Lin *et al.*, 2005). In this study, PRC mRNA levels were slightly decreased in both 10 mM and 25 mM LAC groups. However, due to the lack of antibodies, we were not able to evaluate the expression at protein level.

The underlying mechanisms of lactate treatment inducing PGC-1 β expression in SH-SY5Y cells may involve the activation of p38 MAPK signaling pathway. MAPKs are a family of proteins promoting a phosphorylation signaling cascade, leading to the activation of transcription factors (Turjanski *et al.*, 2007). P38 phosphorylates and activates PGC-1 α (Puigserver *et al.*, 2001), while there is no evidence showing that p38 MAPK directly activates PGC-1 β or PRC. However, activation of p38 MAPK pathway is known to stimulate CREB phosphorylation (Xing *et al.*, 1998), and previous studies have reported that PGC-1 β is a direct transcriptional target of CREB (Ishii *et al.*, 2009; Xia *et al.*, 2013).

A question arises as to whether the increase in mitochondrial respiration is driven by lactate serving as a fuel or is a consequence of lactate modifying bioenergetics-related signaling pathways. As evidenced by immediate increase of mitochondrial OCR after lactate addition to SH-SY5Y cells (Fig. 3A), lactate is likely to enhance mitochondrial respiration by feeding into mitochondria as a fuel first, as the regulation of gene expression presumably would take a longer period of time. However, lactate-induced

modification of bioenergetic infrastructure appears to have effects on mitochondrial respiratory capacity in SH-SY5Y cells. This is supported by the fact that maximal respiration is comparable between untreated cells and cells treated with lactate acutely, while it is significantly increased in cells pre-treated with lactate for 6 hours. It suggests that a longer treatment with lactate possibly increases expression and activities of mitochondrial respiration-related proteins by activating bioenergetic signaling pathways, and therefore it improves the efficiency and capacity of the ETC.

VEGF-mediated brain angiogenesis plays a critical role in neurogenesis by providing the critical neurovascular niches for neuronal remodeling (Fabel *et al.*, 2003a; Latimer *et al.*, 2011), and it has been proposed that lactate may account for exercise-induced angiogenesis/neurogenesis (E *et al.*, 2013a). In fact, subcutaneous administration of lactate promotes VEGF-mediated lung tumor angiogenesis in mice (Sonveaux *et al.*, 2012), and intraperitoneal lactate injection increases VEGF-A expression in mouse brain (E *et al.*, 2013a). In the present study, we confirm that lactate treatment on a human neuronal background also significantly induces VEGF-A expression, further supporting the view that lactate generated from exercising muscles mediates the exercise-induced brain angiogenesis and neurogenesis.

In endothelial cells, PI3K/Akt signaling pathway activation is involved in lactate-induced angiogenesis (Ruan & Kazlauskas, 2013). How exogenous lactate alters Akt signaling pathway in brain has not been defined yet. Interestingly, we found that Akt phosphorylation decreased lactate treatment. Akt signaling pathway regulates the activities of two proteins that play key roles in slowing aging and increasing mammalian lifespan: mTOR and FOXO1. Lower mTOR expression has been considered to slow the

entire aging process in mammals (Wilkinson *et al.*, 2012). In another study, mTOR hypomorphic mice with much less mTORC1 and mTORC2 activity than wild-type mice exhibited an 20% increase in median survival (Wu *et al.*, 2013). On the other hand, increased FOXO1 expression has been consistently linked with longevity, possibly due to its important roles in detoxification of ROS and repair of damaged DNA (Daitoku & Fukamizu, 2007; Kenyon, 2010). In lactate-treated SH-SY5Y cells, reduced phosphorylation of Akt leads to less activation of mTOR but more nuclear translocation of FOXO1. These results suggest that lactate increases expression of key factors of longevity pathway(s) or at least it provides the possibility of functional preservation of brain during aging. Further research is needed to investigate the longevity effects of lactate in an aging model system.

In conclusion, our data demonstrate that lactate treatment shifts bioenergetic fluxes towards a more aerobic state, and induces mitochondrial respiration-related gene expression in human neuronal cells. These effects are accompanied by activation of AMPK, p38 MAPK, and Akt signaling pathways to alter bioenergetic infrastructures to induce mitochondrial biogenesis, and also to increase the expression of proteins that may be involved in longevity (Fig. 10). Our results suggest that lactate may have potential therapeutic implications for neurological disorders with perturbed brain bioenergetic metabolism, such as Alzheimer's and Parkinson's diseases.

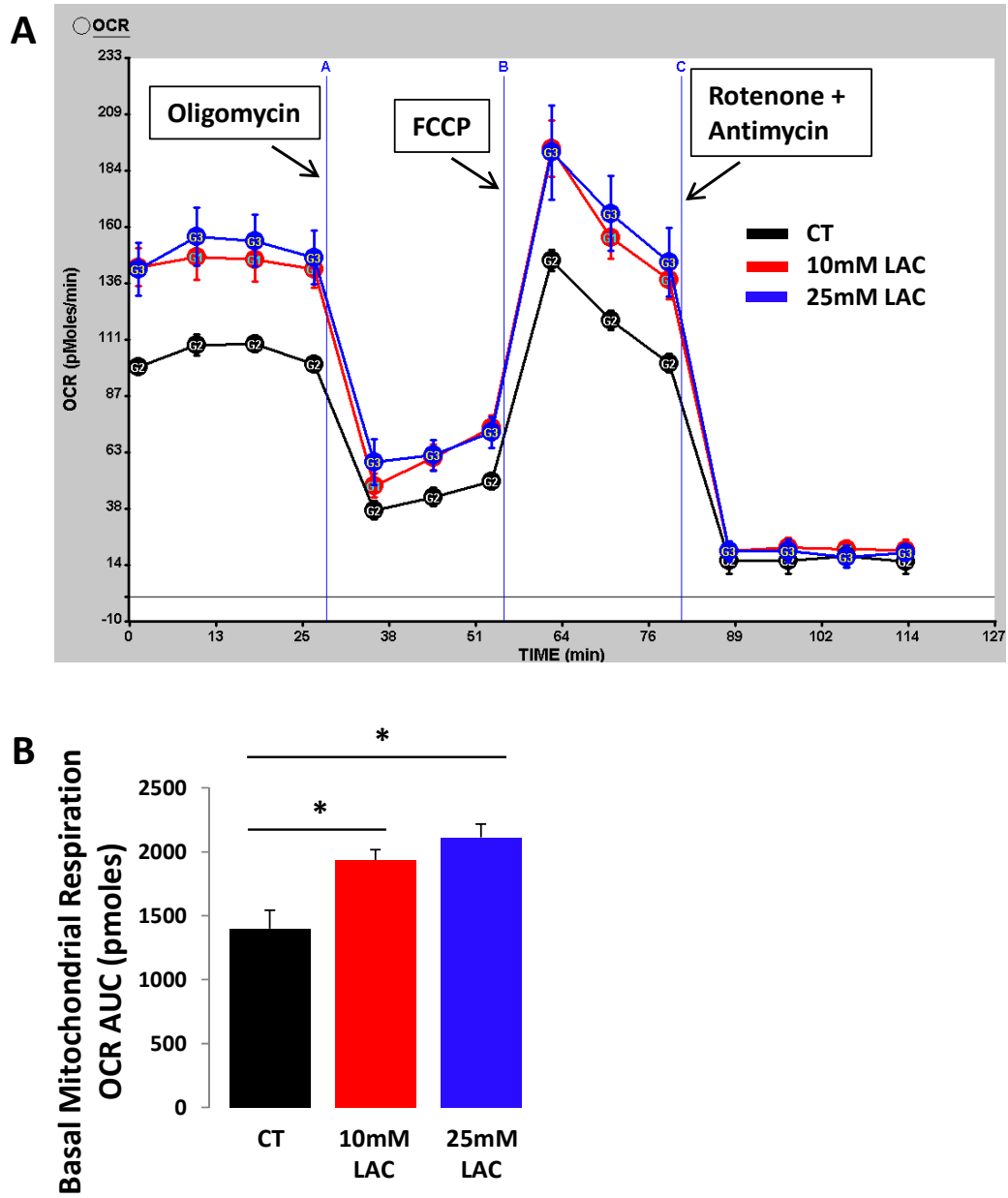


Figure 1. Mitochondrial respiratory flux of SH-SY5Y cells pre-treated with lactate for 6 hours (mitochondrial stress test).

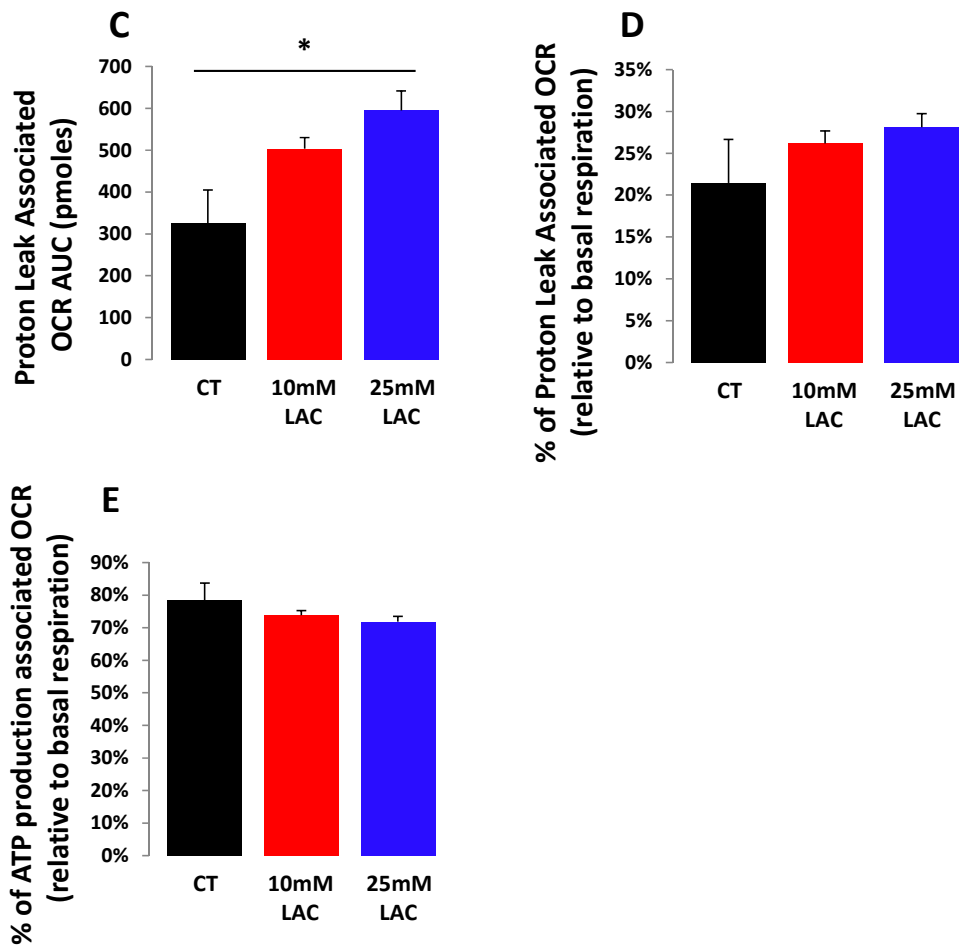


Figure 1. Mitochondrial respiratory flux of SH-SY5Y cells pre-treated with lactate for 6 hours (mitochondrial stress test) (continued).

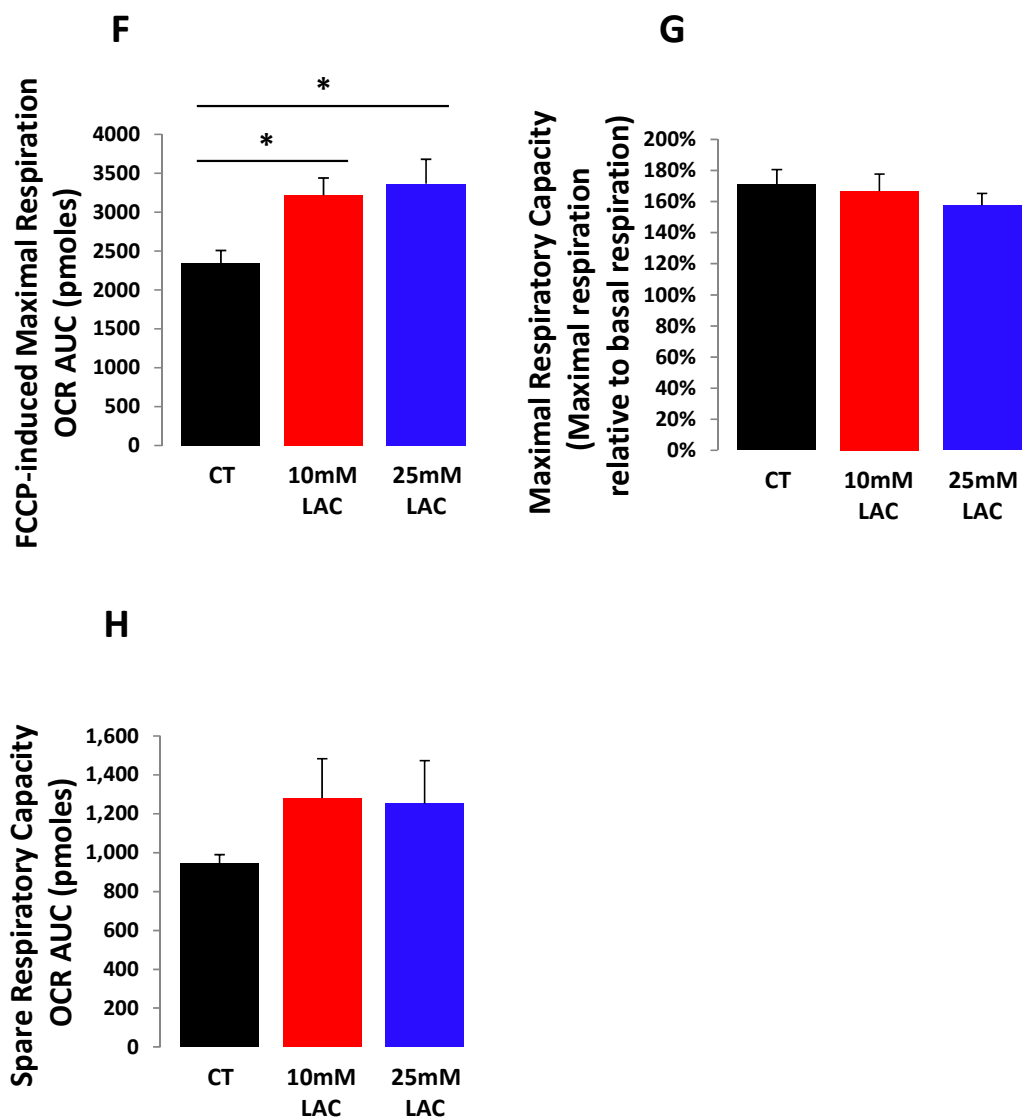


Figure 1. Mitochondrial respiratory flux of SH-SY5Y cells pre-treated with lactate for 6 hours (mitochondrial stress test) (continued). (For figure legend, see next page).

Figure 1. Mitochondrial respiratory flux of SH-SY5Y cells pre-treated with lactate for 6 hours (mitochondrial stress test) (continued). (A) Oxygen consumption rates (OCR) of SH-SY5Y cells untreated, pre-treated with 10 mM lactate, or 25 mM lactate for 6 hours were measured using a Seahorse XF24 Extracellular Flux Analyzer. At the times indicated, oligomycin (1 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) was injected. (B) OCR AUC at baseline was determined from (A). 10 mM LAC and 25 mM LAC groups had significantly higher basal OCR AUC than that in CT group, while no difference was seen between 10 mM LAC and 25 mM LAC groups. (C) After injecting oligomycin to inhibit ATP synthase, AUC of proton leak associated OCR was calculated, and it was elevated only in 25 mM LAC group. (D) Percentage of proton leak associated OCR in basal respiration OCR was comparable between groups. (E) Percentage of ATP production associated OCR in basal respiration OCR was comparable between groups. (F) Maximal respiration OCR AUC was determined with FCCP injection, and it was higher in both 10 mM LAC and 25 mM LAC groups when each group was compared with CT group, but comparable between 10 mM LAC and 25 mM LAC groups. (G) Maximal respiratory capacity was calculated by dividing maximal respiratory OCR AUC by basal respiration OCR AUC, but no inter-group difference was observed. (H) Spare respiratory capacity was calculated by subtracting basal respiration OCR AUC from maximal respiration OCR AUC; no significant inter-group difference was observed. ANOVA analysis with LSD post-hoc test. * $p < 0.05$. AUC, area under the curve; CT, control group; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; LAC, lactate; OCR, oxygen consumption rate.

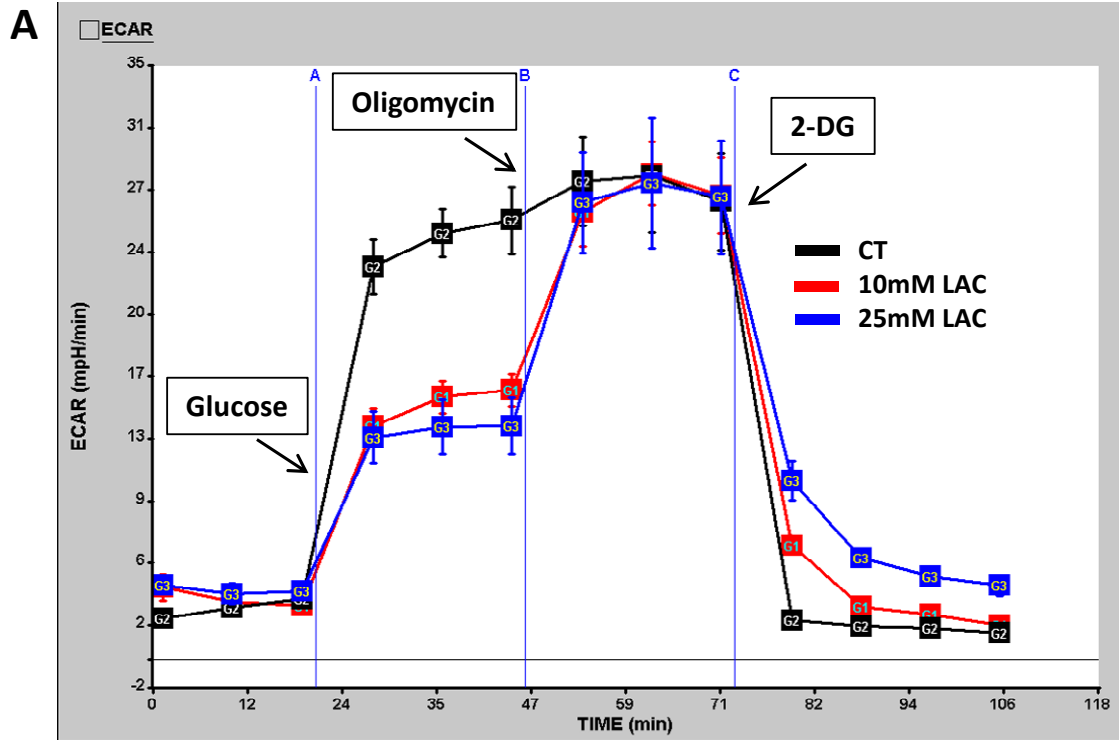


Figure 2. Glycolysis flux of SH-SY5Y cells pre-treated with lactate for 6 hours (glycolysis stress test) (continued).

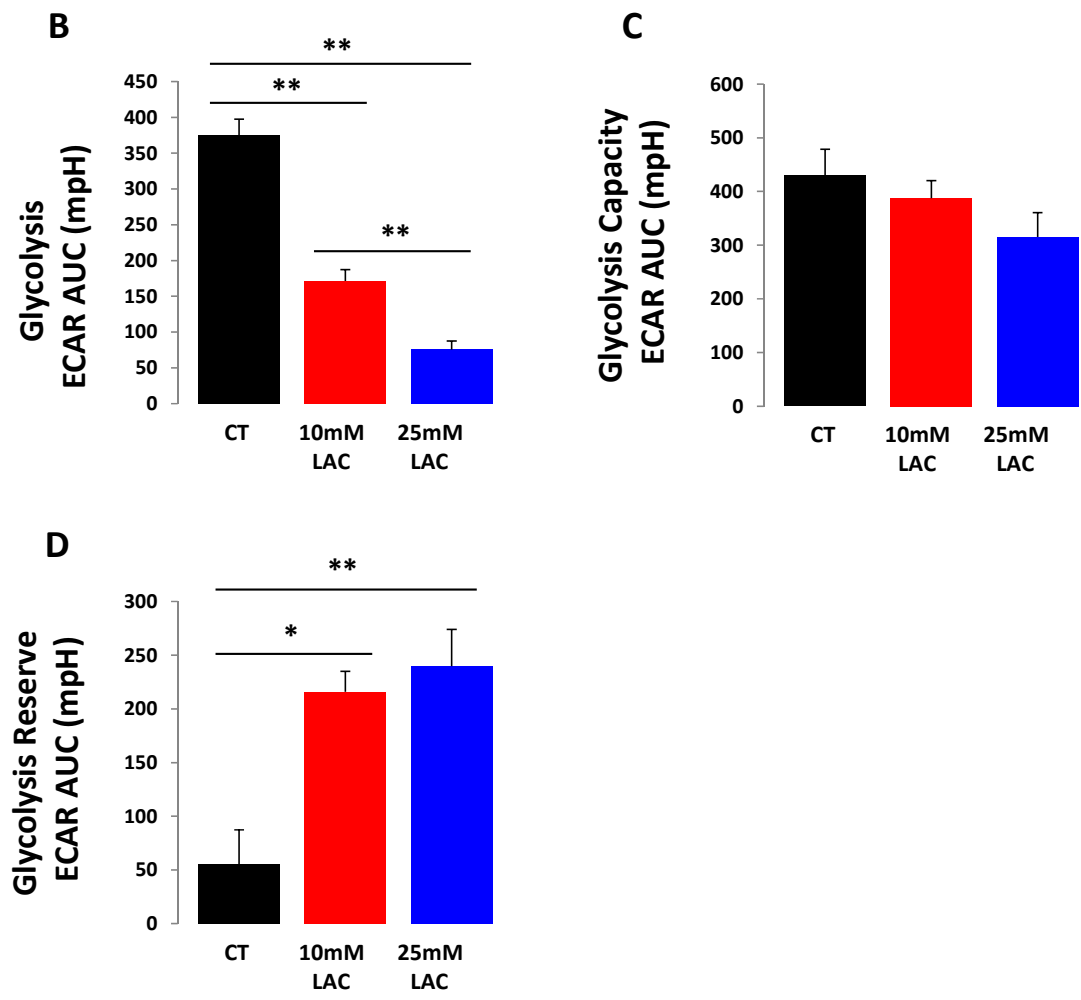


Figure 2. Glycolysis flux of SH-SY5Y cells pre-treated with lactate for 6 hours (glycolysis stress test) (continued). (For figure legend, see next page).

Figure 2. Glycolysis flux of SH-SY5Y cells pre-treated with lactate for 6 hours (glycolysis stress test) (continued). (A) ECAR of SH-SY5Y cells, untreated, pre-treated with 10 mM lactate, or 25 mM lactate for 6 hours were measured using a Seahorse XF24 Extracellular Flux Analyzer. 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) When 25 mM glucose was present in the medium, glycolysis ECAR AUC was significantly lower in both 10 mM LAC and 25 mM LAC groups than that in CT group. The glycolysis ECAR AUC was lower in 25 mM LAC group when compared to 10 mM LAC group. (C) Glycolysis capacity was determined with subsequent oligomycin injection, and no inter-group difference was observed. (D) Glycolysis reserve was calculated by subtracting the glycolysis rate from glycolysis capacity. Both 10 mM and 25 mM lactate treatments significantly increased glycolysis reserve in SH-SY5Y cells. ANOVA analysis with LSD post-hoc test. $*p < 0.05$; $**p < 0.001$. 2-DG, 2-deoxyglucose; AUC, area under the curve; CT, control group; ECAR, extracellular acidification rate; LAC, lactate.

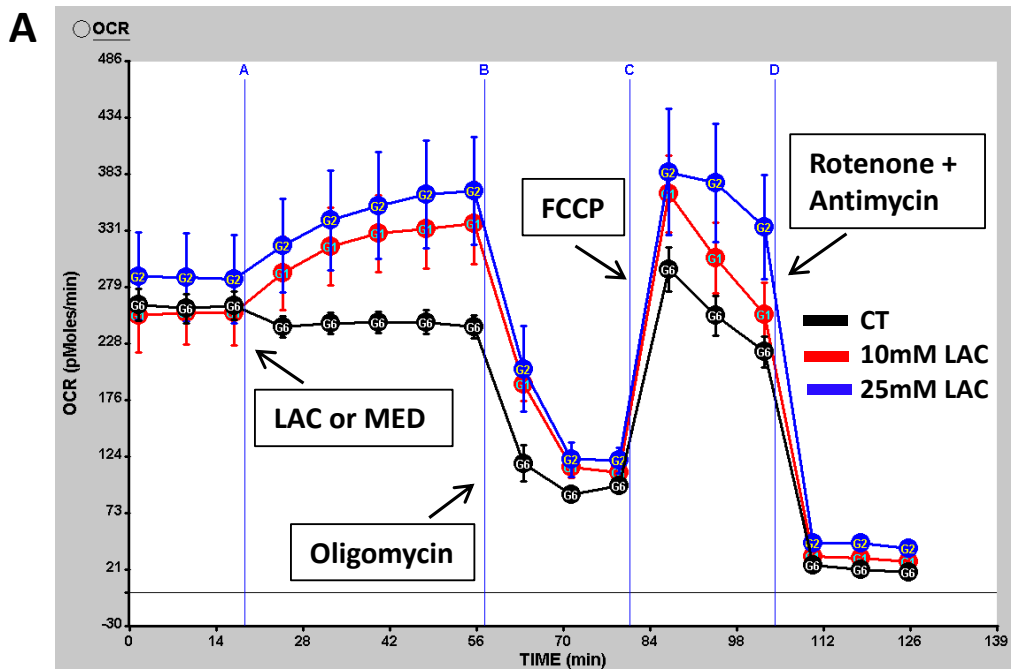


Figure 3. Mitochondrial respiration, glycolysis in SH-SY5Y cells with acute lactate treatment.

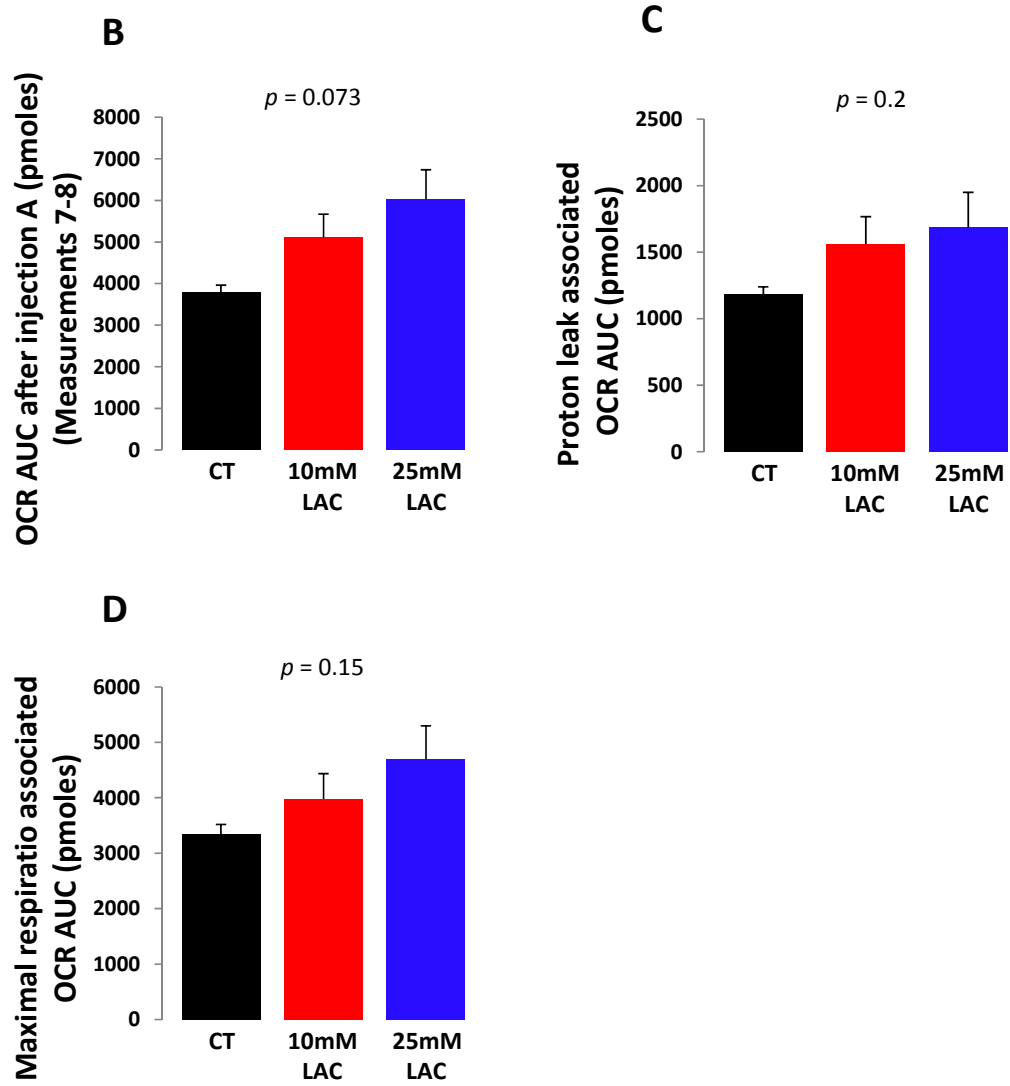


Figure 3. Mitochondrial respiration, glycolysis in SH-SY5Y cells with acute lactate treatment (continued).

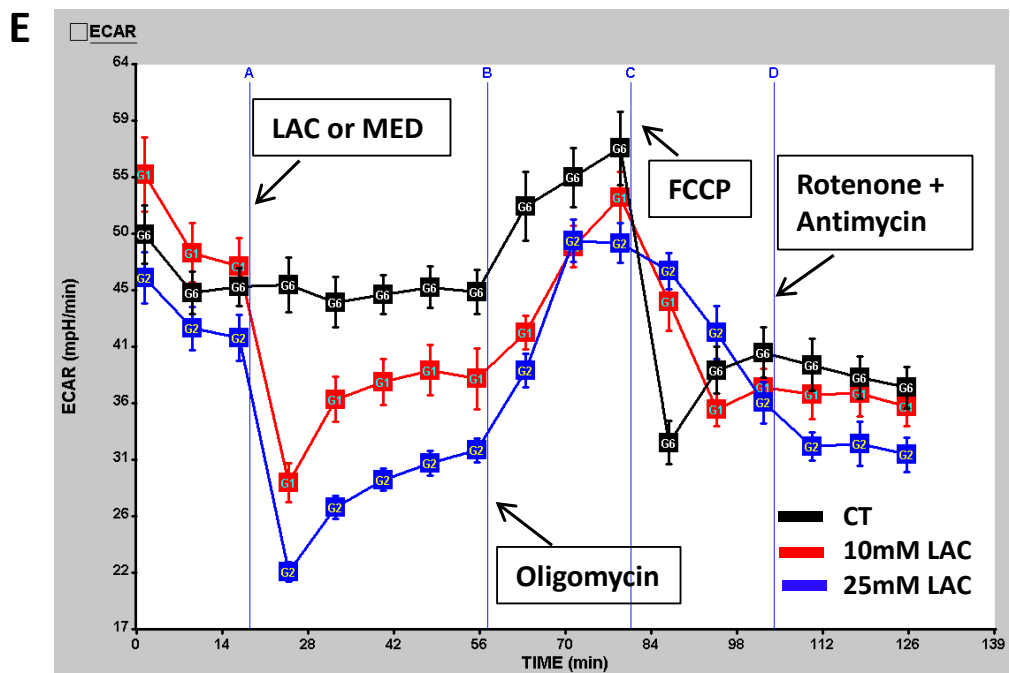


Figure 3. Mitochondrial respiration, glycolysis in SH-SY5Y cells with acute lactate treatment (continued). (For figure legend, see next page).

Figure 3. Mitochondrial respiration, glycolysis in SH-SY5Y cells with acute lactate treatment (continued). (A) OCR of SH-SY5Y cells was measured in a Seahorse Analyzer. At the first injection point indicated, assay medium, or lactate at final concentrations of 10 mM or 25 mM was injected. Subsequently, final concentrations of 1 μ M oligomycin, 0.3 μ M FCCP and 1 μ M rotenone plus 0.2 μ M antimycin were injected automatically. (B) OCR AUC between measuring points 7 and 8 was calculated as basal respiration for each group. Basal respiration was trending higher in lactate injected groups compared to CT group, but no statistically significant inter-group difference was observed. (C) Proton leak associated OCR AUC, and (D) maximal respiration OCR AUC were also comparable between groups. (E) ECAR was measured simultaneously in this experiment. ECAR decreased immediately after lactate injection in 10 mM and 25 mM LAC groups, and the effect was more obvious in 25 mM LAC group than that in 10 mM LAC group. No ECAR changes were observed in CT group where medium was injected. AUC, area under the curve; CT, control group; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; MED, assay medium; LAC, lactate; OCR, oxygen consumption rate.

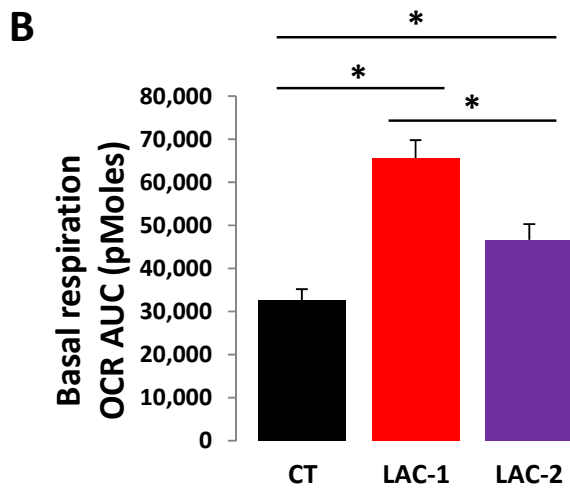
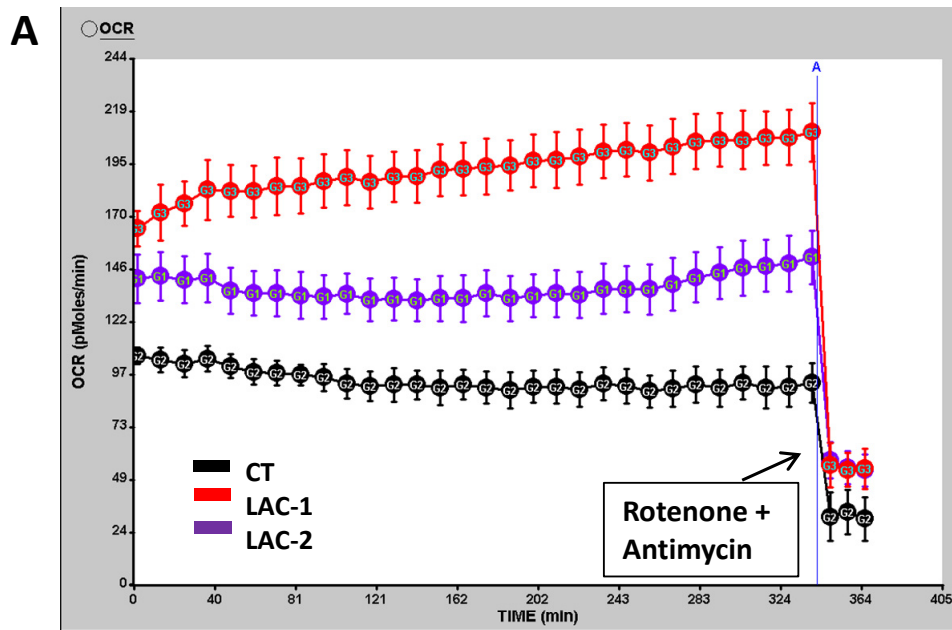


Figure 4. Prolonged effects of lactate pre-treatment on mitochondrial respiration and glycolysis rate in SH-SY5Y cells.

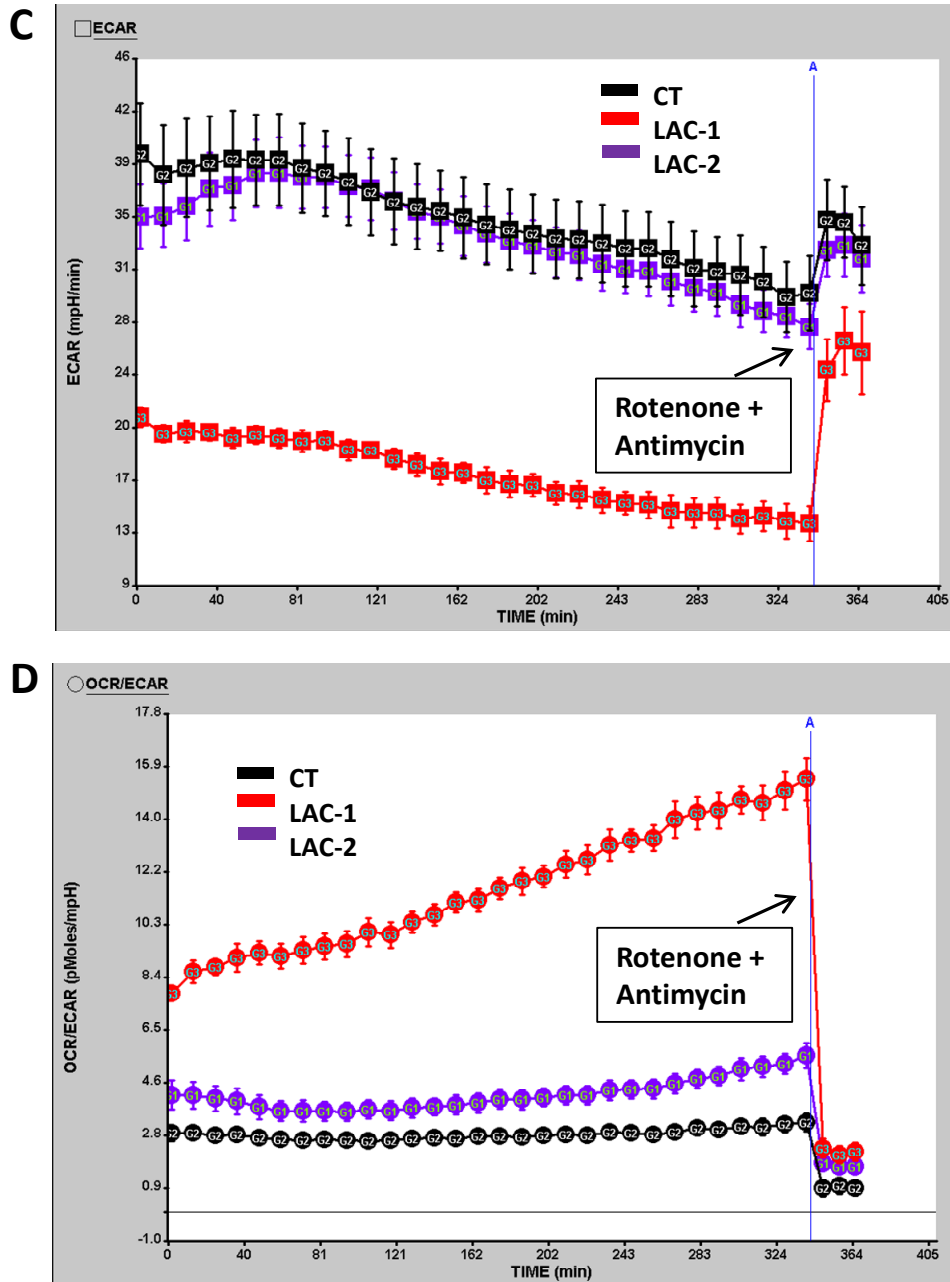


Figure 4. Prolonged effects of lactate pre-treatment on mitochondrial respiration and glycolysis rate in SH-SY5Y cells (continued). (For figure legend, see next page).

Figure 4. Prolonged effects of lactate pre-treatment on mitochondrial respiration and glycolysis rate in SH-SY5Y cells (continued). The cells in group LAC-1 and LAC-2 were pre-treated with 25 mM lactate for 6 hours. Cells in CT group was untreated. Fluxes were measured in a Seahorse Analyzer for ~5.5 hours, and rotenone and antimycin were injected at last at final concentrations of 1 μ M and 0.2 μ M, respectively. In LAC-1 group, 25 mM lactate was present in the assay running medium throughout the flux assay. In LAC-2 group, at the end of 6-hour pre-treatment, lactate was washed out and was absent in the running medium throughout the flux assay. **(A)** OCR in LAC-1 and LAC-2 groups started with higher readings compared to CT group. During the ~5.5-hour assay, OCR in LAC-1 group kept increasing, while LAC-2 and CT groups maintained their OCR level unchanged throughout the assay. **(B)** OCR AUC was calculated between measuring point 1 and 30 for each group. LAC-1 group had significantly higher OCR AUC than those in LAC-2 and CT groups. OCR AUC in LAC-2 group was higher than that in CT group but was lower compared to LAC-1 group. **(C)** ECAR was comparable between LAC-2 and CT groups, while LAC-1 group had considerably lower glycolysis rate than those in LAC-2 and CT groups. **(D)** OCR/ECAR ratio was calculated to evaluate cellular preference for OXPHOS versus glycolysis. While OCR/ECAR ratios of CT and LAC-2 group were maintained around ~3 and ~4.5 pmoles/mpH, respectively, OCR/ECAR in LAC-1 group was constantly increasing over the entire course of the assay. ANOVA analysis with LSD post-hoc test. * $p < 0.05$. AUC, area under the curve; CT, control group; ECAR, extracellular acidification rate; LAC, lactate; OCR, oxygen consumption rate.

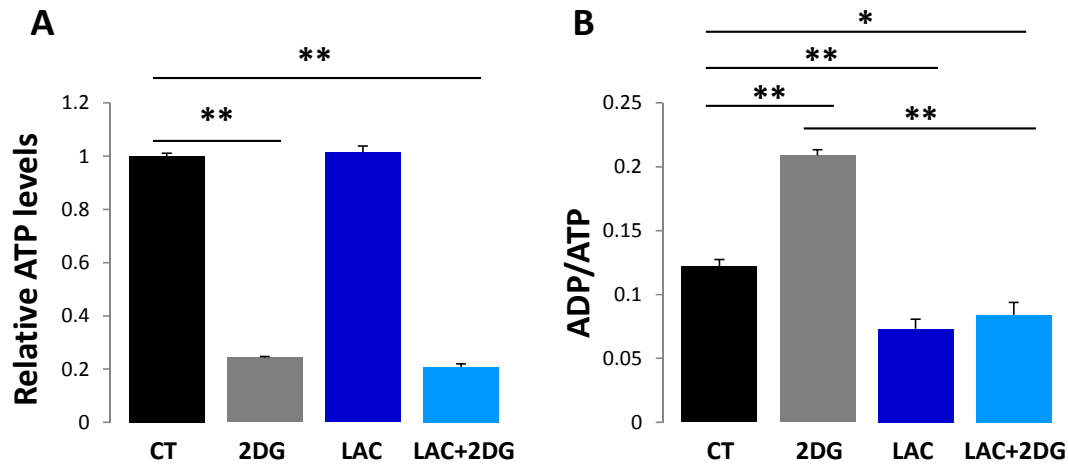


Figure 5. Energy intermediates in lactate pre-treated SH-SY5Y cells. (A) ATP levels in all groups were referenced to the mean value of CT group. Relative ATP levels were significantly reduced in 2-DG group and LAC + 2-DG group, compared to CT group. No difference was observed between CT and LAC groups, as well as between 2-DG and LAC + 2-DG groups. **(B)** ADP/ATP levels were significantly increased in 2-DG group. Lactate treatment lowered ADP/ATP levels compared to CT group. ADP/ATP levels in LAC + 2-DG group were significantly lower than those in 2-DG group. ADP/ATP levels in LAC + 2-DG group were even lower than those in CT group. ANOVA analysis with LSD post-hoc test. * $p < 0.05$; ** $p < 0.001$. 2DG, 2-deoxyglucose; CT, control group; LAC, lactate.

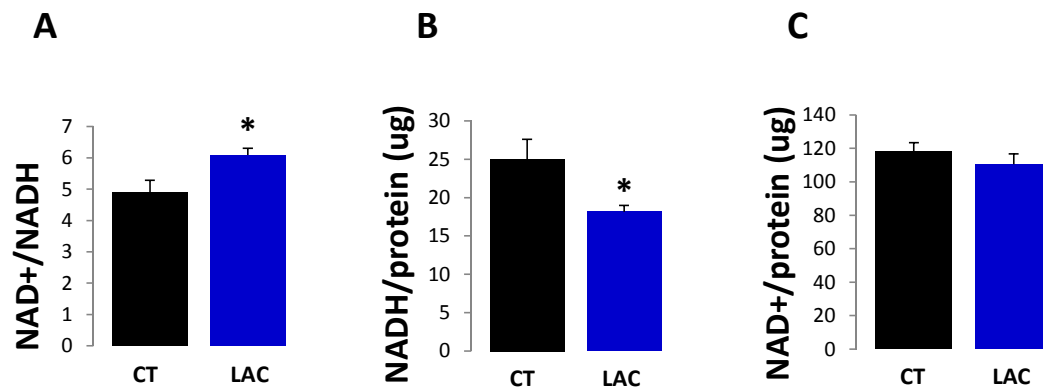


Figure 6. Redox intermediates of lactate pre-treated SH-SY5Y cells.

(A) NAD⁺/NADH ratio was significantly higher in LAC group compared to CT group. (B) NADH levels corrected for protein amount were significantly lower in LAC group compared to CT group. (C) NADH levels corrected for protein amount were comparable between groups. * $p < 0.05$ compared to CT group. CT, control group; LAC, lactate group.

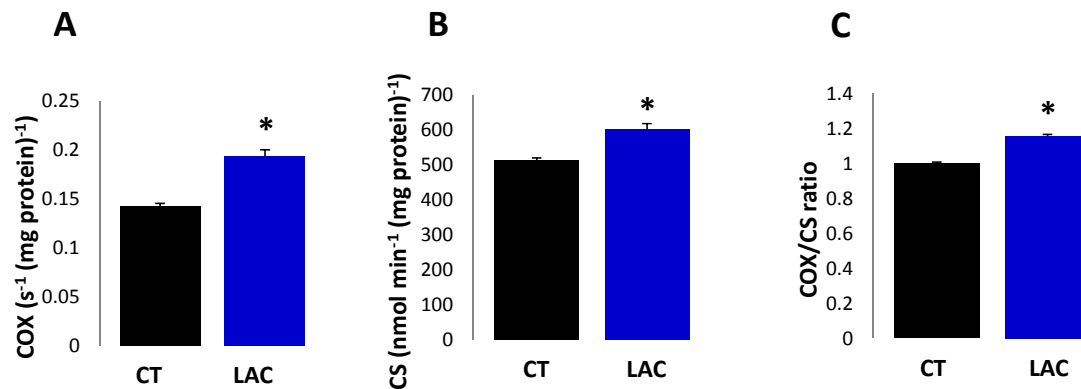


Figure 7. Complex IV and citrate synthase Vmax activities of lactate pre-treated SH-SY5Y cells. (A) COX Vmax activities normalized to protein content were significantly higher in LAC group compared to CT group. (B) CS Vmax activities normalized to protein content were also increased in LAC group compared to CT group. (C) After normalization to CS activity, COX Vmax activities in LAC group were still significantly higher than those in CT group. * $p < 0.05$ compared to CT group. COX, cytochrome *c* oxidase; CS, citrate synthase; CT, control group; LAC, lactate group.

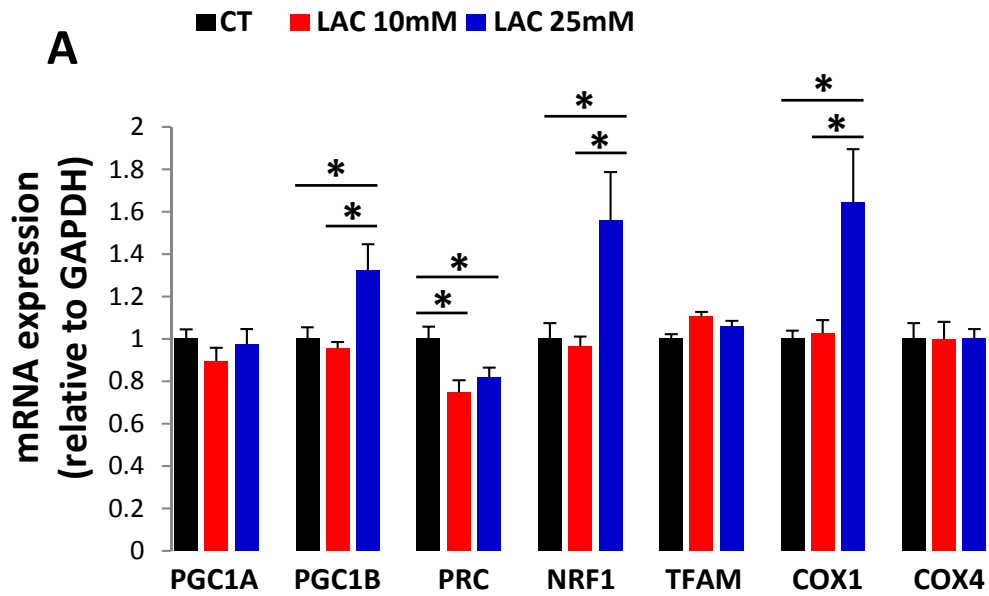


Figure 8. Effects of lactate treatment on the expression of components involved in bioenergetics.

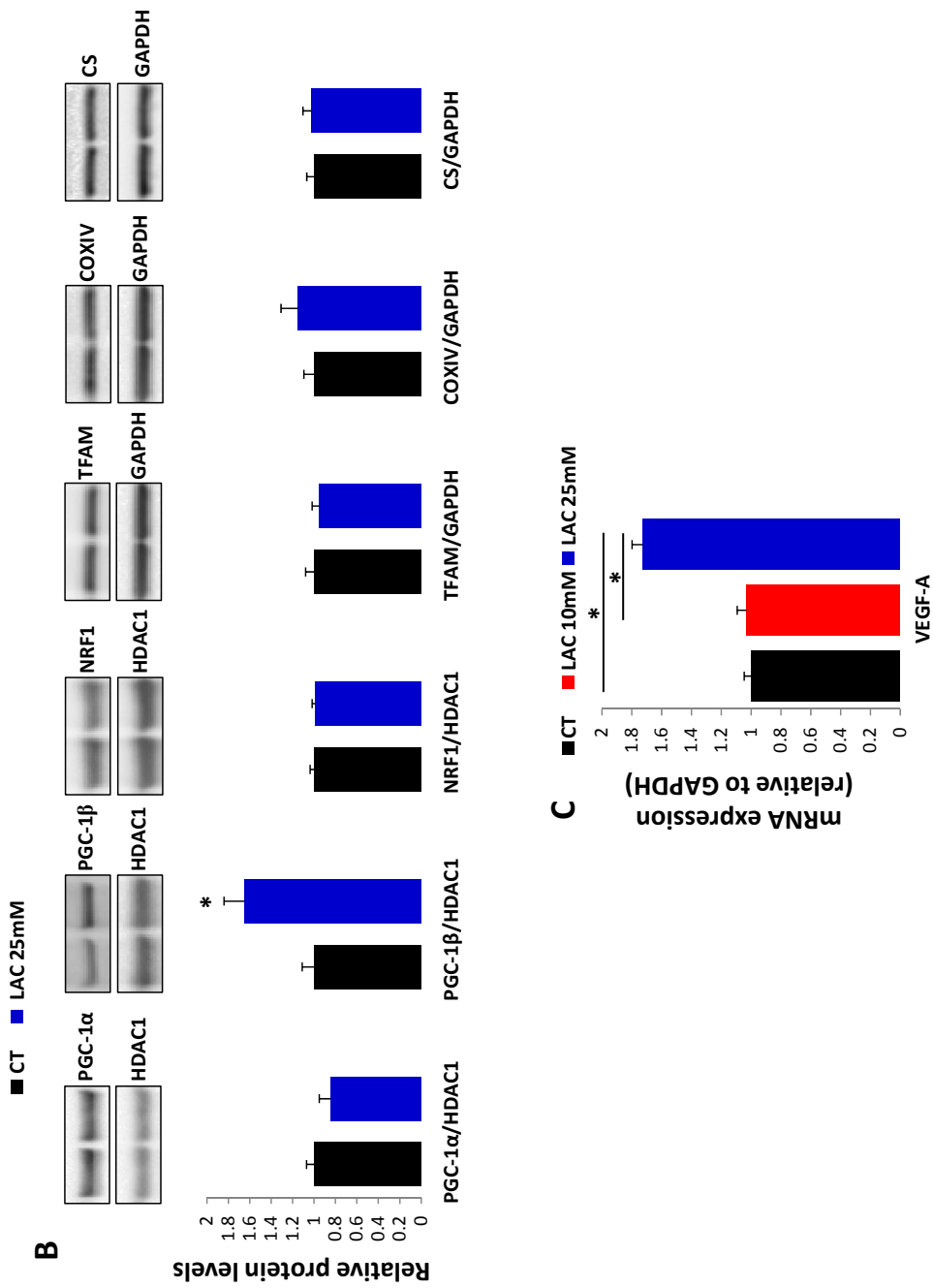


Figure 8. Effects of lactate treatment on the expression of components involved in bioenergetics (continued). (For figure legend, see next page).

Figure 8. Effects of lactate treatment on the expression of components involved in bioenergetics (continued). (A) PGC-1 β mRNA levels were significantly increased in 25 mM LAC group but were comparable between CT and 10 mM LAC groups. PRC mRNA levels were decreased in both LAC groups compared to CT group. NRF-1 and COX4 mRNA levels were both higher in 25 mM LAC group but were comparable between CT and 10 mM LAC groups. No inter-group differences were detected in mRNA levels of PGC-1 α , TFAM, or COX4. ANOVA analysis with LSD post-hoc test. (B) Nuclear PGC-1 β protein levels were significantly higher in LAC group compared to CT group, but inter-group differences were not observed in the protein levels of PGC-1 α , NRF-1, TFAM, COXIV, or CS. (C) VEGF-A mRNA expression was increased with 25 mM lactate pre-treatment but was unchanged with 10 mM lactate treatment. ANOVA analysis with LSD post-hoc test. * $p < 0.05$. COX1, cytochrome *c* oxidase subunit 1; COX4 or COX IV, cytochrome *c* oxidase subunit 4; CS, citrate synthase; CT, control group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC1, histone deacetylase 1; LAC, lactate; NRF1, nuclear respiratory factor 1; PGC-1, peroxisomal proliferator-activated receptor-gamma co-activator 1; PRC, PGC-1 related co-activator; TFAM, mitochondrial transcription factor A; VEGF-A, vascular endothelial growth factor A.

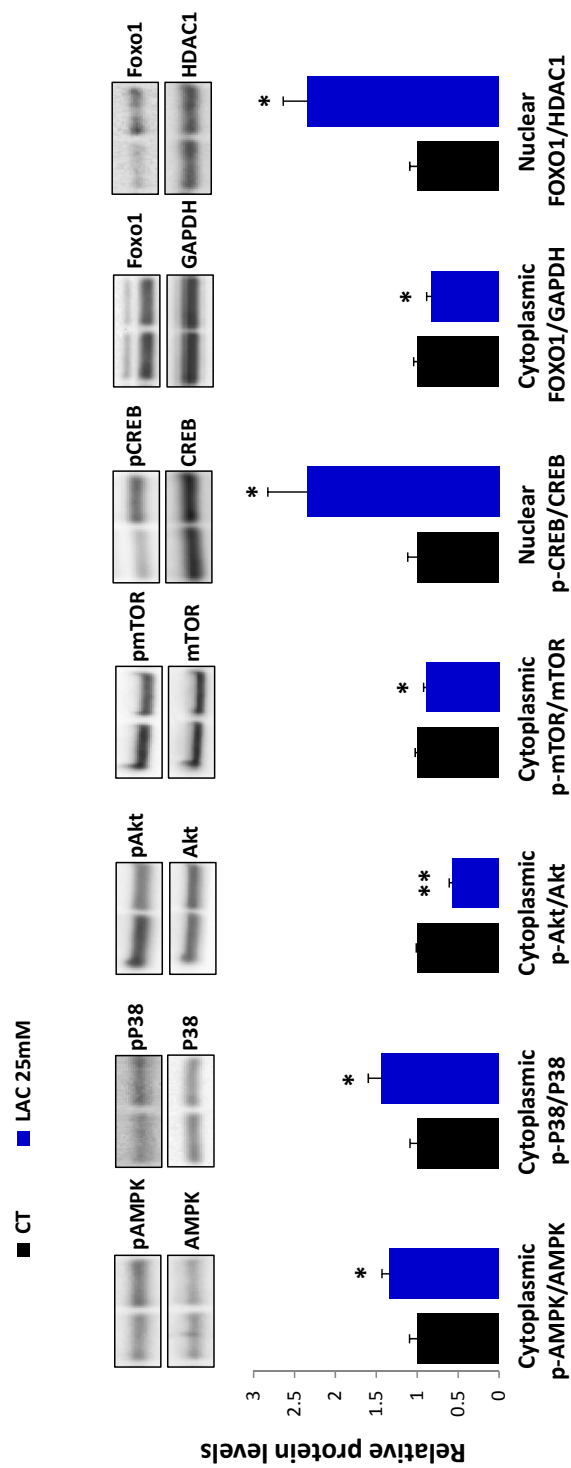


Figure 9. Effects of lactate pre-treatment on energy metabolism sensitive proteins in SH-SY5Y cells. (For figure legend, see next page).

Figure 9. Effects of lactate pre-treatment on energy metabolism sensitive proteins in SH-SY5Y cells. 6 hours of 25 mM lactate pre-treatment significantly increased AMPK phosphorylation and p38 phosphorylation in cytoplasm. Cytoplasmic Akt phosphorylation at Ser 473 and mTOR phosphorylation at Ser 2448 were decreased with lactate treatment. LAC group had higher CREB phosphorylation levels in nuclear fraction when compared to CT group. Lactate pre-treatment induced a significant translocation of FOXO1 protein from cytoplasm to nucleus. $*p < 0.05$; $**p < 0.001$. AMPK, AMP-activated protein kinase; CT, control group; CREB, cAMP-response element binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FOXO1, forkhead box protein O1; HDAC1, histone deacetylase 1; LAC, lactate; mTOR, mammalian target of rapamycin.

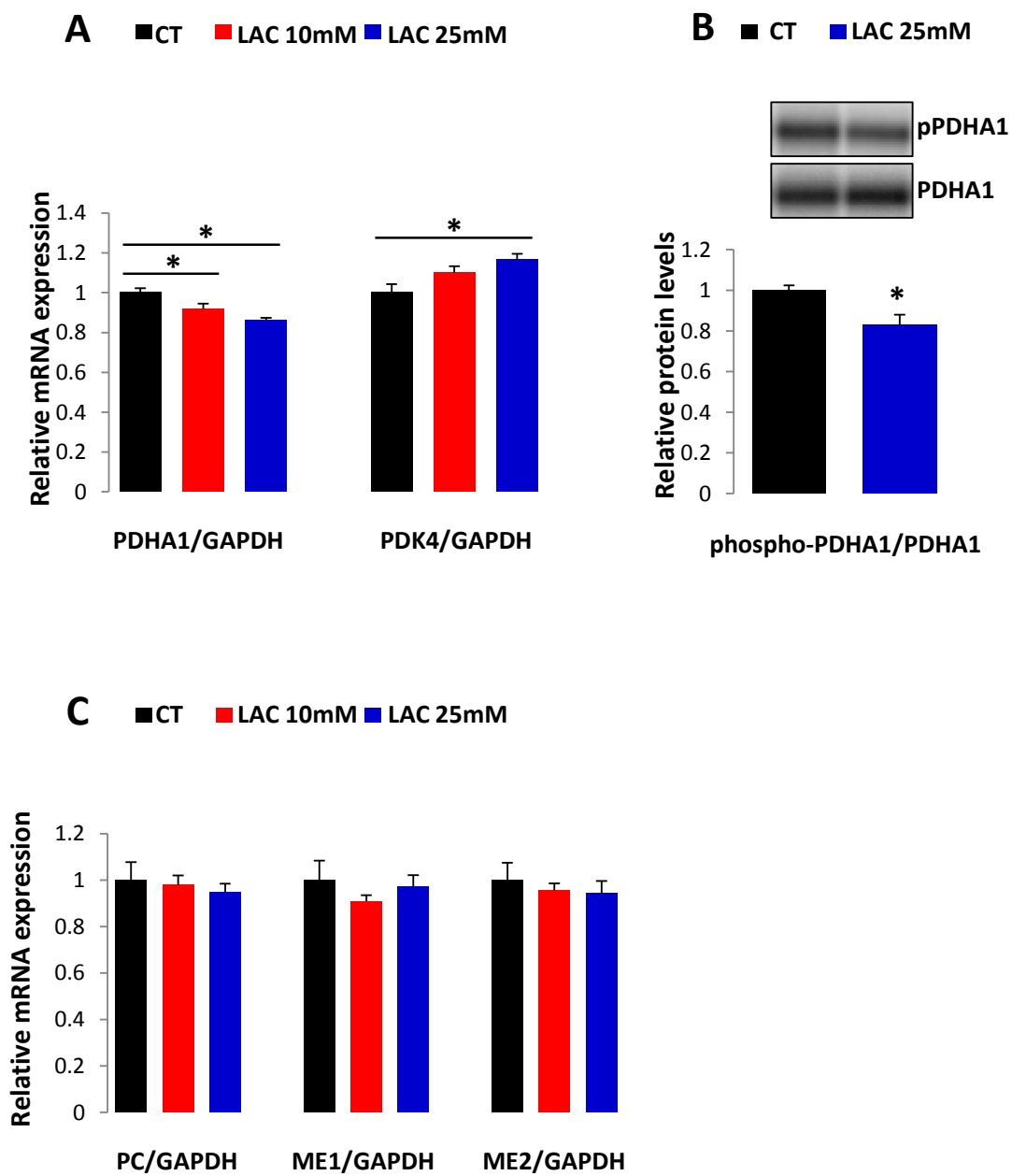


Figure 10. Effects of lactate pre-treatment on pyruvate decarboxylation and pyruvate carboxylation. (For figure legend, see next page).

Figure 10. Effects of lactate pre-treatment on pyruvate decarboxylation and pyruvate carboxylation. (A) PDHA1 mRNA levels were lower in both 10 mM and 25 mM LAC groups compared to CT group. PDK4 mRNA levels were increased in 25 mM LAC group but were comparable between CT and 10 mM LAC groups. **(B)** Phosphorylation of PDHA1 at Ser 293 was significantly decreased with 25 mM lactate pre-treatment. **(C)** No inter-group differences were observed in mRNA expression of PC, ME1 and ME2. * $p < 0.05$. CT, control group; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LAC, lactate; ME, malic enzyme; PC, pyruvate carboxylase; PDHA1, pyruvate dehydrogenase alpha 1; PDK4, pyruvate dehydrogenase kinase 4.

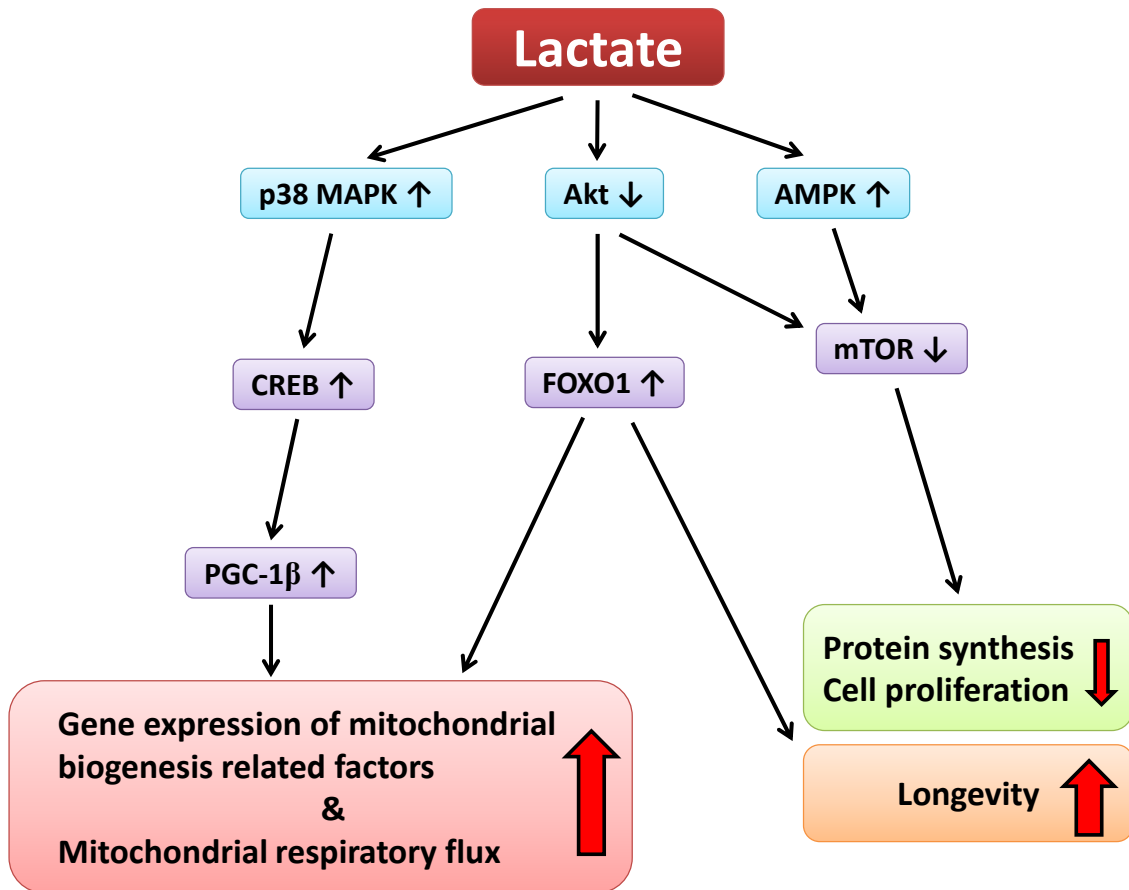


Figure 11. Illustration summarizing lactate-induced activation of bioenergetics-related signaling pathways. AMPK, AMP-activated protein kinase; CREB, cAMP-response element binding protein; FOXO1, forkhead box protein O1; mTOR, mammalian target of rapamycin; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1; peroxisomal proliferator-activated receptor-gamma co-activator 1.

Chapter VI

Conclusion

Summary of Findings

Summary of Chapter II. Effect of Exercise on Mouse Liver and Brain Bioenergetic

Infrastructures

To assess the effects of exercise on liver and brain bioenergetic infrastructures, young, 4-month old C57BL/6 mice were subjected to 6 weeks of treadmill exercise below the lactate threshold. We analyzed pathways and proteins that sense cell energy and redox states. Here, we especially considered the potential role that muscle-generated lactate might play in liver and brain bioenergetics.

Metabolically, we found that body weights were maintained over the 6 weeks in exercised mice, and fasting blood glucose level was lowered by exercise training. At week 6, the decrease of post-running plasma lactate levels, compared to the resting levels in untrained mice, indicated that exercise intensity was below the lactate threshold.

This plasma lactate level decrease was accompanied by a significant increase in the protein level of liver MCT2 that is responsible for liver lactate uptake. Exercise increased liver PGC-1 α expression, likely mediated by the increase in SIRT1 and p38 protein levels, and AMPK phosphorylation. Despite this, liver mtDNA copy number, TFAM and COX2 expression, and CREB phosphorylation, were all reduced. Our results suggest that 6-week treadmill exercise training below the lactate threshold strategically shifts the liver's bioenergetic profile in ways that should promote gluconeogenesis, as well as some aspects of mitochondrial biogenesis, but not oxidative phosphorylation. These effects are possibly lactate-mediated. Although our moderate-intensity exercise training protocol decreased brain TNF- α expression which was the only altered brain parameter in the study, the training below the lactate threshold seemed unable to modify brain bioenergetic infrastructures.

Liver lactate import appears to be favored over brain lactate import, possibly limiting the ability of exercise-generated lactate to modify brain bioenergetics. Therefore, exercise-mediated brain effects may be robust only when lactate production exceeds the lactate threshold.

Summary of Chapter III. Lactate Administration Reproduces Specific Brain and Liver Exercise-related Changes

To better understand how exercise impacts non-muscle tissues, we considered and focused on lactate's ability to mediate exercise-induced changes in liver and brain bioenergetics.

In one set of experiments, young, 4-month old C57BL/6 mice underwent 7 weeks of treadmill exercise sessions at intensities intended to exceed the lactate threshold. Over the course of the study, the mice dramatically increased their lactate threshold, and to ensure plasma lactate accumulated, during the final week the mice were run to exhaustion. In liver, expression of gluconeogenesis-promoting genes and PGC-1 α increased. PGC-1 β and NRF-1 expression decreased. The expression levels of other respiration-related genes were also down-regulated. In brain, PGC-1 α and PGC-1 β were unchanged while PRC expression and mtDNA copy number increased, indicating mitochondrial biogenesis was induced. These changes were accompanied by a decrease of TNF- α expression, and an increase in expression of VEGF-A, an angiogenic/neurogenic factor.

In another set of experiments, exogenous lactate administration, over 14 days consecutively, was found to reproduce some but not all of above-mentioned liver and brain changes induced by intensive exercise training. Specifically, similar to what we

observed in exercise trained mice, exogenous lactate administration enhanced hepatic gluconeogenesis while probably limiting hepatocyte respiration, shown by an increase in expression of gluconeogenic genes and PGC-1 α but a decrease in PGC-1 β and NRF-1 levels. In brain, mtDNA copy number was not altered. However, the exercise-induced increases in brain PRC and VEGF-A expression were reproduced by lactate administration, which implies that exercise-induced brain angiogenesis and neurogenesis may be driven by lactate released from contracting muscles.

In conclusion, our data suggest that lactate, a muscle-generated exercise byproduct, may account for at least some exercise-associated brain and liver bioenergetic infrastructure changes and bioenergetic-associated adaptations. For this reason, we consider that lactate may be a partial exercise mimetic, and it is worth testing in conditions with perturbed brain bioenergetics, such as Alzheimer's and Parkinson's diseases.

Summary of Chapter IV. Supra-lactate Threshold Exercise Training Induces Brain Mitochondrial Biogenesis in Aged Mice: Is There a Connection Between Brain Mitochondrial Bioenergetics, Inflammation, and Neurogenesis?

Intensive exercise training has been shown to improve brain mitochondrial bioenergetics in young, 4-month old mice, while few studies have focused on aging model. In this study, we investigated the effects of supra-lactate threshold intensive exercise training on brain mitochondrial biogenesis in 19-month old C57BL/6 mice. We also assessed the expression of neurogenesis-related factors, and central/systemic levels of inflammatory cytokines/chemokines, to test the relations between mitochondrial

function, inflammation, and neurogenesis, for a better understanding of the connections between different exercise-induced benefits on brain.

8-week supra-lactate threshold exercise did not alter metabolic parameters including blood glucose level, plasma insulin level, and HOMA-IR, while the body weights were lowered in the exercised mice. Our training protocol significantly increased brain expression of PGC-1 α and CS. In addition, brain mtDNA content assessed by ND1, COX2, and ATP6 copy number was also increased in exercise group, suggesting mitochondrial biogenesis was induced.

Brain mitochondrial biogenesis was accompanied by a slight increase in brain VEGF-A expression in exercised mice, possibly mediated by excess lactate in the blood, but our training protocol was not able to alter the brain expression of DCX, and BDNF. A strong positive correlated between brain PRC expression and VEGF-A expression was observed. This result is in agreement with what we reported in *Chapter III*. While mitochondrial biogenesis seemed not clearly associated with neurogenesis in exercise trained aged mice, a transcription factor and a coactivator (FOXO3a and PRC) that play important roles in regulating bioenergetic network were likely influencing a neurogenesis-related factor (VEGF-A).

Central and systemic inflammatory factors were not altered by intensive exercise training, no significant associations between brain mtDNA content and these inflammatory factors were observed. However, plasma CCL11 was negatively correlated with hippocampal DCX expression, supporting the view that systemic inflammation may have a suppressive effect on neurogenesis, while our exercise protocol did not affect the aging-associated increase of plasma CCL11 levels in these aged mice.

Together with the results from *Chapter III*, we conclude that supra-lactate threshold exercise training induces brain mitochondrial biogenesis in both young and aged mice. It certainly has therapeutic implications for neurological disorders with perturbed brain bioenergetics. Yet mechanistic study is needed to identify the causal relationships between mitochondrial biogenesis, neurogenesis, and inflammation under the condition of exercise training.

Summary of Chapter V. Lactate Treatment Modifies Bioenergetic Fluxes and Infrastructures in Cultured Human Neuronal Cells

In this study, we investigated the possibility of lactate treatment as a strategy for bioenergetic flux manipulation using SH-SY5Y human neuronal cells. The underlying mechanisms were also discussed.

Our results showed that, in SH-SY5Y cells, treatment with lactate for 6 hours significantly decreased glycolysis flux and had a prolonged enhancement of mitochondrial respiration. Similar effects were also induced with acute lactate treatment. Interestingly, over time exogenous lactate gradually shifted the bioenergetic metabolism towards a more aerobic state in SH-SY5Y cells. An increase of NAD^+/NADH ratio was observed with lactate treatment, likely induced by a decrease in NADH levels. The changes in these fluxes were not accompanied by alteration of mitochondrial coupling efficiency or overall ATP production, while lactate treatment seemed to improve electron transfer via the mitochondrial ETC.

Although we did not observe changes in mtDNA copy number with lactate treatment, some components of mitochondrial biogenesis were up-regulated. Specifically,

expression of PGC-1 β , NRF-1, and COX1 was significantly increased in lactate-treated cells. Vmax activities of COX and CS were also increased. These lactate-induced effects were likely mediated by activation of AMPK, p38 MAPK, and Akt signaling pathways to modify cellular bioenergetic infrastructure. Similar to what we found in *Chapter III* and *IV*, lactate treatment also increased VEGF-A expression in human neuronal cells, further supporting the view that lactate may account for exercise-induced brain angiogenesis/neurogenesis. Additionally, our results suggest a potential role for lactate in longevity, as lactate treatment decreased the activity of mTOR but induced nuclear translocation of FOXO1.

This study demonstrates that lactate can shift energy metabolism of SH-SY5Y human neuronal cells towards mitochondrial respiration resulting in a more aerobic state, and can activate several signaling pathways to modify bioenergetic infrastructures. Our data provide novel insights into bioenergetics-based pharmacological therapies for neurodegenerative diseases with perturbed brain bioenergetic fluxes.

Limitations and Future Directions

Chapter II, III, IV

For *Chapter II-IV*, our work may lack a strict comparison, between moderate-intensity training and supra-lactate threshold training, as well as between young adult mice and aged mice. We did not include two different exercise intensities, or both young and aged mice in the same study. Similarly, in *Chapter III*, exercise group was subjected to exercise training for 7 weeks, while lactate group and PBS vehicle group received injections for only 14 days. One may argue that these two interventions should not be

compared. As a matter of fact, we did not perform statistical analyses for above mentioned comparisons. Our primary intent was not to compare the intensities, mice of different ages, or exercise versus lactate injection, but rather to provide insights into the potential of exercise or lactate mediated changes of brain bioenergetics.

In *Chapter III*, although we observed that lactate administration reproduced some supra-lactate threshold exercise-induced changes in liver and brain bioenergetic infrastructures, we could not ensure lactate in the blood was able to reach the brain, or remain enriched in the brain for a period of time. Liver gluconeogenesis was enhanced with lactate administration, suggesting considerable amount of blood lactate probably was cleared by liver. This could be the same case for mice subjected to supra-lactate threshold exercise. As a future direction, one may use magnetic resonance spectroscopy techniques to assess the brain lactate levels *in vivo*, after lactate injection of supra-lactate threshold exercise. Administrating specific inhibitors targeting liver MCTs along with lactate treatment, or exercise training may also to some extent reduce this limitation. More specifically, mechanistic studies can be conducted by giving lactate injection and exercise training to mice of neuron-specific conditional knockout of MCTs, to see whether or not supra-lactate threshold exercise-induced benefits on brain would be observed.

In *Chapter IV*, our results and interpretation regarding the relations between brain mitochondrial biogenesis, neurogenesis and inflammatory factors were limited by a technical issue. Neurogenesis is often evaluated by BrdU or DCX staining with quantitative fluorescence microscopy (Wojtowicz & Kee, 2006). We alternatively measured DCX mRNA levels in hippocampus, while currently we do not know to what

extent hippocampal DCX mRNA expression is suggestive of ongoing neurogenesis in the area. We will continue to attempt immunofluorescent estimation of DCX positive neurons in the dentate gyrus of these aged mice.

Chapter V

One limitation in *Chapter V* is that SH-SY5Y cells are neuroblastoma cells. Although undifferentiated SH-SY5Y cells possess some neuronal characteristics, their innate tumor-like features, such as Warburg effect, may confound our study results. Thus, the interpretation of experimental results in this chapter needs to take these factors into account. As a future direction, differentiated SH-SY5Y cells with more mature neuronal characteristics may be used to further explore the effect of lactate from the perspective of bioenergetics. Also, primary mouse neurons may ensure a more appropriate neuronal background to study this topic. Future experiments to apply lactate treatment in cytoplasmic hybrids (cybrids) harboring mtDNA from AD patients may further provide evidence for lactate's therapeutic potentials.

Clinical Implications

The results from this work may provide guidance for clinicians to prescribe customized exercise programs, or provide scientific evidence for designing clinical trials intended to evaluate effects of exercise training in persons with perturbed brain energy metabolism or on preventing these conditions.

The type, duration, frequency and intensity have always been a question when it comes to prescribing or recommending exercise to different populations. In fact, for

different purposes, and different target organs, these parameters may need adjustment. In this study, we have demonstrated that moderate-intensity exercise is sufficient for improving mouse liver gluconeogenesis. However, beneficial effects for brain, in terms of improved mitochondrial bioenergetics, are only robust when exercise intensity is above the lactate threshold. Although verification of these findings is definitely needed in human subject studies, given that altered brain bioenergetics have shown and may play a pathogenic role in many neurodegenerative disorders, our results suggest that regular supra-lactate threshold exercise may be beneficial for healthy population to prevent or delay the onset of neurodegeneration. For persons with neurodegenerative diseases or other neurological disorders with perturbed brain bioenergetics, we hope that this exercise strategy will have therapeutic effects or a potential to delay the disease progression.

However, altered brain energy metabolism, either physiologically or pathologically, are often associated with aging. These populations are not very likely able to tolerate or comply with high-intensity regular exercise training. For this reason and others, exercise mimetics are needed. From this point of view, this body of work provides the mechanistic groundwork for proposing lactate as a partial exercise mimetic or bioenergetic medicine intervention, by virtue of its ability to modify neuronal bioenergetics towards a healthier state. Future studies are also expected to explore lactate's potential for lifespan extension. Meantime, attempts should be undertaken for discovering and developing other pharmacological candidates intended to improve brain bioenergetic balance.

Conclusion

This dissertation work aimed to investigate whether and how, from the perspective of bioenergetics, exercise training influences non-muscle tissues, particularly brain. We have demonstrated that intensive, supra-lactate threshold exercise training robustly induces mitochondrial biogenesis and increases expression of angiogenic/neurogenic factors in the brains of both young and aged mice. Moreover, we have identified a role for lactate in mediating some of the exercise-induced benefits for brain, implying the possibility of lactate serving as a partial exercise mimetic. These results could provide guidance for designing clinical studies intended to investigate the roles of exercise in prevention and treatment of various diseases. At the same time, the benefits of lactate treatment may be further explored not only as a potential exercise mimetic, but also as a therapeutic candidate to modify brain bioenergetic infrastructures towards a healthy, balanced state, especially for persons with perturbed brain energy metabolism, such as neurodegenerative diseases. We believe that this dissertation work will open an insight and avenue for searching and chemically developing more bioenergetics-based therapies for brain health.

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